Towards individualized controlled drug exposure in renal transplantation

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TOWARDS INDIVIDUALIZED CONTROLLED DRUG EXPOSURE IN RENAL TRANSPLANTATION

Proefschrift

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Chapter 1

Scope of the thesis

Kidney transplantation is the preferred solution for most patients with chronic renal failure, who otherwise have to face chronic dialysis, a treatment accompanied by impaired quality of life and excess mortality. After successful renal transplantation, most patients will be able to resume a lot of social and professional activities, which were not possible during dialysis. Due to improved surgical methods, new immunosuppressive drug regimens and better general patient care, the success rates in most centers, measured one year after kidney transplantation nowadays exceed 95%. After this early period, however, a gradual decline of renal function can still be detected in 40 to 50% in the transplant recipients, which is generally an ongoing process ultimately resulting in return to dialysis. The histological substrate for this condition, chronic allograft nephropathy (CAN), is well defined and several factors have been identified, that correlate with the development and progression of CAN. Nephrotoxicity of immunosuppressive drugs and rejection mechanisms, due to insufficient immunosuppression, are known to play a central role in this process. Especially, calcineurin-inhibitors, still serving as cornerstone in current renal transplantation maintenance regimens, are critical drugs, because of their narrow therapeutic index, their inherent nephrotoxicity and their unfavourable cardiovascular side effect profile (hypertension, hyperlipidemia and new onset diabetes mellitus).

In this thesis we focus on the central role of immunosuppressive drugs in the development of CAN. In **Chapter 2** we reviewed the available evidence in the literature of possible pharmacotherapeutic interventions to prevent the development or progression of CAN. Since most novel options are still experimental, we concluded that improvement of therapeutic drug monitoring (TDM) could be the first achievable goal to optimize outcome in the short and intermediate term.
Improved drug monitoring of calcineurin inhibitors and subsequent adequate drug dosing could prevent either over- or underdosing, and in this way optimally prevent structural damage imposed by rejection mechanisms or drug-related nephrotoxicity. With the use of a population based, pharmacokinetic computer program, we developed a simple model to estimate the systemic exposure of cyclosporine (CsA).
In **Chapter 3** we compared several limiting sampling methods in combination of this model to design an optimal monitoring strategy, flexible enough for out-clinic use and robust enough to guarantee reliable estimation of the drug exposure in different groups of transplant recipients.
In **Chapter 4** we extended the model to tacrolimus (Tac)-based clinical immunosuppression. In this study we prospectively tested the model in a cohort of patients, to detect intra-individual pharmacokinetic changes over time and to define optimal monitoring intervals.
In **Chapter 5** we studied the pharmacokinetic interactions between the two current available calcineurin-inhibitors, CsA and Tac, and mycophenolate mofetil (MMF). MMF is commonly used as third immunosuppressive drug, beside steroids and one of the calcineurin-inhibitors. Using a compartmental metabolite PK modelling technique, we investigated if differences in the concentration curves of the main metabolites of MMF, MPA and MPAG could be attributed to one of the co-administrated calcineurin-inhibitors by simulating different levels of interaction.

The population-based model as described in **Chapters 3** and **4** was used to guide drug dosing for 126 renal transplant recipients, included in a trial to compare the early development of fibrosis and CAN in patients randomized to either CsA- or Tac-based immunosuppresion. By using this AUC-guided dosing technique we wanted to rule out that differences in clinical outcome between the drugs could be attributed to the different pharmacokinetic properties of the drugs and in this way make a more fair comparison. By the use of relatively low AUC-targets for both CsA and Tac, we

hypothesized that the intrinsic nephrotoxicity could be minimized, while effective prophylaxis against acute rejection could be maintained for both drugs, both leading to a reduced amount of interstitial fibrosis. As primary read-out for this trial we obtained surveillance biopsies at 6 and 12 months, in which interstitial fibrosis was evaluated by quantitative digital analysis of Sirius red staining. This method has been validated in earlier reports and is generally accepted as a reliable surrogate marker for the development of CAN and ultimately graft-survival.

The results of this trial are reported in **Chapter 6** and the impact of the TDM strategy with either CsA or Tac on other relevant end-points such as incidence of acute rejection episodes, prevalence of subclinical acute rejection (SAR), allograft function as well as side effects were compared.

In **Chapter 7** we investigated the clinical relevance of the presence of SAR in 6 month surveillance biopsies. We evaluated the relationship of SAR with pharmacokinetic data, donor and recipient variables and histological parameters. Finally, since by protocol, SAR was not treated, we investigated if the presence of SAR at 6 months negatively influenced renal histology in follow-up biopsies and renal function up to two years after transplantation.

In **Chapter 8** we summarize the studies described in this thesis. We discuss the importance of drug monitoring in the field of renal transplantation and its possible role in further developments in the near future.

Chapter 2

Pharmacotherapeutic approach to prevent or treat chronic allograft nephropathy

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Introduction

Chronic allograft nephropathy and cardiac transplant vasculopathy are the leading causes of graft failure in kidney and heart transplants respectively, while it seems less prevalent in liver transplants.

In death censored graft survival studies, chronic allograft nephropathy is the most prevalent cause of graft failure. Clinically, it is characterized by a slow but variable loss of function, often in combination with proteinuria and hypertension. The histopathology is not specific and consists of graft atherosclerosis, multilayering of the peritubular capillaries; transplant glomerulopathy and glomerulosclerosis, interstitial fibrosis and tubular atrophy (Figure 1).

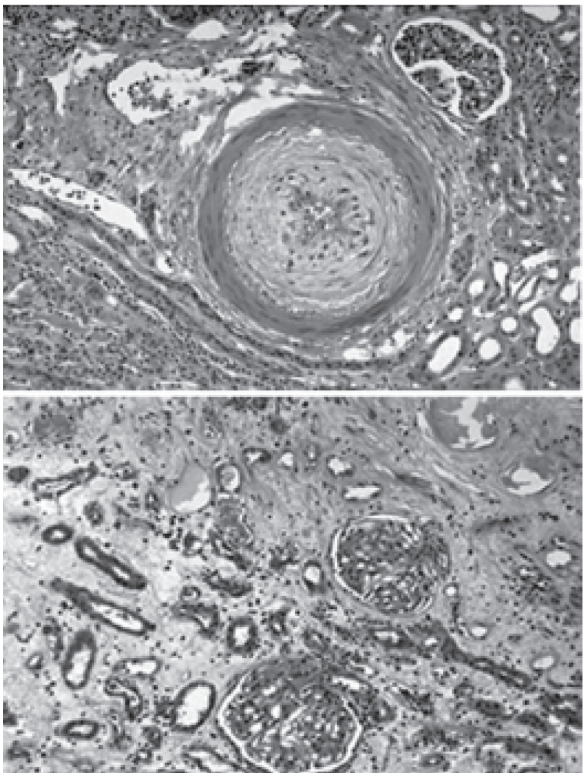

Figure 1: Photomicrograph of a human kidney transplant removed because of chronic transplant failure. There is an extensive graft atherosclerosis, with narrowing of the vascular lumen, interstitial fibrosis, tubular atrophy and glomerular sclerosis.

Graft vasculopathy is characterized by intimal proliferation of myofibroblasts and deposition of extra-cellular matrix proteins in the vascular intima [1]. Cardiac graft vasculopathy is angiographically detected in 20% of heart transplants at 1 year and at 3 years the prevalence is nearly 50% [2,3]. Graft vasculopathy may present as a silent myocardial infarct, congestive heart failure, ventricular arrhythmias or sudden death; angina pectoris is uncommon as the grafted heart lacks neural innervation.

Tissue injury and remodeling are characteristic features of chronic rejection. The initial response is inflammation and involves the attraction of lymphocytes, macrophages and mesenchymal cells [4], cells that contribute to the inflammatory and remodeling response. Cytokines, growth factors and chemokines play important regulatory roles in these processes. Myofibroblasts migrate and proliferate to form scar tissue. In chronic rejection, there is disruption of the normal tissue architecture due to excessive fibrointimal changes.

Immunosuppression and long-term graft outcome

Immunosuppressive drugs are probably the most important ingredient in the prevention of acute rejection although their role in the prevention of late graft loss is less clear. Experimental studies have shown that immunetolerance protocols in animals can prevent chronic allograft failure in some combinations [5,6] but there is no evidence yet in humans, that such protocols are clinically feasible. Prior to 1980s, standard immunosuppression consisted of the combination of corticosteroids and azathioprine, complemented in some centers with the prophylactic administration of anti-lymphocyte antisera in the first few post-transplant weeks. Although most clinical studies had patient and graft survival as primary outcomes, many surviving grafts had signs of dysfunction and fibrosclerotic changes. The strong correlation of acute rejection episodes with later dysfunction [7-9] lead to the hypothesis that these late changes may have an immune pathogenesis. We will therefore examine the role of immunosuppression on long-term outcome and chronic allograft nephropathy.
The introduction of the calcineurin inhibitor cyclosporine A (CsA) has resulted in a decreased incidence of acute rejection episodes as well as an improved 1-year graft survival rate but it has been disputed whether this has had a beneficial effect

on the rate of graft attrition after the first post-transplant year [10]. Only recently has long-term graft survival improved but no single factor has been identified as the responsible cause [11]. Questions have been raised whether the immunosuppressive efficacy of calcineurin inhibitors is off-set by their nephrotoxic adverse effects [12], whether drug exposure has been adequate [13,14], or whether these drugs are effective to prevent or treat chronic allograft nephropathy.

T**able 1:** Putative sites of anti-atherogenic action of drugs used in the prevention of chronic allograft nephropathy.

Drug	**Site of action**
Corticosteroids	Anti-inflammatory actions,Immune suppressant
Azathioprine	Immune suppressant
Anti-lymphocyte antibodies	Immune suppressant
Cyclosporine	Immune suppressant
Tacrolimus	Immune suppressant
Mycophenolate Mofetil	Immune suppressant
Sirolimus, Everolimus	Immune suppressant, Inhibits smooth muscle cell proliferation
HMG CoA Reductase Inhibitors	Lipid lowering. Immune suppressant, Inhibits vascular smooth muscle cell proliferation
Calcium Channel Blockers	Blood pressure lowering, direct tissue effect, indirect effect on CsA metabolism
ACE-inhibitors, Angiotensin II receptor blockers	Blood pressure lowering, direct tissue effect
15-Deoxyspergualin	Immune suppressant
Vitamin D analogs	Immune suppressant
Soluble complement receptors	Immune suppressant
FTY 720	Immune suppressant
Ganciclovir	Anti viral drug, inhibits vascular smooth muscle cell replication
Superoxide dismutase	Ischemia/reperfusion damage
Endothelin receptor blocker	Tissue protective
Insulin-like growth factor analogs	Tissue protective
Tyrosine kinase inhibitors	Tissue protective
Low molecular weight heparin	Tissue protective
Estrogens	Tissue protective
Somatostatins	Inhibits smooth muscle cell proliferation

In addition to the nephrotoxic side effects from calcineurin inhibitors, other non-immune toxicity side effects from several drugs may affect graft structure and function. In renal ablation models, glucocorticoids may accelerate the development of proteinuria and glomerulosclerosis [15]. CsA, tacrolimus and corticosteroids aggravate hypertension, and have detrimental effects on lipid metabolism.
The lack of randomized trials with robust long-term follow-up information makes it difficult to assess the impact of various immunosuppressive drug regimens on long-term outcome although some information is available from registry data and mostly underpowered single center studies. Registry data on immunosuppressive drug therapies should be regarded, however, with caution as they are not derived from randomized trials and often reflect a treatment or reporting bias. The evidence of the relative efficacy of various immunosuppressive drugs is largely based on early events and the choice of an agent for the long-term is made without hard evidence.

Currently used immunesuppressive drugs

Corticosteroids
In the Collaborative Transplant Study database, the 5-year graft survival and projected graft half-life was significantly better in patients who had been switched from CsA, steroids, and azathioprine to a steroid-free maintenance immunosuppression with CsA with or without azathioprine [16,17]. However, only 10% of patients reported to this registry were treated with steroid-free maintenance regimens and it is likely that they represent a group of low risk patients in whom steroids can be safely withdrawn.
A Canadian multicenter steroid-withdrawal study of patients treated with CsA, azathioprine and corticosteroids found a significantly better 5-year graft survival in patients who continued on prednisone (0.3 mg/kg on alternate days) compared with patients who, starting at 3 months after transplantation, were treated with an "ultra-low" dose of prednisone (2 to 3 mg on alternate days). The graft survival curves of the two groups dissociated 1.5 to 2 years after the study enrollment and suggest a beneficial effect of steroids on long-term prognosis. When risk factors confounding graft survival were included in the analysis, no significant influence of assigned treatment was found [18] but the direction was still in favor of maintenance

of corticosteroids. In a single centre study from Leiden, prednisone was withdrawn 1 year after transplantation; this resulted in an increase in the incidence of acute rejection episodes, a larger but non-significant loss of renal function and a higher proportion of patients with proteinuria in the prednisone withdrawal group [19]. Although there was no difference in graft survival, it remains to be seen whether the deterioration of function is an early sign of chronic allograft nephropathy.

In a meta-analysis that included nine studies that examined the effects of prednisone withdrawal, this was associated with a higher incidence of acute rejection and graft failure after a mean follow-up of 29 months [20].

Several recent studies have shown that steroids can be withdrawn safely in patients treated with mycophenolate mofetil (MMF) and cyclosporine [21], MMF and tacrolimus, or rapamycin albeit that no long-term follow-up data are available. In two of the prednisone withdrawal trials in patients who were on MMF [22,23] the difference in acute rejection episodes between prednisone withdrawal and control did not seem to be different compared with trials that did not use MMF. Both trials reported only 12 months of follow-up, so it may be too early to tell whether the effect of prednisone withdrawal will be different in these studies.

Azathioprine

Data from the Collaborative Transplant Study registry show no difference in the estimated half-life of kidney transplants in patients treated with CsA (Sandimmune) plus corticosteroids compared with CsA, corticosteroids, and azathioprine [18]. A meta-analysis of five trials comparing these regimens also failed to show a difference in patient or graft survival [24], suggesting that azathioprine does not add anything to improve long-term outcome. In a British trial, no differences in graft survival were found in patients treated with triple therapy, dual therapy with CsA and azathioprine, or monotherapy with CsA only [25]. A recently published randomized study of patients treated with dual therapy consisting of 10 mg/kg/day of CsA plus prednisone or triple therapy consisting of 6 mg/kg/day of CsA plus azathioprine and prednisone found a similar incidence of chronic graft dysfunction in both groups (15 to 17% in both groups but the 4-year graft survival was better in the triple therapy group (83% compared with 71% in the double therapy group, $p = 0.089$) [26]. In a multicenter study from Italy, CsA alone was compared with CsA plus steroids or CsA plus steroids and azathioprine; after 9 years of follow up actuarial patient and

graft survival did not differ among the three groups [27], suggesting that there is no benefit in the addition of azathioprine or for that matter corticosteroids.

Anti-lymphocyte antibody prophylaxis

Many investigators, especially in North America, have explored the benefits of the prophylactic use of antibodies or antisera against lymphocytes in the immediate post transplant period, a regimen often referred to as "induction therapy". Although most cohort studies and all randomized controlled trials individually have failed to demonstrate a benefit of anti-lymphocyte preparations, data from large registries show a graft survival benefit [28,29]. Cecka *et al.* reported an 8.1% greater 1-year survival rate in the antibody-treated group [28] and Opelz found a 3.9 and 2% survival advantage at 3 and 5 years respectively [29]. Unfortunately, in the antibody-treated group there is a higher incidence of lymphoproliferative disease [28]. A recently published meta-analysis of seven randomized controlled trials of prophylactic anti-lymphocyte antibody treatments demonstrated a 6% greater 2-year graft survival rate in the treated group [30]. This benefit became smaller with time and was not detectable after 2 years of follow-up, except in a subgroup of sensitized patients [31].

Comparison of the U.S. and the Tricontinental Mycophenolate Mofetil Renal Transplant Studies [32,33] provide some insight in the efficacy of antibody induction in conjunction with MMF. In the U.S. study, MMF in combination with CsA, corticosteroids and anti-thymocyte gamma-globulin gave a 3-year graft survival of 86.0% compared with 83.1% in the Tricontinental Study in which patients received MMF, CsA and corticosteroids without antibody induction, suggesting a small beneficial effect of anti-thymocyte antisera.

Cyclosporine

Cyclosporine (CsA) has improved the short-term results of kidney transplantation but it is not clear whether it has decreased the incidence of chronic allograft nephropathy. In the 1992 UCLA/UNOS database, the introduction of CsA has not affected the graft half-lives [34] although other studies reported some long-term benefit. In the Eurotransplant registry, there was a slightly longer half-life in CsA-treated patients transplanted between 1981 and 1987 compared with patients transplanted between 1981 and 1984 who did not receive CsA. Some individual centers reported similar data [35] .

In the European Multicenter Study [36] and in the data from Basel [37], graft survival was slightly better in patients allocated to CsA than in those on conventional therapy, but there were no differences when only grafts surviving more than 1 year were analyzed [36] and in both studies, approximately 40% of patients were switched to azathioprine and steroids. In another study, in which patients treated with CsA and prednisone were compared with azathioprine and prednisone, graft survival was not different in the groups if only considering grafts that functioned at 1 year [38]. Ponticelli *et al.* [39] performed a randomized study in which the majority of patients were on CsA at the end of follow-up and found 56% graft survival at 10 years in the CsA group and 35% in the azathioprine arm.

The Collaborative Transplant Study database showed that patients receiving CsA 3 – 6 mg/kg/day at 1 year post-transplantation had the best graft survival rate at 10 years. CsA doses of < 2 mg/kg/day were least beneficial and doses > 6 mg/kg/day was associated with over-immunosuppression [40].

A receiver operating characteristic analysis of the correlation between the % coefficient variation in CsA exposure on a concentration-controlled CsA dosing regimen versus the risk of chronic rejection showed that the cohort of patients with less variable exposure displayed a significantly longer time to the occurrence of chronic rejection compared with the cohort with a high variability [41].

A recent USRDS analysis has shown that cyclosporine microemulsion (Neoral) use was associated with a significantly lower risk (RR = 0.6, CI = 0.5 – 0.7) for chronic allograft failure as opposed to conventional cyclosporine formulation [42]. Similar data have been reported by the Collaborative Transplant Study [40].

Concerns regarding CsA nephrotoxicity initiated several trials of CsA withdrawal after transplantation. An analysis of more than 12,000 patients reported to the Collaborative Transplant Study showed the worst 5-year graft survival in patients who initially received CsA, azathioprine and corticosteroids and who were switched in the first year to corticosteroids and azathioprine; the best results were obtained in patients who had remained on CsA plus azathioprine or CsA only [16]. The half-lives were 10.5 years for grafts treated with steroids and azathioprine, 30.0 years for grafts treated with CsA only and 26.6 years for grafts treated with CsA and azathioprine [17]. These observations and projections are at variance with the results of a meta-analysis involving 10 randomized and 7 non-randomized trials of elective CsA discontinuation at some time point after surgery. Although these trials showed

a greater acute rejection rate among patients from whom the drug was withdrawn compared with patients who continued to receive CsA, there were no differences in graft loss or mortality attributable to CsA withdrawal [43]. A later study with additional trials and a longer follow-up confirmed that CsA withdrawal is associated with acute rejections but unlike prednisone withdrawal, CsA withdrawal does not seem to increase the rate of graft failure [20].

A prospective CsA conversion study in our center of 128 patients who were randomly assigned to either continuation of CsA or switching to azathioprine at 3 months showed an 8-year patient survival of 75.3% in patients who had remained on CsA and of 85.9% in the azathioprine group ($p = 0.14$); the graft survival rates were 64.0% and 76.6%, respectively ($p = 0.38$) [44]. Chronic allograft nephropathy was diagnosed in 12% of CsA-treated patients and in 8% of azathioprine treated patients.

In a non-randomized study of 20 patients who were 2 to 5 years post-transplant and were on microemulsion CsA in combination with azathioprine and steroids, azathioprine was discontinued and MMF introduced followed by CsA withdrawal 4 months later. After CsA withdrawal only 1 patient experienced a reversible rejection episode. Inuline clearance improved significantly but at the end of follow-up at 40 weeks after inclusion, histological worsening was observed in 50% of patients [45].

In a recent multicenter trial 84 patients on Neoral, corticosteroids and MMF were at 3 months randomly assigned to be withdrawn from either Neoral or MMF. Both the creatinine clearance and calculated GFR were significantly better in MMF-treated patients at 1 year. Conversion to MMF was associated with a decline of blood pressure and with a more favorable lipid profile. There was no difference in patient or graft survival; and acute rejection episodes occurred more frequently after withdrawal of CsA.

Tacrolimus or FK 506

Tacrolimus is a macrolide that, like CsA, inhibits the intracellular enzyme calcineurin. As a result, calcium-dependent signaling from the T cell receptor to the nucleus is impeded, and transcription factors required for cytokine gene transcription are not activated.

Phase III studies comparing tacrolimus with CsA (gel capsules) in both the United States and Europe showed in the tacrolimus group a significantly lower rate of acute rejections in general as well as steroid-resistant rejections [46,47]. No differences were found in graft survival rates at 1 and 3 years or in serum creatinine at 3 years. The group on tacrolimus required less antihypertensive therapy but had more post-transplant diabetes mellitus. About 40% of grafts in the US trial that were still functioning at 2 years underwent a biopsy and in 8.9% of tacrolimus treated patients there was evidence of acute rejection versus 9.2% in CsA treated patients. Chronic allograft nephropathy was found in 62% of tacrolimus biopsies and 72.3% of CsA biopsies ($p = 0.155$) [48]. In a recent meta-analysis, tacrolimus treatment reduced acute rejection (odds ratio: 0.52; 95% confidence interval 0.36 – 0.75) but not graft loss at 1 year [49]. A small, prospective, randomized study showed less acute rejection in the tacrolimus group (33% versus 40% in the Neoral group) [50].
In the UNOS kidney transplant registry, the projected half-life of 544 transplants treated with tacrolimus was 15.3 years compared with 8.5 years in more than 35,000 grafts treated with CsA [51]. Caution is warranted because the tacrolimus data were scarce and centre-restricted and virtually all patients treated with CsA received the old formulary of gel capsules. However, using the same database updated with additional information, it was confirmed that tacrolimus improves graft survival and half-life [52]. A recent analysis of a large number of primary renal transplants reported to the United States Renal Data System between 1994 and 1997 confirmed that tacrolimus treatment is associated with a lower risk of chronic allograft failure compared with conventional cyclosporine formulation (RR = 0.7, CI = 0.6 – 0.8). Treatment with conventional gel capsules CsA formulation was associated with a 87.6% adjusted chronic allograft failure free survival rate at 4 years while tacrolimus and cyclosporine microemulsion (Neoral) are both associated with a significantly better adjusted chronic allograft failure-free survival at 4 years (91.4 and 92.4%, respectively) [42].

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is a semi-synthetic ethyl ester prodrug of mycophenolic acid (MPA). MPA is a non-competitive, highly selective inhibitor of inosine monophosphate dehydrogenase, which results in a deficiency of guanine nucleotide and inhibition of de novo purine synthesis, which pathway is required by proliferating

B and T lymphocytes. MMF in conjunction with CsA decreases the incidence of acute rejection in the first 6 months after transplantation by about 50% compared with placebo or azathioprine [53-55]. At 3 years, both the intention-to-treat and on-study analyses of graft and patient survival showed a non-significant trend towards an advantage for MMF. Renal function and urinary protein excretion rates were not different among the groups [33].

The addition of MMF to either CsA- or tacrolimus-based immunosuppression has been suggested for managing chronic allograft dysfunction [56-58] but the data are unconvincing to the extent that MMF gives clinicians confidence to reduce calcineurin inhibitor doses, which may improve renal function [59-61]. Recently, 17 patients were converted from Neoral and steroids to MMF and steroids after 6 months with benefits to blood pressure, glomerular hemodynamics and lipids [62].

A multivariate analysis of risk factors for chronic allograft failure within the US Renal Transplant Scientific Registry involving approximately 8,500 recipients treated with MMF showed that the drug decreased the relative risk to develop chronic allograft failure by 27%. This effect was independent of its outcome on acute rejection. Censored graft survival at 4 years using MMF versus azathioprine was significantly improved (85.6 % versus 81.9%) [63]. However, a study of only 45 stable renal transplant patients who had MMF withdrawn after 1 year showed no evidence of an increased incidence of proteinuria or increased creatinine levels [64].

In a small non-randomized study of patients who developed chronic rejection while on azathioprine maintenance therapy, azathioprine was discontinued and MMF was started [65]. After MMF introduction, the overall GFR decrease attenuated; in particular in 7 of 12 patients a significant reduction of mean creatinine value was recorded. A similar beneficial effect was observed in children who developed chronic rejection while on azathioprine who were switched to MMF [66]. In a pilot study of four renal transplant recipients with chronic humoral rejection, the combination of MMF together with tacrolimus gave a decrease in donor specific antibody titers [67] .

Sirolimus (Rapamycine) and everolimus

Rapamycine is the most recently introduced immunosuppressant used to prevent acute rejection. Its main mechanism of action as an immunosuppressive drug is to interfere in the IL-2 receptor signal transduction pathway in T-lymphocytes. The

drug alone, or in combination with CsA or tacrolimus, is effective to prevent acute rejection. In addition to its effect on T lymphocytes, it inhibits antigen-presentation [68] and smooth muscle cell migration and proliferation in response to growth factors [69,70]. Although no clinical data are available on the efficacy of rapamycine to prevent chronic rejection, its effects on smooth muscle cells may be of particular benefit. In non-immune models of vascular injury, rapamycine inhibits intimal smooth muscle proliferation [71] and rapamycine-coated stents inhibit neointimal formation in human coronary arteries [72]. In a rat cardiac allograft model, rapamycine alone or in combination with MMF resulted in a diminution in the number of vessels affected by graft atherosclerosis as well as the degree of atherosclerosis [73,74]. In an allergenic vessel interposition model in cynomolgus monkeys the drug was given after the establishment of chronic vascular rejection and this resulted in a halt of progression of the disease [75]. Finally, in a model of allogenic kidney transplantation rapamycine gave significant inhibition of chronic rejection [76].
Everolimus is a close relative of rapamycine, with a hydroxyethyl chain at position 40, conferring increased polarity. Everolimus inhibits the growth factor drive proliferation of lymphoid- and nonlymphoid-cells *in-vitro* and *in-vivo*. It is developed primarily as adjunctive therapy in combination with calcineurin inhibitors in the prophylaxis of acute and chronic rejection. Everolimus is comparable to sirolimus in animal studies and from published data from the phase I and phase II rapamycine trials. Side effects in humans include thrombocytopenia, infections and hyperlipidemia.

Currently used non-immune therapies

In addition to immunologic factors, there are many non-immunologic factors that act on the graft and cause damage. There is an increasing use of older and marginal donors [77] and it remains to be seen whether more potent immunosuppression will be of any therapeutic benefit in such situations.

Hydroxy-methyl-glutaryl Coenzyme A reductase inhibitors
Hypercholesterolemia as well as hypertriglyceridemia occur frequently after transplantation and are risk factors for chronic allograft vascular disease [78,79]. The cholesterol lowering agent pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme

A reductase inhibitor, is effective and safe in lowering serum cholesterol levels after transplantation. In a randomized trial of cardiac transplant patients, the drug lowered the cholesterol levels, the incidence of vasculopathy and it resulted in an improved survival. Somewhat surprisingly, the drug also decreased the incidence of severe cardiac rejection [80]. In a subgroup of patients, intracoronary ultrasound measurements at baseline and at one year showed less progression in maximal intimal thickness in the pravastatin group [80]. Pravastatin was also successful in lowering the mean serum cholesterol and triglyceride levels in renal transplant patients, which was also associated with a reduction in the incidence of biopsy-proven acute rejection episodes, the incidence of multiple rejection episodes and the use of anti-rejection treatments [81].

Simvastatin together with a low cholesterol diet in heart transplant patients lowered LDL-cholesterol, gave a better survival and gave and a lower incidence of graft vascular disease on angiography (16.6% versus 42.3%) [82]. The incidence of graft rejection did not differ between the groups although there was a tendency towards a lower number of serious rejections in the simvastatin group. Intracoronary ultrasound performed after 4 years in a subgroup of 27 patients showed less intimal thickening in patients with low cholesterol.

Not all statins seem to have this anti-rejection activity as fluvastatin reduced the lipid parameters significantly but it had no effect discernable on the acute rejection rate, the severity of acute rejections, the incidence of steroid resistant rejections, or the renal function [83].

Calcium channel blockers

Since the report that verapamil prevents vitamin D-induced injury of rat aortas, [84] many studies have reported on the vasculoprotective effects of calcium channel blockers (CCB). Amlodipine, a third generation long-acting CCB of the dihydropyridine class, inhibits graft vascular disease in a rat heterotopic heart transplant model. The amlodipine-treated animals had significantly less coronary artery narrowing compared with controls but this beneficial effect was entirely accounted for by inhibition of graft vasculopathy in the epicardial vessels whereas the intramyocardial arteries were not different compared with controls [85].

Using the LEW to F344 rat heart transplant model without immunosuppressive medication, Takami *et al.* investigated the efficacy of diltiazem [86]. After 4 months,

the extent of vasculopathy or graft survival was not different between the treated and non-treated group. Whereas no difference in either baseline coronary artery resistance or response to acetylcholine was found, a significantly greater vasodilatory response to nitroglycerin was observed in the diltiazem-treated group.

In a rat model of chronic kidney graft rejection in which the recipient received transiently CsA, the CCB lacidipine gave a non-significant improvement in renal function and graft survival and histopathological studies showed numerically less vascular lesions in the lacidipine-treated group but the difference was not significant [87].

MacDonald *et al.* randomized 60 heart transplant patients to receive diltiazem or not; the 2 year actuarial survival was 87% in the diltiazem group compared with 83% in the control group. The prevalence of graft vasculopathy was 17% in the diltiazem group and 32% in the control group [88]. An intravascular ultrasound study from New Orleans investigated 32 consecutive patients who were treated with either a CCB, an ACE-inhibitor or a combination, whereas a control group did not receive any of these drugs. At 1 year, coronary artery intimal thickness was significantly greater in the untreated control group compared with the groups that received treatment with either a CCB or an ACE-inhibitor [89].

The Stanford group conducted an open-label, prospective randomized study comparing diltiazem with a treatment regimen that contained no CCB [90]. Fifty-two patients received diltiazem and 54 patients received no CCB. In the no CCB group, the average coronary artery diameter decreased from 2.41 ± 0.27 mm at baseline to 2.19 ± 0.28 mm at one year, and to 2.22 ± 0.26 mm at two years ($p < 0.01$). In contrast, the average diameter in the diltiazem group changed little from the baseline value of 2.32 ± 0.22 mm to 2.32 ± 0.27 mm at one year and 2.36 ± 0.22 mm at two years. New angiographic evidence of graft vasculopathy developed in 14 patients not given CCB compared with 5 diltiazem-treated patients ($p = 0.082$). Patient survival at two years was not different between the groups but the treatment effect became significant with extension of the study and longer follow-up [90]. Survival, retransplantation, and angiographically visible accelerated graft vasculopathy three years after transplantation was evident in 82% of diltiazem and 62% of no CCB patients ($p = 0.01$); 5 years after transplantation, these combined events were absent in 56% of the diltiazem group and 30% in the no CCB group ($p = 0.004$). Thus, whereas the animal data regarding the efficacy of CCB to prevent graft vasculopathy

are not very strong, this study, together with other less robust studies, support the hypothesis that CCB are beneficial to prevent vasculopathy. The clinical benefit of diltiazem could have resulted from the drug's effect on CsA pharmacokinetics leading to altered blood levels [90,91] which in turn could have provided better protection against immune-mediated graft damage.
The use of the CCB isradipine in kidney transplantation was investigated in a multi-center study in the Netherlands. In a study of 210 patients it was shown that in the isradipine group renal function was better at 3 and 12 months compared with the placebo group but there were no differences in delayed graft function or acute rejection [92]. Whether the better function in the isradipine group reflects a better structure or it has to do with renal hemodynamics associated with its use is unknown. No information is available on its long-term benefit in kidney transplantation.

Angiotensin-converting enzyme inhibitors

Experimental studies have shown the involvement of the renin-angiotensin system in atherosclerotic vessel wall remodeling [93]. Angiotensin influences endothelial and smooth muscle cell migration [94], cytokine expression and extra-cellular matrix metabolism [95]. Angiotensin-converting enzyme inhibitors (ACE-inhibitors) inhibit neointimal proliferation of carotid arteries following balloon injury, very likely through inhibition of angiotensin II generation [96,97]. In a rat aortic transplant model an ACE-inhibitor decreases graft intimal thickening [98].
The efficacy of the ACE-inhibitor cilazapril to inhibit vasculopathy was investigated in a rat heterotopic heart graft model [99]. Graft vasculopathy was more extensive in allogeneic grafts removed from recipients that had not received CsA compared with syngeneic grafts whereas CsA treatment increased the extent of graft vasculopathy in syngeneic grafts. Cilazapril had no effect on the number of vessels affected but it decreased the degree of intimal thickness, suggesting that ACE-inhibitors do not affect the initiating mechanism but influence the intimal proliferation.
Kobayashi *et al*. examined the efficacy of captopril in the same animal model. LEW hearts were transplanted into non-immunosuppressed F344 recipients and followed for graft survival and histopathology [100]. Graft survival was better in the captopril-treated group and histopathology at 3 and 6 months showed less cellular infiltration and less perivascular edema. Whereas most graft vessels in the control group showed moderate to severe myointimal proliferation with significant luminal stenosis, most

epicardial and intramyocardial vessels in the captopril-treated group showed mild smooth muscle cell proliferation and the luminal patency was maintained [100].
Both captopril and the angiotensin II type I receptor blocker TCV-116 showed a beneficial effect in the DBA/2 to B10.D2 mouse cardiac allograft model, a combination that differs in background genes only and allows long-term graft survival in most recipients without the use of immunosuppressive drugs. Both captopril and TCV-116 tended to improve day 70 graft survival and ameliorated intimal thickening, perivascular and interstitial fibrosis [101].
The ACE-inhibitor cilazapril and an angiotensin II receptor blocker were tested in a rat kidney graft model of chronic rejection and a significant beneficial effect was found on graft survival, degree of proteinuria and extent of glomerulosclerosis and graft vessel disease. Although the combination of reserpine, hydrochlorothiazide and hydralazine gave a similar degree of protection, the ACE-inhibitor and the angiotensin II blocker gave a more robust protection against glomerulosclerosis and intimal proliferation [102]. Other investigators have confirmed the beneficial effect of angiotensin receptor blockers in this model [103]. Thus, in experimental models of aortic, heart and kidney graft rejection, ACE-inhibitors or angiotensin receptor blockers administered from the day of transplantation inhibit graft vascular disease. Recently, data were published that show that treatment with an ACE-inhibitor for more than 20 weeks prevents glomerulosclerosis but gave a massive increment in vascular intimal hyperplasia [103].
A retrospective clinical study supports the hypothesis that angiotensin converting enzyme inhibitors are beneficial in chronic allograft nephropathy [104], but controlled clinical trials are needed. In a small study losartan was administered to proteinuric renal transplant patients [105] and it decreased the proteinuria, but it is not known whether it affected the rate of decline of the renal function.

Experimental immune suppressive drugs

15-Deoxyspergualin

15-Deoxyspergualin (DSG) is an immunosuppressive molecule that was initially developed as an anti-tumor agent. Its main target was thought to inhibit monocyte/macrophage function. DSG inhibits lysosomal enzyme release, superoxide

production and class II induction on monocytes in response to immunologic stimuli. Later evidence has shown that DSG also inhibits the production of a T cell-derived factor that activates macrophages. DSG seems to have little effect on proliferating B-lymphocytes, but it suppresses selectively the early differentiation process of human B-lymphocytes [106].

In a rat aortic allograft model of graft atherosclerosis it reduces the adventitia inflammation, the media necrosis and the intimal thickening [107] whereas it has no inhibitory effect on smooth muscle proliferation *in-vitr*o or in the carotic denudation model *in-vivo*. Thus, the inhibitory effect of the drug seems to work via suppression of the immune/inflammatory response rather than via a direct antiproliferative effect on smooth muscle cells. In rats it has a small therapeutic window and there are no clinical data on its effectiveness in the clinical situation.

Vitamin D analogs

An active form of vitamin D, 1a,25-dihydroxycholecaliciferol (1a,25-(OH)2D3) has immunoregulatory properties and modifies the course of experimental autoimmune encephalomyelitis, lupus and diabetes. However, treatment of patients with 1a,25-dihydroxycholecaliciferol in doses required for immunosuppression is impossible because of its hypercalcemic effect. Therefore, vitamin D analogs have been developed that are immunosuppressive but give less hypercalcaemia. Some of these analogs are highly immunosuppressive *in-vitro* and *in-vivo*. One of these analogs, MC 1288 (20-epi-1a,25-(OH)2D3) differs from 1a,25-(OH)2D3 by its stereochemistry in the carbon 20 position but binds to the vitamin D receptor with the same affinity as 1a,25-(OH)2D3. MC1288 is a potent inhibitor of T lymphocyte activation and it prolongs the survival of both cardiac and small bowel allografts [108]. In a rat aortic allograft model, MC 1288 administration inhibited adventitia inflammation and the intima thickening [109]. No data are available on its clinical effectiveness.

Soluble complement receptor 1

Of endogenous complement regulatory proteins, complement receptor type 1 (CR1) has the best inhibitory potential to block C3 and C5 convertases. Recombinant soluble receptor type 1 (sCR1), which has a complement inhibitory and anti-inflammatory activity, prolongs graft survival in models of hyperacute allo- and xenograft rejection. In rat model of primary renal allograft rejection, sCR1 treatments partially inhibited

vascular injury and leukocyte infiltration [110]. Although sCR1 has not been tested in heart or kidney models of chronic rejection, it has a beneficial effect in a tracheal model of lung transplantation in the rat [111].

FTY720

FTY720 induces apoptosis in lymphocytes and changes in lymphocyte homing, resulting in a profound reduction in the number of peripheral lymphocytes. In various allograft models, FTY720 prolongs allograft survival, particularly when combined with low doses of other immunosuppressants. No data are available regarding its efficacy in the prevention of chronic rejection.

Experimental non-immune drugs

9-(1,3-Dihydroxy-2-propoxymethyl) guanine

Several lines of evidence exists that cytomegalovirus infection (CMV) can act as an accelerating factor in the development of cardiac allograft vasculopathy. Treatment with 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG) (ganciclovir) significantly reduced intimal thickening in rat CMV-infected and CsA-treated animals and the number of vessels affected was reduced. The drug is also effective to inhibit the enhancing effect of CMV infection on aortic allograft atherosclerosis when given prophylactically, whereas DHPG treatment initiated during the infection partly inhibited the development of intimal lesions [112]. Ganciclovir prophylaxis treatment delays the onset of chronic rejection in heart-lung and lung transplant patients [113].

Superoxide dismutase

Prolonged cold ischemia time and the generation of free oxygen radicals have been suggested to be risk factors of chronic rejection. Ischemic damage has been shown to lead to vascular changes in syngeneic rat aortic transplants [114] and prolonged ischemia enhances chronic rejection and shortens graft survival [115]. Hypoxia causes an accumulation of free oxygen radicals and during reperfusion toxic oxygen metabolites such as superoxide anion are formed. The generation of free radicals during reperfusion is a likely mechanism leading to endothelial damage and smooth muscle replication in the arteries.

Superoxide dismutase is an enzyme that catalyses dismutation from a superoxide anion into hydrogen peroxide and thus renders it biologically less harmful. The role of intravenous recombinant human superoxide dismutase administration in preventing ischemia-induced reperfusion injury and improving long-term outcome has been demonstrated in experimental studies and in clinical trials [116]. In a rat aortic transplant model of graft atherosclerosis induced by prolonged ischemia of syngeneic grafts, no therapeutic benefit of recombinant SOD could be established [117]. This drug is currently not commercially available.

Endothelin receptor blockers

Endothelin (ET)-1 is a vasoconstrictor with strong mitogenic and proinflammatory properties. Mice that express the human ET-1 transgene develop chronic renal failure characterized by severe glomerulosclerosis and interstitial fibrosis without changes in systemic blood pressure, changes that resemble chronic rejection. Rats with chronic rejection show an increase in ET mRNA and ET protein content [118-121]. In a chronic kidney graft rejection model in the rat, treatment with the ET-A receptor antagonist LU135252 resulted in a significant improvement in survival after 24 weeks. Creatinine clearance was higher in animals treated with the selective ET-A receptor antagonist. LU135252 had no influence on blood pressure and proteinuria [122].

Insulin-like Growth Factor analog

A myriad of smooth muscle growth promoting substances are produced in the vascular wall following injury, including interleukin-1, eicosanoids, and several peptide factors like insulin-like (IGF-1) growth factor. A synthetic D-aminoacid peptide that structurally resembles the D-domain of IGF-1 has been tested in a balloon injury model where it reduces the intimal proliferation by 60 – 70% [123].

Tyrosine kinase inhibitors

CGP 53716 is a selective PDGF-receptor protein tyrosine kinase inhibitor that reduces the incidence and intensity of atherosclerotic lesions in rat cardiac and aortic allograft recipients [124]. In primary rat smooth muscle cell cultures, a dose-dependent inhibition of PDGF-AA and -BB induced migration and thymidine incorporation of smooth muscle cells was seen [125]. When rat coronary smooth muscle cells

were stimulated *in-vitro* with PDGF-AA or -BB in the presence of interleukin-1ß or TNF-α, CGP 53716 significantly inhibited only AA-ligand-induced but not BB-ligand-induced replication. Concomitantly, interleukin-1ß or TNF-α stimulation specifically upregulated the expression of PDGF-Ra mRNA but not of other ligand or receptor genes in cultured smooth muscle cells. Thus, a PDGF-AA/Ra-dependent cycle is induced in the generation of allograft atherosclerosis that may be inhibited by blocking of signaling downstream of PDGF-R [124].

Low molecular weight heparin (LMWH)

Heparin consists of components with molecular weights ranging from 3000 to 40,000. It is well known for its anticoagulant properties but it also possesses the ability to enhance other immunosuppressants, inhibit smooth muscle cell proliferation and interact with the endothelium [126]. Heparin mediates the conversion of L-arginine to endothelium-derived NO, which regulates endothelin-1 activity through cyclic GMP in the endothelium. It may regulate the repair of vessels and low dose heparin is associated with the prolongation of allograft survival time and a reduction of chronic rejection. Low molecular weight heparin (LMWH) is depolymerized heparin, has a molecular weight of approximately 6000 and a longer half-life. It has less antithrombin activity than heparin and it reduces bleeding. LMWH has a beneficial effect on graft atherosclerosis in cardiac and aorta transplant models [127,128].

Estrogens

The vasculoprotective effect of estrogen was first shown in population studies in women, where estrogen replacement therapy demonstrated a protective effect on atherosclerotic vascular disease, and later confirmed in ovariectomized monkeys. Estrogen inhibits intimal thickening after mechanical carotid balloon injury in rabbits, rats, and mice as well as after allogeneic transplantation [129]. *In-vitro*, it has been demonstrated that estrogen inhibits migration and replication of vascular smooth muscle cells. Estrogen-based drug therapy in cardiovascular diseases has been difficult because it has not been possible to separate the desired vasculoprotective effect from the unwanted effects of the hormone to the reproductive system. However, following endothelial denudation of rat carotid artery, the mRNA of the novel estrogen receptor (ERb) in the smooth muscle cells in the media and neointima increases > 40-fold whereas the classical ERa mRNA remains low [130]. Treatment of ovariectomized

female rats with the isoflavone phytoestrogen genistein, which has a 20-fold higher binding affinity to ERß than to ERa, or with 17ß-estradiol, which does not differentiate between the two receptors, provides similar dose-dependent vasculoprotective effect in the rat carotid injury model. On the other hand only treatment with 17ß-estradiol, but not with genistein, is accompanied with a dose-dependent uterotrophic effect. These data suggest that preferential targeting to ERß will provide vasculoprotective estrogen analogs devoid of effects to the reproductive system [130].

Somatostatin analogues

Somatostatin (SST) is a neurohormone that is widely produced in the body and acts systematically via the circulation as well as locally to inhibit cell proliferation and the secretion of various hormones, growth factors and neurotransmitter substances. SST and its metabolically stable synthetic analogs like the octapeptides SMA201-995 (octreotide), and BIM23014 (lanreotide, angiopeptin) exert a number of vascular effects. Lanreotide and angiopeptide have been tested successfully in experimental transplantation models [128,131,132]. To optimize the vasculoprotective effect of SST, the pattern of expression of all five SST receptor (SSTR) subtypes following vascular injury was examined in a rat thoracic aorta model [133]. In this model, all five SSTRs were expressed and displayed a time-dependent, subtype-selective response to endothelial denudation. mRNA for SSTR1 and 2 increased acutely on days 3 and 7, coincident with smooth muscle proliferation, and declined to basal levels by day 14. SSTR3 and 4 displayed patterns with delayed, more gradual increase in mRNA beginning at days 3 − 7 and continued to increase thereafter. SSTR5 mRNA was constitutively expressed at a low level and showed no change during the 2 weeks post injury period. The five SSTRs are predominantly localized in the smooth muscle cells. It is speculated that the SSTR1 may be the optimal subtype to target for inhibition of myointimal proliferation and SSTR 3 and 4 for migration and remodeling [133].

Conclusion

While none of the recommendations proposed to avoid chronic allograft nephropathy have been tested formally, it would seem prudent to recommend the

following strategies. First, avoid graft damage as a result of ischemia-reperfusion injury. While superoxide dismutase given at the time of surgery seems effective to prevent chronic allograft nephropathy [134], the drug does not universally prevent chronic allograft nephropathy changes [125]. Secondly, acute rejection episodes should be treated aggressively to ensure complete reversal of graft function [134]. Surveillance biopsies are perhaps useful to diagnose vascular rejections which, if diagnosed should be treated promptly [135]. It remains to be seen whether more aggressive treatment of late acute rejection episodes will decrease the incidence of chronic allograft nephropathy. The impact of anti-viral therapy on chronic allograft nephropathy is also unknown. At this stage there are also no clinical data to show that lipid- or blood pressure-lowering through medication will decrease graft loss from chronic allograft nephropathy but the lack of clinical data is no justification to ignore post-transplant hypertension and hyperlipidemia as they will very likely impact on patient cardiovascular morbidity and mortality [136,137]. Based on animal data, the use of antihypertensive drugs, especially angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, prolongs graft survival in chronic allograft nephropathy [87,102] but careful monitoring of graft function and electrolytes is necessary. Strategies to treat post-transplant hyperlipidemia have recently been reviewed [138,139]. With the current financial pressures on health care systems there is a tendency to transfer long-term follow up of renal transplant patients to peripheral health care physicians but careful long-term follow up with appropriate counseling on medication and monitoring of drug adherence should remain a high priority [140].

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Chapter 3

A compartmental pharmacokinetic model of cyclosporine and its predictive performance after bayesian estimation in kidney and simultaneous pancreas-kidney transplant recipients

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Abstract

Background: Therapeutic drug monitoring of cyclosporine (CsA) is an obvious necessity because of its unpredictable absorption and narrow therapeutic window. The use of Limited Sampling Methods (LSMs) has improved the estimation of the systemic exposure (area under the curve, AUC) compared to C_{0h} monitoring, but these equations are rigid and not reliable in patients with an abnormal absorption profile. We developed and validated a limited sampling (t = 0, 2 and 3 h) strategy, based on a compartmental population PK model for CsA after kidney transplantation alone (KTA) and simultaneous pancreas-kidney transplant (SPKT) recipients, a group of patients with unpredictable absorption kinetics.

Methods: A two-compartment model with lag-time and first order absorption was calculated using a PK software package from data of 20 KTA and SPKT recipients and validated prospectively in 20 KTA and 20 SPKT recipients. Calculated population PK parameters were individualized for each of the remaining 40 patients based on their CsA dosing and on one or a combination of measured CsA blood concentrations using the Bayesian fitting method. AUCs were calculated from individualized PK parameters. AUCs were also calculated using previously published LSMs. Relationships between AUCs calculated by the models and the "golden standard" AUC (trapezoidal rule) were investigated by Pearson correlation test.

Results and conclusion: A population two-compartment model is presented to reliably estimate the CsA AUC in KTA and SPKT recipients. The performance of the model to estimate the AUC is comparable to the performance of two published LSMs in KTA patients, but markedly better in SPKT patients. Combined with Bayesian fitting, the model is very flexible since sampling times are not rigid and can be varied as long as dosing and sampling times are recorded accurately. The model has already proven to be clinically useful, and is currently used to further investigate CsA in an integrated pharmacokinetic/pharmacodynamic model.

Introduction

Since the introduction of cyclosporine A (CsA), it was apparent that defining the appropriate dose was problematic because of its poor and unpredictable absorption and narrow therapeutic window. Therapeutic drug monitoring (TDM) is therefore an obvious necessity and, by convention, dosing adjustments became targeted to trough levels (C_{0h}), i.e., the CsA blood concentration immediately before the next scheduled dose. Although the correlation between C_0 level and its efficacy to prevent acute rejection or chronic nephrotoxicity is poor, this parameter is still widely used to guide CsA dosing after transplantation [1,2]. Despite maintaining CsA trough levels within the therapeutic range, substantial groups of patients experience either acute rejection episodes or nephrotoxicity [3,4]. The CsA area under the drug concentration versus time curve (AUC) seems a better measure of systemic drug exposure and efficacy to prevent acute rejection episodes [5], but AUC monitoring has not gained popularity, largely because of the inconvenience of multiple blood samplings over a 12-hour period and cost considerations.

Because AUC reflects systemic drug exposure, several groups have used sparse-sampling algorithms (Limited Sampling Models, LSMs) as a way of predicting AUCs without the need for large numbers of blood-level measurements [6,7]. Exposure during the first 4 hours (AUC_{0-4}), the area of greatest inter- and intrapatient variability, is a reliable estimate of total drug exposure throughout the dosing interval (AUC_{0-12}) [8]. Recently dosing based on a single drug level measurement, i.e. C_2, within this absorption phase has been associated with improved results after de novo heart, liver and kidney transplantation [9]. It should be stressed, however, that estimation of the true AUC will always be more reliable when more samples are taken. This may be particularly true, or even mandatory, in patients with an unpredictable absorption profile such as diabetic recipients [10].

Compared with LSMs, a compartmental population pharmacokinetic model for CsA in renal transplant recipients combined with the maximum a posteriori Bayesian fitting method offers in clinical practice the important advantage of flexibility [11,12]. The exact sampling time is no longer an issue as long as the exact times of drug administration and blood drawing are recorded. In the present study we describe the development and prospective validation of such a model in non-diabetic patients who received a kidney transplant alone (KTA) and simultaneous pancreas kidney transplant (SPKT) recipients.

Materials and methods

Patients

Sixty (34 KTA and 28 SPKT) transplant recipients (38 male; 22 female) treated with CsA-based immunosuppression were studied. Thirty patients were early (< 3 months) post transplantation, while the other thirty had been transplanted at least three months before the present study. All patients received the microemulsion formulation of CsA (Neoral, Novartis Pharmaceuticals, Basel, Switzerland) with prior dosing based on trough levels. Patients who used drugs known to interact with CsA pharmacokinetics were excluded. Patient characteristics are listed in table 1. Prior to and after the morning dose of CsA, blood was taken at t = 0, 1, 2, 3, 4, 6, 8 and 12h. Blood was drawn using an indwelling catheter and was collected in a vacutainer containing EDTA. Blood was stored at 4°C until analysis, usually the same day.

Table 1: Patient characteristics

	Model building (n = 20)	**KTA recipients (n = 20)**	**SPKT recipients (n = 20)**
Male/female	13 / 7	12 / 8	13 / 7
Weight (kg)	70 ± 15	70 ± 13	75 ± 16
Length (cm)	173 ± 9	169 ± 11	175 ± 9
GFR (ml/min)*	46 ± 19	52 ± 18	59 ± 25
Cause of renal failure			
Cystic kidney disease	3	5	0
Hypertension	3	3	0
Glomerulonephritis	3	7	0
Unknown, other	3	5	0
Diabetes Mellitus (DM)	8	0	20
KTA/SPKT	12 / 8	20 / 0	0 / 20

* Cockcroft and Gault

Pharmacokinetics

Using the Kinpop module of the pharmacokinetic software package MW/Pharm version 3.33 (Mediware, Groningen, the Netherlands), a population 2-compartment model with a lag-time and first order absorption pharmacokinetics was calculated from the CsA dosing and the blood concentration values of the first 20 patients (12 KTA and

8 SPKT recipients). This program uses an iterative two-stage Bayesian procedure, and calculates means, medians and standard deviations of the pharmacokinetic parameters [11]. During the iterative two-stage Bayesian procedure pharmacokinetic parameters were set to be distributed log-normally, and bioavailability was fixed at 0.5.

The calculated mean population pharmacokinetic parameters were individualized for each of the remaining 40 patients (20 non-diabetic KTA recipients and 20 SPKT recipients) based on their CsA dosing and one or a combination of measured blood concentrations (0h; 1h; 2h; 3h; 4h; 0+1h; 0+2h; 0+3h; 0+4h; 2+4h; 0+1+2h; 0+1+3h; 0+2+3h; 1+2+3h; 0+1+2+3h; 0+1+2+4h; 0+1+2+3+4+6+8+12h) according to the maximum a posteriori (MAP) Bayesian fitting method [12], using the MW/Pharm computer program. By means of MAP Bayesian fitting any available information, i.e. 'a priori' population parameters, drug dosage regimen, and measured blood concentrations, can be used to estimate the 'a posteriori' pharmacokinetic parameters of the individual patients. These 'a posteriori' pharmacokinetic parameters of the individual patient are the maximum likelihood estimates obtained by MAP Bayesian fitting, minimizing the deviations of measured and predicted concentrations, and of population pharmacokinetic parameters and pharmacokinetic parameters of the individual patient [12]. This approach is very flexible and it ensures an optimal use of available information, both from a population and from the individual patient. From the individualized pharmacokinetic parameters the area under the CsA blood concentration time curve ($AUC_{0\text{-}12h}$) was calculated for each combination of measured blood concentrations.

The $AUC_{0\text{-}12h}$ of the 40 patients was also calculated using the 2-point limited sampling strategy as described by Wacke *et al.* [7]: $AUC_{0\text{-}12h} = 343.57 + 1.22*C1h + 4.62*C3h$; and using the 2-point limited sampling strategy as described by Amante *et al.* [6]: $AUC_{0\text{-}12h} = 195.8 + 2.4*C2h + 7.7*C6h$.

As the golden standard the $AUC_{0\text{-}12h}$ of the remaining 40 patients was calculated from all CsA blood concentrations using the trapezoidal rule (Kinfit module, MW/Pharm). Using the trapezoidal rule the $AUC_{0\text{-}4h}$ was also determined.

Drug analysis

Whole blood concentrations of CsA in the first 20 patients were determined by Radio ImmunoAssay RIA (Cyclotrac, IncStar, Stillwater, MN, USA). Because of a change

of equipment, whole blood concentrations of CsA in the remaining 40 patients were determined by Fluorescence Polarization Immuno Assay FPIA (Axsym, Abbott Diagnostics, Abbott Park, IL, USA).

Statistics

Statistical analysis was performed three times. First the KTA recipient group was investigated. Secondly the SPKT recipient group was investigated. Finally these two groups were analyzed together.

The AUCs calculated by the different methods were compared to the golden standard AUC by linear regression analysis and Pearson correlation coefficient. Calculations were carried out by means of the SPSS software (version 9.0). Predictive performance of the different methods was also investigated by calculating the prediction precision and bias according to Sheiner and Beal [13]. Prediction bias was calculated as the mean prediction error (MPE), i.e. the mean of differences between the AUC according to the different methods and the golden standard AUC. Prediction precision was calculated as the mean absolute prediction error (MAPE), i.e. the mean of the absolute differences between the AUC according to the several different methods and the golden standard AUC. Smaller values for MPE and MAPE indicate less bias and greater precision.

The actual description of blood concentration in time by the model was investigated prospectively by calculating residuals between measured and predicted CsA blood concentrations in the 40 patients. The concentrations were predicted for each patient using either the whole data set or only limited sampling at t = 0, 2 and 3h after drug administration.

Results

CsA pharmacokinetics was adequately described by a two-compartment model with a lag-time. Pharmacokinetic parameters as calculated by Kinpop are listed in table 2. A representative example of Bayesian fitting based on measured CsA concentrations at t = 0, 2 and 3h is shown in figure 1.

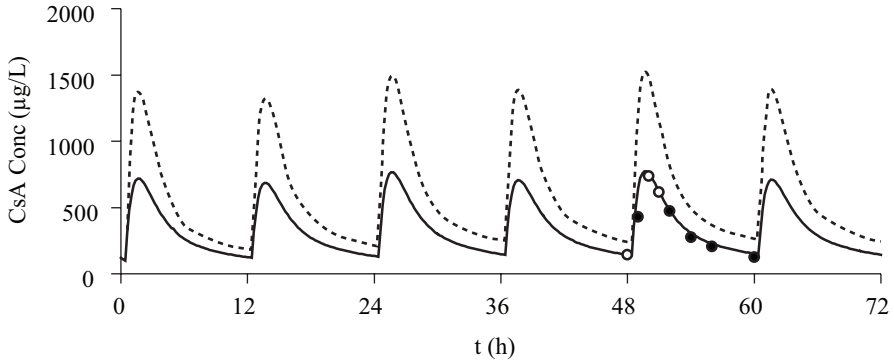

Figure 1: CsA blood concentration time curve according to the population-model (dashed line), the actual measured CsA blood concentrations at t = 0, 2, 3 (open circles), 1, 4, 6, 8 and 12h (closed circles), and the CsA blood concentration time curve according to the model (solid line) after fitting the population parameters to the measured concentrations at t = 0, 2 and 3h after administration, in a 45 year old female 1 year after renal transplantation (Dose = 225/200 mg).

Table 2: Cyclosporine pharmacokinetics in renal transplant recipients (n = 20)

Parameter	**Mean (SD)**
t_{lag} (h)	0.576 (0.485)
F	0.5 (fixed)
Ka (h^{-1})	0.741 (0.273)
V_1 (l/kg)	0.491 (0.135)
K_{elm} (h^{-1})	0.559 (0.045)
K_{12} (h^{-1})	0.567 (0.215)
K_{21} (h^{-1})	0.149 (0.114)

In figure 2, 3 and 4 the predictive performance in both KTA and SPKT recipients is shown of several methods of CsA TDM. In figure 2a the relationship is plotted between trough levels and the $AUC_{0\text{-}12h}$ calculated using the trapezium method. Figure 2b and 2c, which show the relationships between systemic exposure and the $AUC_{0\text{-}12h}$ calculated according to the compartment model with blood concentration time points taken at 0 and 2h (b), and 0, 2 and 3h (c), illustrate that using the compartmental model the $AUC_{0\text{-}12h}$ is estimated well in both patient groups, and that the predictive performance improves when more concentration time points are used. Figure 3a and b show the relationship between the $AUC_{0\text{-}12h}$ calculated with the trapezium rule and the AUC according to the two published Limited Sampling Models [6,7]. The

variation of the actual AUC is less when estimating the AUC using any of the models, when compared with the estimation of the systemic exposure from the trough levels. Predictive performance differs between the models, which is predominantly caused by values obtained in SPKT recipients. In figure 4a and b the relationships are shown between C2h and $AUC_{0\text{-}4h}$ and between C2h and $AUC_{0\text{-}12h}$ respectively. In figure 4c the relationship between $AUC_{0\text{-}4h}$ and $AUC_{0\text{-}12h}$ calculated by the trapezoidal rule is shown. These figures illustrate that absorption profiling has a good predictive performance in KTA recipients, but it is significantly worse in SPKT recipients.

Bias and precision of the different models are listed in table 3 and 4. In general, when using the compartmental model, bias as well as precision improve when the Bayesian estimations are made based on more blood concentration data with a bias of –2.2% and precision of 2.6% when based on all data, indicating that the model tends to slightly underestimate the AUC as determined by the trapezoidal rule. Bias and precision of the Limited Sampling Models are comparable to bias and precision of the compartmental model, if the latter is used with limited sampling at two or more blood concentration data.

In KTA recipients (table 3) bias and precision of the limited sampling models are comparable to bias and precision of the compartmental model, if the latter is used with limited sampling at two or more blood concentration data. The best relationships were found with the blood concentration data 0+2h, 0+1+3h and 0+1+2+3h, when only data within 3 hours after drug administration are taken into consideration.

In SPKT recipients (table 4) it appears that the first limited sampling model, LSM [1,3], is less useful to estimate the $AUC_{0\text{-}12h}$. Bias and precision and correlation of the compartmental model are also worse in several combinations of blood concentration data compared to KTA recipients. The best relationships are found with the blood concentration data 0+3h, 0+2+3h, an 0+1+2+3h when only data within 3 hours after drug administration are taken into consideration. Bias and precision of these combinations are good and comparable to the good performance of the second LSM [2,6].

When the performance of the models is validated on both KTA and SPKT recipients together (data not shown), the combination of blood concentration data that best describe the systemic exposure are 0+2, 0+2+3h and 0+1+2+3h. The performance of LSM [2,6] is very well, the performance of LSM [1,3] seems less useful.

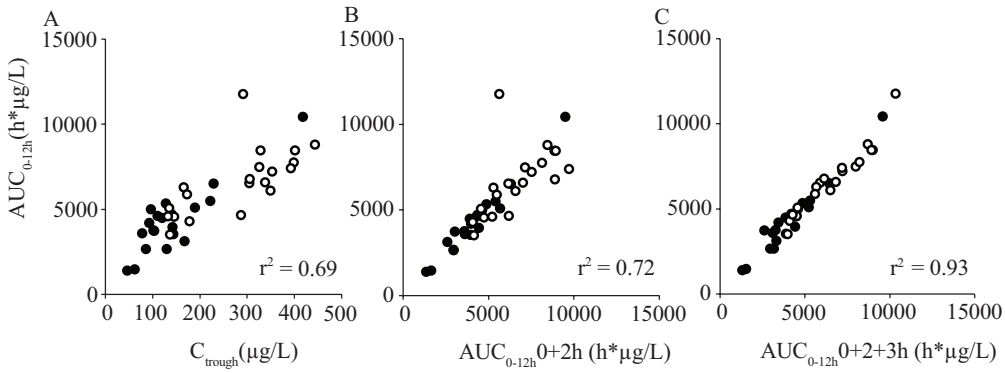

Figure 2: A) Relationship between C_{trough} and AUC calculated with the trapezium rule (golden standard); Relationship between the AUC calculated according to the compartment model with blood concentration time points taken at 0 and 2h (B) and 0, 2 and 3h (C) and the golden standard AUC. All relationships are shown for 20 KTA (closed circles) and 20 SPKT (open circles) recipients. The r^2 is based on all 40 patients.

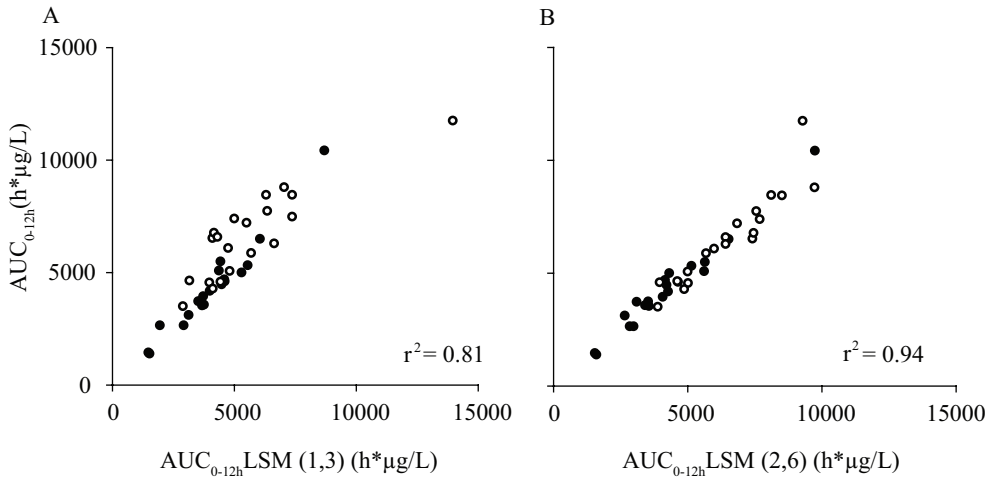

Figure 3: A) Relationship between the AUC according to the LSM with sampling points at 1 and 3h [7] and the golden standard AUC; B) Relationship between the AUC according to the LSM with sampling points at 2 and 6h [6] and the golden standard AUC. All relationships are shown for 20 KTA (closed circles) and 20 SPKT (solid circles) recipients. The r^2 is based on all 40 patients.

Table 3: Bias, precision (%) and Pearson correlation coefficients (r) of different combinations of blood sampling time-points used with the population model, to estimate the CsA AUC, compared with the AUC calculated according to the trapezium rule in 20 KTA recipients.

Time points blood sampling (h)	MPE (%)	(95% CI)	MAPE (%)	(95% CI)	r^2
0 (trough)	–	–	–	–	0.78
0 (with model)	−9	(−21; 4)	23	(17; 30)	0.75
2	−8	(−17; 1)	16	(11; 22)	0.80
3	−13	(−22; −4)	19	(13; 25)	0.82
0,2	−1	(−6; 4)	9	(6; 11)	0.96
0,3	−9	(−16; −1)	15	(11; 20)	0.90
0,1,2	3	(−1; 7)	8	(5; 10)	0.97
0,1,3	−1	(−6; 3)	6	(3; 9)	0.98
0,2,3	−3	(−9; 2)	11	(7; 14)	0.96
0,1,2,3	0	(−3; 3)	5	(3; 7)	0.99
0,1,2,3,4	−1	(−4; 2)	5	(3; 6)	0.99
0,1,2,3,4,6,12	−3	(−4; −1)	3	(2; 4)	1.00
LSM (1,3)	−3	(−8; 1)	7	(4; 11)	0.95
LSM (2,6)	−1	(−5; 3)	7	(5; 10)	0.97

CI = Confidence Interval

Table 4: Bias, precision (%) and Pearson correlation coefficients (r) of different combinations of blood sampling time-points used with the population model, to estimate the CsA AUC, compared with the AUC calculated according to the trapezium rule in 20 SPKT recipients.

Time points blood sampling (h)	MPE (%)	(95% CI)	MAPE (%)	(95% CI)	r^2
0 (trough)	–	–	–	–	0.46
0 (with model)	15	(4; 27)	25	(19; 31)	0.38
2	−13	(−24; −1)	21	(13; 28)	0.13
3	−6	(−12−0)	11	(8; 15)	0.83
0,2	3	(−5; 12)	13	(7; 19)	0.35
0,3	3	(−1; 7)	9	(7; 11)	0.87
0,1,2	−2	(−11; 6)	12	(5; 19)	0.50
0,1,3	−2	(−6; 1)	6	(3; 9)	0.88
0,2,3	−1	(−4; 2)	6	(4; 7)	0.93
0,1,2,3	−2	(−5; 0)	5	(3; 7)	0.94
0,1,2,3,4	0	(−3; 3)	5	(3; 8)	0.86
0,1,2,3,4,6,12	−1	(−2; −1)	2	(1; 2)	0.99
LSM (1,3)	−15	(−23; −8)	18	(12; 24)	0.75
LSM (2,6)	−1	(−3; 5)	6	(4; 9)	0.87

CI = Confidence Interval

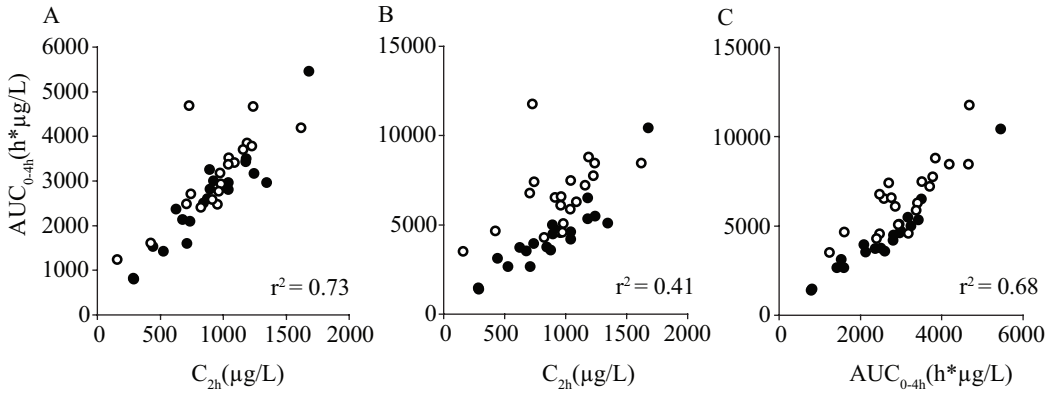

Figure 4: A) Relationship between C_{2h} and the $AUC_{0\text{-}4h}$; B) Relationship between C_{2h} and the $AUC_{0\text{-}12h}$; C) Relationship between the $AUC_{0\text{-}4h}$ and the $AUC_{0\text{-}12h}$. All relationships are shown 20 KTA (closed circles) and 20 SPKT (solid circles) recipients. The r^2 is based on all 40 patients.

Figure 5 displays the residuals of the model as a function of time after the MAP Bayesian fitting procedure based on all concentrations and based on limited sampling at t = 0, 2 and 3h in the 20 KTA and 20 SPKT recipients. In both groups the concentrations are scattered around the x-axis indicating a good description of the blood concentrations by the model. The largest deviations from the measured concentrations are around t = 1h. The peak concentration measured is usually reached at 1 or 2 h. Limited sampling at t = 0, 2 and 3 h causes somewhat more deviation from the measured concentrations. However, the residuals remain more or less distributed around 0 µg/L, especially at t = 1 and 2 h. The residuals at the other time points do not appear to change during limited sampling at 0, 2 and 3 h. The residuals in the SPKT recipients are also scattered around the x-axis. However, the pattern is somewhat different, indicating different pharmacokinetics in this group.

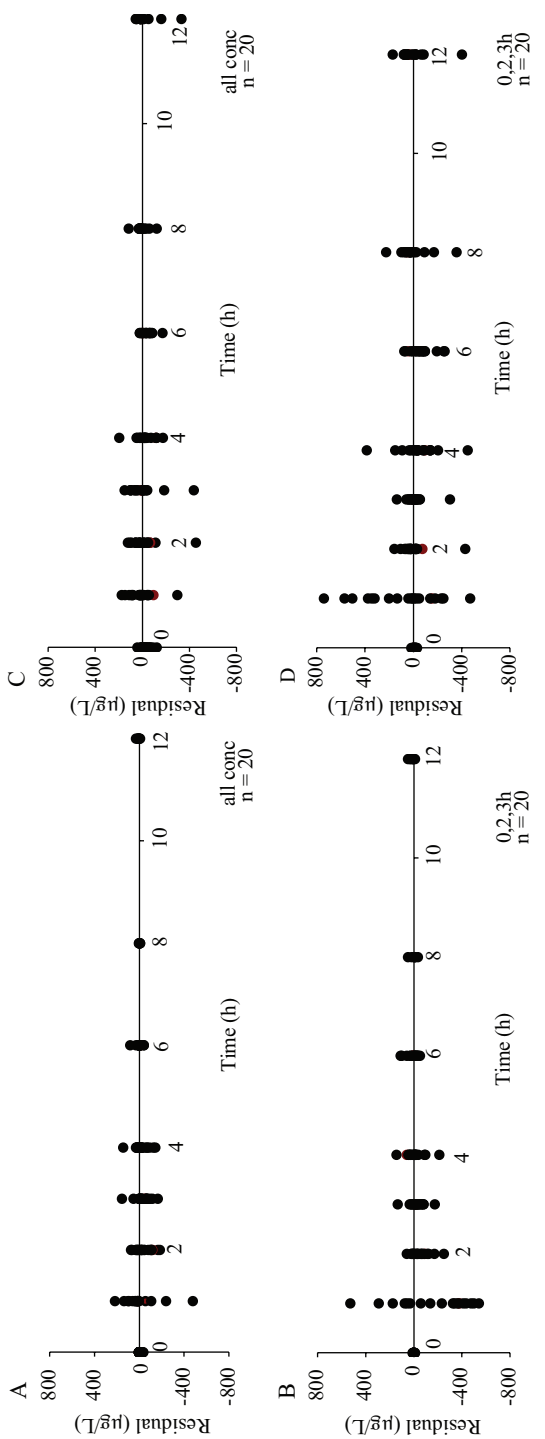

Figure 5: Distribution of residuals versus time in 20 KTA (A and B) and 20 SPKT (C and D) recipients. The concentrations predicted are based on either the whole data set (A and C) or a limited sampling data set 0, 2 and 3 h (B and D).

Discussion

At present, calcineurin inhibitors still constitute the cornerstone of immunosuppressive regimens for the prevention of allograft rejection in renal transplant recipients. Recent data have indicated that the long-term outcome of both cadaveric and living donor renal allografts has improved in the past decade, especially if acute rejection episodes could be prevented [14]. In healthy volunteers and stable renal transplant recipients, the CsA microemulsion formulation under the name Neoral has increased dose linearity with AUC and has resulted in a better correlation between C_{0h} and $AUC_{0\text{-}12h}$ than cyclosporine gelcaps or Sandimmune [15]. However, in de novo transplant recipients, the inter-patient variability of pharmacokinetic parameters is not different between these formulations during the first postoperative weeks, and in clinical practice, C_{0h} has an equally poor correlation with $AUC_{0\text{-}12h}$ [16]. Conversely, AUC estimates correlate with early clinical events [17], but there is little information about the usefulness of AUC monitoring to improve long-term outcome [18].

Estimating the systemic exposure to CsA with LSMs, but also with C_2, is rigid. It is not allowed to deviate too much from the predefined time points. Moreover, once a time-point is missed, a LSM formula becomes useless, since then one of the determinants in the formula is lacking. Deviations from predefined time points and missing of samples occur frequently in daily clinical practice. In contrast to the LSMs, these everyday problems are dealt with easily by a Bayesian fitting procedure. For example, the pharmacokinetic model can be used to calculate systemic exposure to CsA in a data set that consists of 0h, 1.5h and 3.4h time-points instead of 0h, 2h and 3h, or a data set that consists of only 2h and 3h time-points, illustrating the flexibility of the model, and the clinical applicability.

The performance of the current population model to estimate the $AUC_{0\text{-}12h}$ was investigated using several sampling strategies. As expected, the estimation of the $AUC_{0\text{-}12h}$ always improves when the number of sampling points is increased, as more information is introduced. However, using the time points t = 0, 2 and 3 hours post-dose with the population model and MAP Bayesian fitting, the AUC was estimated with a mean accuracy of –2.3% and a mean precision of 8.3%. This is well within accepted and clinical relevant limits.

Using individualized population pharmacokinetic models, CsA exposure in renal transplant patients can be estimated well. However, intrapatient pharmacokinetics is

known to change in the early period (6 to 8 weeks) after transplantation. The model was developed and validated in a population with varying time after transplantation. Consequently, the model is able to deal with the changes in pharmacokinetics after transplantation, mainly because this change has been incorporated into the range of the pharmacokinetic parameters. However, using the population pharmacokinetic model as described, the systemic exposure can be estimated, but actual prediction of the systemic exposure during a period of great changes in pharmacokinetics, is not possible. In order to ensure an adequate systemic exposure several approaches can be made. Either the estimation of the AUC of CsA is performed frequently in the early period after transplantation, or a PK model is used in which factors specifically influencing this change in PK are incorporated. One of those factors is probably time after transplantation. Clinically applicable pharmacokinetic models though, in which time after transplantation has been incorporated, enabling the actual prediction of systemic exposure in a period of changing pharmacokinetics of CsA, are scarce. For our own approach, in which we thus estimate the $AUC_{0\text{-}12h}$ of CsA at a certain moment, we are currently investigating still the optimal frequency of drug monitoring, both short- and long-term after transplantation. In our prospective study we use every other week estimations of the $AUC_{0\text{-}12h}$ during the first 12 weeks after transplantation, which is followed by an estimation of the $AUC_{0\text{-}12h}$ every three to six months.

The study population consisted of both patients that received kidney transplant as well as SPKT recipients. Patients with insulin-dependent diabetes mellitus often suffer from (severe) gastrointestinal dysfunction and in these patients CsA pharmacokinetics is often variable and unpredictable [10]. Inclusion of these patients may have skewed the pharmacokinetic parameters of the population model, leading to deviation of predicted from measured CsA concentrations in the KTA patients. The advantage, however, is that the model is suitable to describe CsA pharmacokinetics in the population of interest, i.e. recipients with an abnormal absorption profile. The differences in CsA pharmacokinetics between KTA and SPKT recipients were adequately detected by the compartment-model and by one of the previously described LSMs [6]. The performance of the other LSM was significantly worse [7], indicating that caution should be taken when monitoring CsA in various patient groups with defined sampling time points. This is illustrated especially by the lack of correlation between C_2 and $AUC_{0\text{-}4h}$ or $AUC_{0\text{-}12h}$ in SPKT recipients as compared

with the patients without diabetes mellitus, who received a kidney transplant. In figure 6 two clinical examples are given illustrating the above-mentioned issues and how the model deals with them.

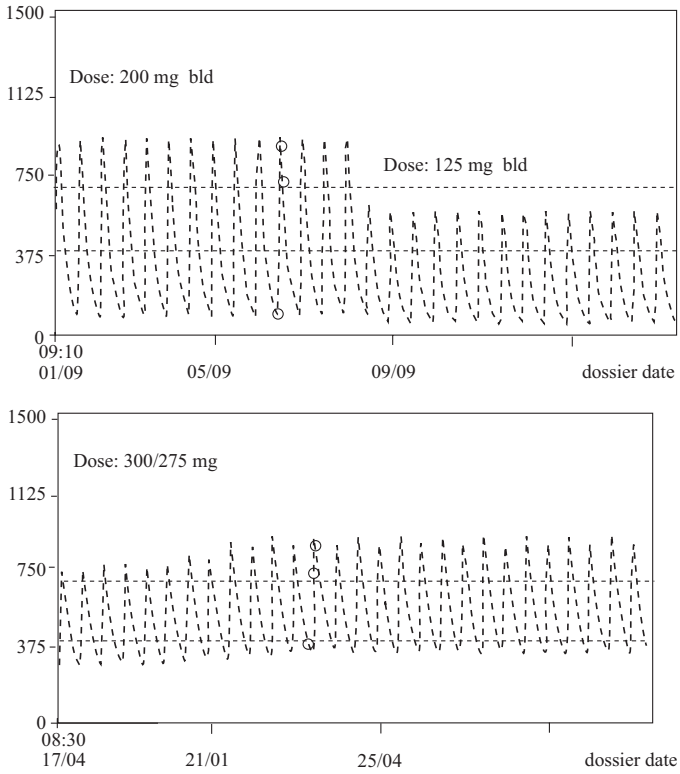

Figure 6A: CsA blood concentrations measured at 0, 2 and 3h after drug administration (open circles) and according to the model after fitting (solid line) in a 60 year old stable female KTA recipient (sCr = 70 µmol/L; Body Weight = 46 kg). C_{0h} was 97 µg/L, the $AUC_{0\text{-}12h}$ according to the model was 4596 h*µg/L, while the golden standard $AUC_{0\text{-}12h}$ was 4997 h*µg/L. The dose adjustment was made to achieve a target $AUC_{0\text{-}12h}$ of 3250 h*µg/L.

Figure 6B: CsA blood concentrations measured at 0, 2 and 3h after drug administration (open circles) and according to the model after fitting (solid line) in a 50 year old SPKT recipient (sCr = 272 µmol/L; Body Weight = 64 kg). C_{0h} was 393 µg/L, $AUC_{0\text{-}12h}$ according to the model was 7201 h*µg/L, C_{1h} was 480 µg/L, C_{2h} 744 µg/L and C_{3h} 882 µg/L. The $AUC_{0\text{-}12h}$ according to Wacke *et al.* [7] was 5004 h*µg/L, and according to Amante *et al.* [6] 7679 h*µg/L, while the golden standard $AUC_{0\text{-}12h}$ was 7391 h*µg/L.

Although the drug is metabolized to a least 25 metabolites, blood concentrations of CsA after oral administration as determined by the immunoassays, are adequately described by a two-compartment model with a lag-time and first order absorption pharmacokinetics. The model was superior to a one-compartment model, while an extra compartment did not add significantly to the description of the data. Fitting procedures by the calculation algorithm were improved by fixing the oral availability at 0.5. Absorption pharmacokinetics therefore is characterized mainly by the lag-time and the absorption rate-constant. This may also explain why deviation of the CsA concentrations predicted by the model was largest at 1 hour post-dose. A recent study by Debord et al has applied a gamma distribution model of absorption to the pharmacokinetics of cyclosporine in stable renal transplant recipients, and indeed yielded a better fit during the absorption phase compared with a classical exponential model with lag-time [19]. An underestimation of the C_{max} using the latter model, however, was not observed in the current population with scattering of the residuals around zero at both 1 and 2 hours after ingestion of the oral dose. Compared with the FPIA, the RIA method slightly overestimated the data in our laboratory. Recently, Keown *et al.* pointed out that differences between assays may have consequences for trough-level-based monitoring, while these assays performed quite comparable during the absorption phase of CsA in renal transplant recipients [9]. The presented PK model however, is flexible enough to deal with the apparent differences. The model therefore performs adequately despite the immunoassay used.

Calcineurin-inhibitor trough levels are a poor indicator of drug exposure, and drug exposure should be quantitated by means of more accurate methods. To prevent structural underimmunosuppression or graft dysfunction due to chronic calcineurin-inhibitor toxicity, a compartmental population pharmacokinetic model with maximum a posteriori Bayesian fitting method offers several advantages. The presented model is reliable and more flexible than LSMs and does not depend on exact blood sampling time points. In addition, it can be used in transplant recipients with gastrointestinal dysfunction and even offers the possibility to integrate pharmacodynamic parameters [20]. These practical and theoretical advantages argue in favor of this approach for future therapeutic drug monitoring.

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Chapter 4

AUC-guided dosing of tacrolimus prevents progressive systemic overexposure in renal transplant recipients

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Abstract

Background: Tacrolimus has a narrow therapeutic window and bioavailability is known to vary considerably between renal transplant recipients. Most centers still rely on measurement of trough levels, but there are conflicting reports on the correlation between tacrolimus trough levels and systemic exposure, as measured by the area-under-the-concentration-over-time curve ($AUC_{(0\text{-}12h)}$).

Methods: We developed and validated a 2-compartmental population-based pharmacokinetic model with Bayesian estimation of tacrolimus systemic exposure**.** Subsequently we used this model to apply prospectively AUC-guided dosing of tacrolimus in 15 consecutive renal transplant recipients. The main objective was to study intra-patient variability in the course of time.

Results: Bayesian forecasting with a two-point sampling strategy, a trough level and a second sample obtained between 2 and 4 hours post-dose, significantly improved the squared correlation with the $AUC_{(0\text{-}12h)}$ ($r^2 = 0.94$). Compared with trough level monitoring only this approach reduced the 95%-prediction interval by 50%. The Bayesian approach proved to be feasible in clinical practice and provided accurate information about systemic tacrolimus exposure in individual patients. In the AUC-guided dosing cohort the apparent clearance of tacrolimus decreased gradually over time, which was not reflected in corresponding trough levels.

Conclusions: This simple, flexible method provides the opportunity to tailor immunosuppression and should help minimize tacrolimus-related toxicity, such as nephrotoxicity and post-transplant diabetes mellitus.

Introduction

The currently available calcineurin inhibitors cyclosporine microemulsion and tacrolimus both have a narrow therapeutic window, which makes regular drug monitoring necessary. Most transplantation centers adjust the dose of these drugs to reach or maintain a defined trough level range. Especially for cyclosporine several studies have already documented that estimates of systemic drug exposure, and the absorption profile in particular, represented by the area-under-the-concentration over time curve (AUC), correlate better with clinical events (acute rejections, nephrotoxicity) as compared with trough levels [1-3]. Prospective studies in de novo renal transplant recipients have shown, that targeting certain predefined cyclosporine $AUC_{(0\text{-}4h)}$ or C2 levels was associated with a very low incidence of acute rejection episodes [4,5].

For tacrolimus most centers still rely on trough levels monitoring, but there are conflicting data about the correlation with systemic exposure. Some studies reported a reasonable squared correlation coefficient between trough levels and tacrolimus $AUC_{(0\text{-}12h)}$ (r^2 0.60 to 0.85) [6-8], others found poor correlations ((r^2) < 0,50) [9,10]. The observed differences may originate from at least three factors, including differences in sample sizes, type of correlation tests used and time interval after transplantation, at which the studies were done.

In general, a considerable variation in $AUC_{(0\text{-}12h)}$ can be expected in relation to a single trough level, which of course is augmented by the range of trough levels that is thought to be acceptable in clinical practice (e.g. tacrolimus trough levels between 10 − 20 ng/ml early post transplant and 5 − 10 ng/ml in stable transplant recipients). Similar to cyclosporine, patients with low systemic tacrolimus exposure in relationship to tacrolimus trough levels could be expected to have an increased risk of developing acute or chronic rejection, whereas patients with a high AUC/ C_0 ratio are likely to be overdosed.

Prevention of tacrolimus overexposure by AUC monitoring may be relevant to reduce nephrotoxicity, hypertension and hypercholesterolemia, but the impact of controlled systemic exposure on these side effects has not been studied. The increased risk for tacrolimus treated patients to develop post-transplant diabetes mellitus [11] and polyoma-virus associated nephropathy [12] in comparison with cyclosporine treated patients, may theoretically be reduced by AUC monitoring. Before the impact of

tacrolimus drug exposure can be studied over longer periods of time, a simple and flexible strategy is needed to estimate systemic drug exposure, since "full" 12 hrs AUC sampling is not a realistic option in daily practice. The major disadvantage of limiting sampling models, with a mathematically derived equation [9], is the imperative of accurate timing of the blood samples. When a sample is taken 15 minutes too late, the mathematic equation is no longer valid [13].

Bayesian forecasting is a therapeutic drug monitoring tool, which uses pharmacokinetic parameter estimates (such as mean population drug clearance and volume of distribution) along with expected associated variability and information about the patient (e.g. body weight, renal function), to predict drug concentrations achieved with specific doses [14]. Pharmacokinetic parameters for each patient become individualized and the influence of the population parameters decrease [15]. Optimally these techniques also inform the clinician of the next appropriate dose to maintain or reach the desired drug concentration. The number of blood collections needed and the time to reach the required drug concentrations can be reduced [15-17].

We previously described a population based 2-compartmental computer model for cyclosporine [18], which uses Bayesian forecasting combined with a limited sampling strategy, and now used the same program to analyze pharmacokinetic data obtained in tacrolimus treated renal transplant recipients. After building and validating a new model for tacrolimus, we prospectively applied AUC-guided dosing to a cohort of de novo patients during the first post-operative year. The main objective was to study intra-individual pharmacokinetic changes in this for systemic exposure standardized cohort, in order to be able to design an optimal pharmacokinetic monitoring strategy for tacrolimus treated renal transplant recipients.

Materials and methods

Population based model

Using the kin pop module of the pharmacokinetic software package MW/Pharm version 3.33 (Mediware, Groningen, the Netherlands) [16], a population 2-compartment model with a lag-time and first order absorption pharmacokinetics was calculated using the tacrolimus dose and the blood concentration values of 20 tacrolimus curves (blood concentration at t = 0,1,2,3,4,6,8,12h) obtained from

17 renal transplant recipients (6 females, 11 males; mean age 45.4 year, mean body weight 73.1 kg; 30% living donation) and taken at different time points post transplantation (11 curves between 2 and 6 weeks, 9 curves between 6 and 52 weeks post transplantation). Whole blood concentrations (ng /ml) were determined by micro particle enzyme immunoassay (MEIA, Abbott Laboratories, Abbott Park, IL, USA). The MW/Pharm program uses an iterative two-stage Bayesian procedure, to calculate means and standard deviations of the relevant pharmacokinetic parameters as shown in Table 1.

Table 1: Tacrolimus pharmacokinetic parameters derived from the model building set of 20 curves, obtained in 17 renal transplant recipients.

Parameter		Mean (SD)
t_{lag} (h)	lag time	0.956 (0.161)
F	oral bioavailability	0.23 (fixed)
Ka (h^{-1})	absorption rate constant	0.580 (0.524)
V_1 (l/kg)	apparent volume of distribution of central compartment	0.180 (0.063)
K_{elm} (h^{-1})	elimination rate constant	0.517 (0.096)
K_{12} (h^{-1})	distribution rate constant (central to peripheral compartment)	2.850 (2.219)
K_{21} (h^{-1})	distribution rate constant (peripheral to central compartment)	0.384 (0.410)

Validation of the model and different limited sampling strategies

The population-based model was validated in another cohort of 26 renal transplant recipients. The characteristics of this validation group are summarized in Table 2. The calculated mean population pharmacokinetic parameters were individualized for each of 64 curves (blood concentration data points at t = 0,1,2,3,4,6,8h), based on the tacrolimus dose and different sampling methods, using the maximum a posteriori (MAP) Bayesian fitting method. Twenty-two of the curves were obtained in the early postoperative phase (i.e. within 2 weeks after transplantation), 42 curves were obtained between 6 weeks and 52 weeks post transplantation. For each of the combination of time points individualized pharmacokinetic parameters were calculated with the model, from which the $AUC_{0\text{-}12h}$ was derived (Figure 1). For comparison the $AUC_{0\text{-}12h}$ of the 64 curves was also calculated using the equation based strategy as described by Wong *et al.* [9]: $AUC_{0\text{-}12h}$ = 16.2 + 2.4*C2h + 5.9*C4h. The "standard" or reference $AUC_{0\text{-}12h}$ of the 64 curves was calculated from all tacrolimus blood concentrations using the trapezoidal method (Kin fit module, MW/Pharm).

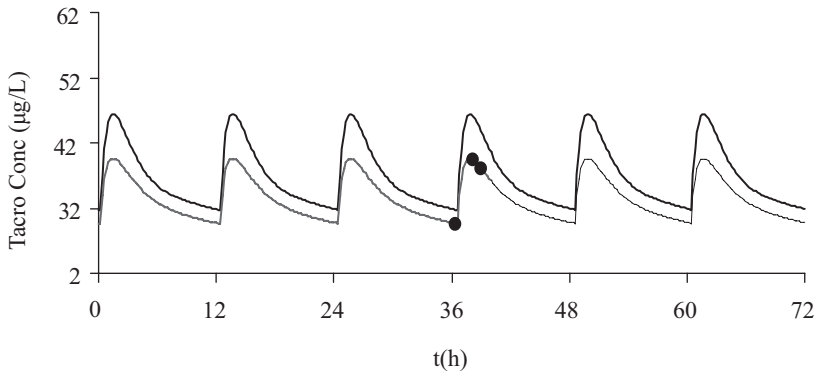

Figure 1: Tacrolimus blood concentration time curve according to the population-based model (continuous line), the measured tacrolimus blood concentrations at t = 0h, 2h, 3h in a patient (●), and the tacrolimus blood concentration time curve according to the model after fitting the population parameters to the measured concentrations (dotted line), after which the $AUC_{0\text{-}12h}$ is calculated by the model.

Statistics

The AUCs calculated by the different methods were compared to the standard AUC by Pearson's correlation coefficient. C0h (trough levels), C1h, C2h, C3h were also correlated to the standard AUC by Pearson's correlation coefficient. Predictive performance was investigated by calculating the prediction precision and bias according to Sheiner and Beal [19].

AUC-guided dosing

This study was approved by the Medical Ethic Committee of the Leiden University Medical Center. Fifteen consecutive de novo renal transplant recipients were included and their characteristics are summarized in Table 2. These patients were prospectively treated, according to the following AUC-guided dosing protocol: Tacrolimus (starting dose 0,1 mg/kg) was given in a twice-daily schedule starting 3 hours before surgery. In the first week target trough levels were 12,5 ng/ml (range 10 − 15 ng/ml). Tacrolimus "full"$AUC_{0\text{-}12h}$ was determined at weeks 2, 6, 12, 26, and 52 using the pharmacokinetic model. Limited sampling estimates of $AUC_{0\text{-}12h}$ were obtained at weeks 4, 8, 10, 17, 21 and 39. After each AUC-assessment dose adjustments were made to reach the predefined target $AUC_{0\text{-}12h}$: 210 ng.h /ml within the first 6 weeks (corresponding with a trough level of 12.5 ng/ml, derived from

the model using mean population based PK parameters), and 125 ng.h/ml thereafter (corresponding with a trough level of 7.5 ng/ml). Since according to the model there was a linear correlation between dose and AUC, dose adjustments were made by the model according to the formula $D_{new} = D_{current} \times AUC_{target} / AUC_{current}$. Concomitant immunosuppressive medication consisted of prednisolon (100 mg day 1 − 3, 50 mg day 4, 20 mg day 5 − 14, 15 mg day 15 − 21, 10 mg after day 22), mycophenolate mofetil, 500 mg b.i.d. and basiliximab prophylaxis, 20 mg on days 0 and 4. Drugs, that are known to alter concentrations of tacrolimus, were prohibited.

Table 2: Characteristics of a) renal transplant recipients, whose tacrolimus curves (n = 64) were used for validation of the model and of b) patients, treated with AUC-guided dosing.

	Validation set (n = 26)	**AUC-dosing set (n = 15)**
	Mean (range)	**Mean (range)**
Age (years)	46.9 (20 − 65)	45.9 (33 − 65)
% Male	65	80
Renal disease		
Hereditary	27%	33%
Glomerulonephritis	31%	27%
Hypertension, nephrosclerosis	19%	20%
Other or unknown	23%	20%
Race		
Caucasian	80%	87%
African	8%	13%
Oriental	12%	0%
Body weight (kg)	78.5 (53 − 114)	81.5 (70 − 108)
Procedure		
Cadaveric, Heart-beating	39%	47%
Cadaveric, Non Heart-beating	15%	13%
Living related donation	31%	27%
Living unrelated donation	15%	13%
Delayed graft function (need for dialysis)	− − −	20%
Cockroft clearance (ml/min)	60.4 (12 − 96)	65.6 (16 − 95) at 1 yr

Statistics

Mean pharmacokinetic variables (± SD) resulting from this strategy (C_{trough}, [AUC/dose], Tmax, Cmax) were calculated from data obtained at time points of full 0-12h

AUC sampling (t = 2, 6, 12, 26 and 52 week post transplantation) and analyzed by repeated measurements ANOVA (SPSS version 11.0).

Results

AUC-monitoring

The relationship between tacrolimus trough levels and standard $AUC_{(0\text{-}12h)}$, as calculated by trapezoidal rule is plotted in Figure 2a. We found a squared correlation coefficient of 0.79, which was comparable with previous studies [6-8]. As a single sample strategy the 3h post dose tacrolimus level had an improved correlation ($r^2 = 0.88$) with the standard AUC, but still a wide range of the 95% prediction interval, as is illustrated in figure 2b, indicating that the precision is not optimal. The correlation between the Bayesian estimates of $AUC_{(0\text{-}12h)}$ using the tacrolimus concentrations at 0h and 3h and the standard $AUC_{(0\text{-}12h)}$ was significantly better ($r^2 = 0.96$, Figure 2c). This strategy resulted in a markedly improved precision for an individual measurement. The squared correlation coefficients, bias and precision of all the strategies tested to estimate systemic exposure, compared with the standard $AUC_{(0\text{-}12h)}$ as determined by trapezoidal rule are summarized in Table 3. All two-point strategies including a trough level with either a 2h, 3h or 4h sample had a strong correlation with the standard AUC ($r^2 = 0.94$, 0.96 and 0.95 respectively). Introduction of more samples further improved the estimation of systemic exposure. The performance of the limited sampling model as described by Wong was comparable. ($r^2 = 0.92$), but the imperative of exact timing to draw samples at 2h and 4h post dose make this approach inflexible and less attractive for daily practice. When only the curves obtained two weeks after transplantation were evaluated, the correlation of trough levels with the standard AUC was even worse ($r^2 = 0.67$). In contrast, the correlations of Bayesian estimates derived from all limited sampling procedures were hardly affected (Table 3). This underlines not only the large inter-patient variability especially in the early post-transplant period, but also the robustness of the model.

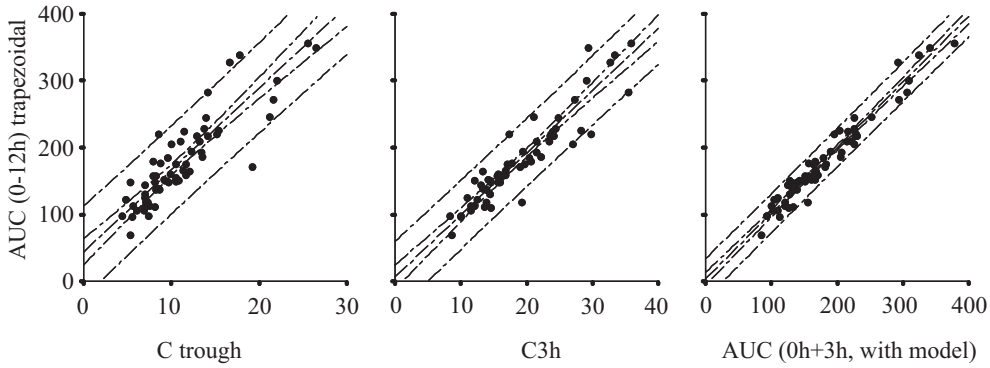

Figure 2: Relationship between either a) pre-dose level (C_{trough}, ng/ml, b) a single sample at 3 hours post dose (C3h, ng /ml) or c) Bayesian estimates of $AUC_{0\text{-}12h}$ using blood concentrations at 0h and 3h post dose (AUC 0h +3h, ng.h/ml) and the $AUC_{0\text{-}12h}$ calculated by trapezoidal rule. The inner lines (− · −) demonstrate the 95% confidence interval, the outer lines the 95% prediction interval.

Table 3: Pearson correlation coefficients (r^2), mean prediction error (MPE) and mean absolute prediction error (MAPE) of different strategies to estimate systemic exposure, compared with $AUC_{0\text{-}12h}$ determined by trapezoidal rule, based on 64 curves of 26 renal transplant recipients treated with tacrolimus.

Sampling strategy	r^2 (All curves)	MPE (%)	MAPE (%)	r^2 < 2wks post tx (22 curves)	r^2 > 2wks post tx (42 curves)
0h	0.79	−3.0	13.4	0.67	0.78
1h	0.48	−7.6	21.2	0.77	0.36
2h	0.77	−3.4	13.3	0.86	0.74
3h	0.88	−1.3	9.5	0.82	0.91
0,2h[a]	**0.94**	**1.4**	**7.6**	**0.93**	**0.95**
0,3h[a]	**0.96**	**0.3**	**7.1**	**0.97**	**0.96**
0,4h[a]	**0.95**	**−1.3**	**6.7**	**0.94**	**0.96**
0,2,3h[a]	0.96	0.8	6.4	0.96	0.97
0,1,3h[a]	0.97	3.7	6.5	0.97	0.98
0,2,4h[a]	0.97	−1.1	5.2	0.96	0.98
0,1,2,3 h[a]	0.97	2.7	6.0	0.97	0.98
0,1,2,3,4h[a]	0.98	1.4	4.8	0.98	0.99
0,1,2,3,4,6,8,12h[a]	0.99	−0.4	2.2	0.99	0.99
16.2+2.4*C2h+5.9*C4h	0.92	−6.5	8.2	0.96	0.92

[a] Bayesian estimation

AUC-guided dosing

A total of 15 consecutive de novo renal transplant recipients received tacrolimus based immunosuppression and were prospectively dosed using the described model and predefined AUC-targets. Cumulative incidence of biopsy proven acute rejection at one year was 6,6% (1 of 15 patients). The only acute rejection episode, we encountered, occurred at day 4 post transplantation in a living unrelated donation procedure, and could not be attributed to a low tacrolimus AUC. Patient and graft survival at one year both were 100%. At one year post transplantation mean GFR as calculated by the Cockroft formula was 66 ml/min (SD ± 21), mean total cholesterol 5,3 mmol/l (SD ± 1.3, 32% of patients used statins), mean systolic blood pressure 139 mmHg (SD ± 16), mean diastolic blood pressure 82 mmHg (SD ± 9), mean number of antihypertensive drugs per patient 1,6 (SD ± 1.0). In figure 3 for every time point (weeks) at which tacrolimus AUC was estimated, both the mean actual AUCs (± SD) and dose corrections (mg/12 hrs) needed to reach the predefined AUC-targets (dotted lines) are plotted. It is important to stress that no dose corrections were made in between these time points. There were no episodes of suspected nephrotoxicity resulting in dose reductions. Despite the corrections made at week 2 and week 4 ("early phase") the standard deviation of tacrolimus AUCs at week 4 and week 6 was considerable. These data indicated that intra-individual changes in pharmacokinetic parameters are still occurring in this early phase after renal transplantation. Between week 6 and 12 by protocol the target AUC was stepwise reduced to 125 ng.h/ml. The AUCs obtained at 12 weeks and later, were defined as the "steady phase" and showed stabilization of the intra-patient variability. This was also reflected in a decrease of dose corrections in the "steady phase" needed to maintain the AUCs within the target range.

Further analysis of the tacrolimus curves, obtained at week 2 and week 6 indicated an increase in the AUC, that was predominantly determined by the absorption phase of the curve (figure 4a and 4b). Not only in the "early phase", but also between week 12 and 52 there was a rise in the for tacrolimus dose corrected concentration curves. (figure 4c and 4d). The corresponding pharmacokinetic variables are summarized in table 4, showing a steady and significant increase over time of [AUC/dose], while this was not reflected in a change of [C_{trough}/dose] in the "stable phase". ($p = 0.01$, repeated measurements ANOVA analysis, linear test of within subjects contrast)

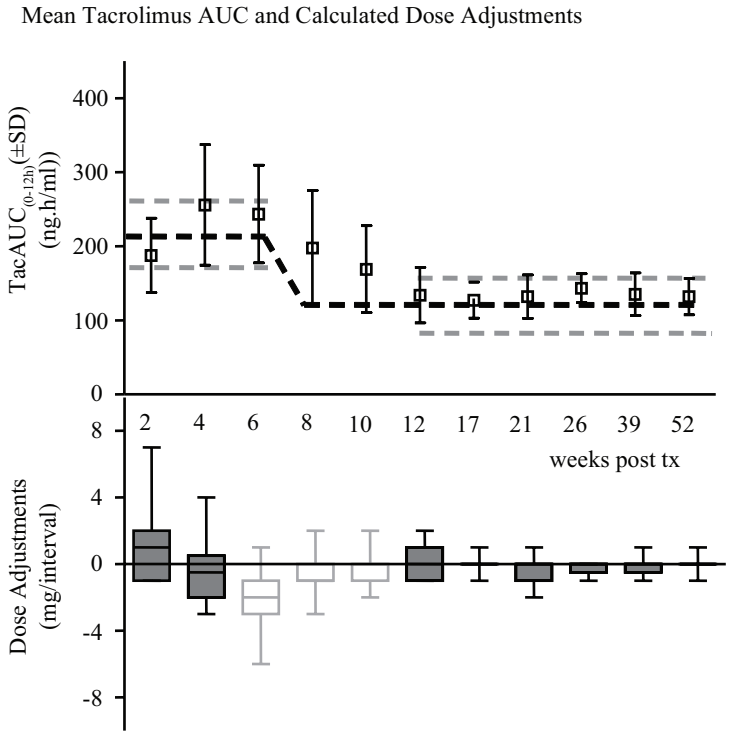

Figure 3: AUC-guided tacrolimus dosing in de novo kidney transplant recipients. Upper graph: Mean tacrolimus AUCs (±SD) in ng.h/ml and target AUC (dotted line) ± 20% (gray dotted lines).

Lower graph: Dose corrections (± SD and range) in mg, calculated by the model, to reach these targets. Beyond 6 weeks (according to the protocol) the target AUC was lowered, which was implemented gradually between 6 and 10 weeks post transplantation for safety reasons. For this reason the dose corrections at these time points are shown in gray.

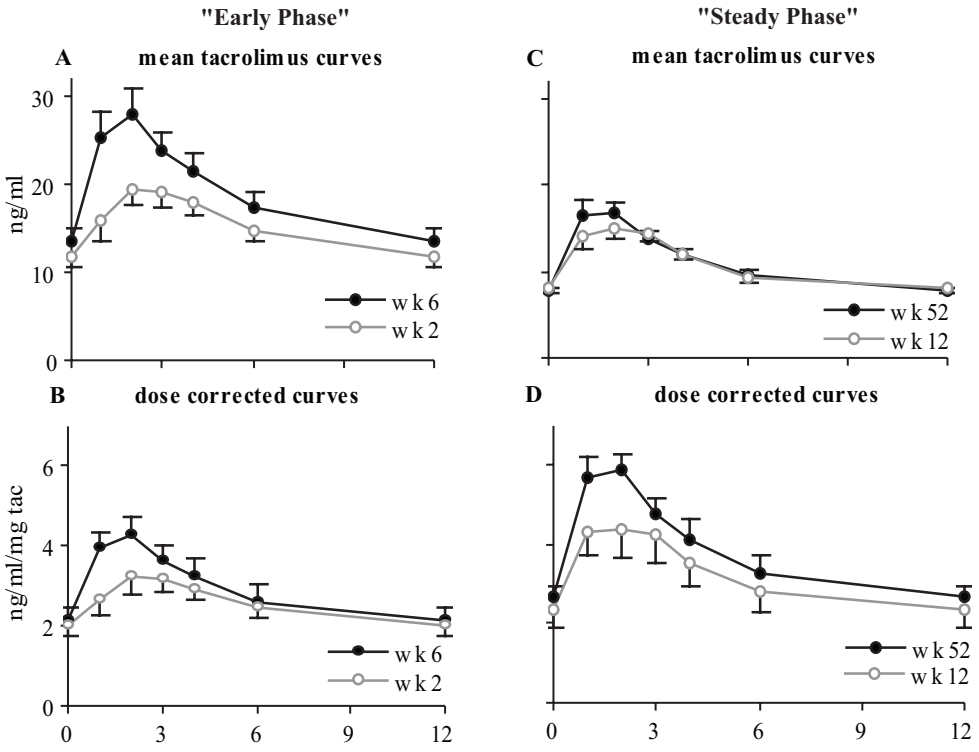

Figure 4A, B, C, D: Tacrolimus concentrations (mean ± SE) (upper panels) and dose corrected tacrolimus curves (lower panels) in the early phase post transplant (target AUC: 210 ng.h/ml) (left panels), and in the "steady" phase (target AUC: 125 ng.h/ml) (right panels) in de novo kidney transplant recipients.

Table 4: Mean values of pharmacokinetic variables with time

Wks Post tx	**AUC (0-12h)**	**C_{trough}**	**Tmax**	**Cmax**	**Dose tacro**	**Body weight**	**Dose / weight**	**C_{trough}/ Dose**	**AUC/ Dose**
	ng.h/ml (± SD)	ng/ml (± SD)	minutes post dose	ng/ml	mg/12h	kg	mg/kg	conc./mg	AUC/ mg
(Early phase)									
2 wk	181 (± 50)	11,9 (± 4,7)	130	23,1	6,0	77,5	0,077	2,1	30*
6 wk	224 (± 67)	13,6 (± 5,5)	121	29,5	6,5	74,8	0,087	2,5	34*
(Steady phase)									
12 wk	129 (± 35)	8,0 (± 2,4)	108	17,5	3,4	74,5	0,046	2,8	38*
26 wk	139 (± 20)	8,1 (± 2,1)	92	19,1	3,4	76,0	0,044	2,8	42*
52 wk	130 (± 23)	7,7 (± 1,6)	78	18,5	2,9	76,8	0,038	2,9	45*

* = significant, $p < 0,01$ by repeated measures ANOVA analysis.

Discussion

There is increasing evidence that dosing of cyclosporine microemulsion, guided by estimation of the absorption profile of the drug is superior, as compared with trough level monitoring. Adequate systemic exposure was associated with a reduced incidence of acute rejection episodes, cyclosporine related nephrotoxicity and cardiovascular risk factors, including hypertension and hypercholesterolemia. A critical appraisal of tacrolimus monitoring by trough levels only has thus far not received much attention, partly because earlier reports suggested that tacrolimus trough levels correlated better with systemic exposure [8]. Our data indicate that also in case of maintenance therapy with tacrolimus, a trough level is not a reliable tool to estimate systemic exposure in renal transplant recipients. Until date no conclusive prospective trials [10,20] have been published that evaluated the correlation between tacrolimus exposure and rejections or side effects. Kuypers *et al.* [21] reported a tendency for a lower incidence of acute rejection in relationship with simultaneous, adequate AUCs for both tacrolimus (> 150 ng.h/ml) and mycophenolate mofetil (> 45 ng.h/ml) at day 7, compared with patients who did not reach both targets. Although no differences in AUC were found in patients with versus patients without hypertension, hyperlipidemia and nephrotoxicity, they did report a significant higher AUC in patients with infectious complications, compared to patients without infections.

Our population-based computer model with Bayesian fitting improved significantly the estimation precision to predict the AUC, requiring a trough level in combination with only one additional, but timed, sample. For illustration: a trough level of 7.5 ng/ml corresponded with a mean $AUC_{(0\text{-}12h)}$ of 135 ng.h/ml, with a 95% prediction interval of 69 to 202 ng/ml. This interval could be reduced to 108 − 166 ng /ml when a two point (0h and 3h) strategy was followed. By analogy, the 95% prediction interval of a trough level of 12,5 ng/ml could be improved from an AUC range from 162 − 294 to a range of 198 − 256 ng.h/ml.

The major advantage of this model is that it can handle sampling at any time point between 2 and 4 hours post dose, without influencing its predictive performance. This approach is simple and feasible in the outpatient clinic setting. In contrast, limited sampling strategies based on a mathematical formula become useless when the obligatory predefined time points are not met. Also monitoring guided by a single

C2h or C3h level is very much dependent on accurate timing of the intake of the drug and the drawing of the samples [13].

We tested our model-based approach prospectively in a cohort of renal transplant recipients at various time points in the first post-operative year. Early after transplantation there was a change in the absorption phase in at least 50% of the patients we studied. The performance of the model combined with two-point sampling however was not influenced by these time-dependent changes in individual pharmacokinetics. In contrast, the worst correlation of trough levels with the trapezoidal AUCs was found in the early period after transplantation ($r^2 = 0.67$). Changes in absorption or elimination of tacrolimus, resulting in suboptimal systemic exposure, are not reliably identified by trough level monitoring. This is illustrated by the fact that in our data (validation set and AUC guided dosing set) only a limited fraction of curves with a trapezoidal AUC deviating more than 20% from the target AUCs would have been detected using trough levels. Using Bayesian estimates with sampling at 0h and 3h post dose this proportion is considerably higher (Table 5). To narrow the range of accepted trough levels would only result in a limited improvement of the proportion, and of course impair the test specificity. For example in the stable period, the sensitivity and specificity of a trough level > 9 ng/ml to predict an AUC > 150 ng.h/ml were 0.65 respectively 0.88 (for comparison: trough level > 10 ng/ml: sensitivity 0.50, specificity 0.90; AUC(0h3h) > 150 ng.h/ml: sensitivity 0.95, specificity 0.98).

In the first months after transplantation we observed a considerable standard deviation of the actual reached AUCs despite dose adjustments. This indicated that early after transplantation an AUC-measurement has a limited predictive value for drug exposure in the following weeks, which still makes in our opinion frequent monitoring necessary. After this period only minor dose corrections were needed to maintain patients within the defined AUC target range (100 − 150 ng.h/ml). The difficulty to reach the target AUC in the first weeks post transplantation reflects the changes in intra-individual PK parameters in this time period. Repeated measurements every 3 or 4 days could help to signalize these changes earlier, whereas the simplicity of the model facilitates these kinds of strategies. Also the system could theoretically be improved by putting the time related PK-changes into the model. However the disadvantage of this kind of strategies would be that the simplicity of the model is affected.

Table 5: Relationship of trough levels and of Bayesian estimates of $AUC_{0\text{-}12h}$ using blood concentrations at 0h and 3h post dose (AUC 0h3h, ng.h/ml) with trapezoidal AUCs and their performance as a test to detect "abnormal" exposure of tacrolimus.

Early Period					*n = 65 curves*			
		AUC trapezoidal ng.h/ml				AUC trapezoidal ng.h/ml		
		< 170	170 − 250	> 250		< 170	170 − 250	> 250
Ctrough ng/ml	< 10	14	8	0	<170	22	2	0
	10 − 15	7	15	4	170 − 250	1	26	0
	> 15	2	5	10	> 250	0	0	14
Test performance:			**sens**	**spec**	**Test performance:**		**sens**	**spec**
high Ctrough > overexposure:			0.71	0.86	high AUC(0h3h) > overexposure:		1.00	1.00
low Ctrough > underexposure:			0.61	0.81	low AUC(0h3h) > underexposure:		0.96	0.95
Stable period					*n = 72 curves*			
		AUC trapezoidal ng.h/ml				AUC trapezoidal ng.h/ml		
		< 100	100 − 150	> 150		< 100	100 − 150	> 150
Ctrough ng/ml	< 5	1	2	0	< 100	6	1	0
	5 − 10	7	37	10	100 − 150	2	42	1
	> 10	0	5	10	> 150	0	1	19
Test performance:			**sens**	**spec**	**Test performance:**		**sens**	**spec**
high C_{trough} > overexposure:			0.50	0.90	high AUC(0h3h) > overexposure:		0.95	0.98
low C_{trough} > underexposure:			0.13	0.97	low AUC(0h3h) > underexposure:		0.75	0.98

sens: sensitivity, spec: specificity. Overexposure was defined as a (trapezoidal) $AUC_{0\text{-}12h}$, more than 20% above the target AUC (> 250 ng.h/ml, early period; > 150 ng.h/ml, stable period), underexposure as an AUC0-12h, more than 20% under target (< 170 ng.h/ml, early period; < 100 ng.h/ml, stable period).

In this intensively monitored group of patients with controlled systemic exposure, the most striking change in pharmacokinetic parameters was the consistent increase of dose corrected AUC ([AUC / dose]) of tacrolimus with time. Since AUC / dose is equal to F / CL, this is a reflection of a decrease in apparent clearance, which can be a result from either an increasing bioavailability, by improving of absorption, or

a decrease in the actual elimination clearance of tacrolimus over time. Especially early post transplantation the change of the absorption profiles as shown in Figure 3 suggests that changing absorption kinetics in individual patients may be the principal cause for this phenomena. Presystemic metabolism of tacrolimus by the gastrointestinal cytochrome P450 3A (CYP3a) isoenzymes and removal by P-glycoprotein transport is extensive and likely to contribute significantly to large variability in the rate and extent of drug absorption [22,23]. CYP3A4 expression is highly variable between individuals, with up to 30-fold differences in small intestine expression [24]. Expression of CYP3A4 in the gut mucosa varies along the intestinal tract, the upper small intestine being the major site for CYP3A4-mediated first-pass metabolism in humans [25,26]. P-glycoprotein lowers intracellular concentrations of tacrolimus by pumping absorbed drug back into the intestinal lumen. P-glycoprotein may regulate access of tacrolimus to CYP3A enzymes preventing these enzymes from being overwhelmed by high drug concentrations [24]. Tacrolimus is repeatedly transported out of the intestinal mucosal cells and then passively reabsorbed. At least theoretically this continuous repeated exposure could lead to more efficient metabolism [27]. P-glycoprotein shows significant interindividual variability, with 2- to 8-fold variation found in small intestine biopsies from kidney transplant recipients and healthy volunteers [28]. P-glycoprotein mRNA levels increase longitudinally along the intestine [29]. In addition factors such as the poor aqueous solubility of tacrolimus and alterations in gut motility may cause intraindividual variability in tacrolimus exposure to CYP450 and P-glycoprotein systems and hence random intraindividual variability in tacrolimus bioavailibility.

Using trough levels as monitoring tool, this effect would not have been appreciated since the corresponding trough levels remained consistently within the generally accepted target range of 5 to 10 ng/ml. These data suggest that there may be a “silent” and progressive increase in the systemic exposure in tacrolimus treated patients, despite stable trough levels. It is important to note that all patients in the present study received a standard dose of 10 mg prednisolon beyond day 22 after transplantation. The observed pharmacokinetic changes can therefore not be attributed to a change in induction of CYP3A as a result of steroid tapering [30,31].

In conclusion, we present a simple and reliable model-based approach, which with only one additive sample, significantly improved estimation of tacrolimus exposure. A trough level in combination with a second sample, obtained somewhere between

2 and 4 hours post dose is sufficient to accurately and reliably estimate tacrolimus AUC, even in the early post transplant period. Especially in the first months ("early phase") post transplantation this method can serve as a tool to prevent under- or overexposure, compared to trough level monitoring, but changes in intra-individual pharmacokinetics with time make it necessary to repeat AUC-estimations frequently in this phase. After the first 3 months, the intra-individual changes in AUC are minimal, and in stable outpatients AUC estimates can be done every 3 months to prevent systemic overexposure with time. The applicability of AUC-guided tacrolimus dose adjustments can be expanded to overt tacrolimus associated complications, such as post transplant diabetes mellitus, polyoma virus associated nephropathy or unpredictable changes in tacrolimus exposure in relation to diarrhea [32]. A prospective study, in which AUC-guided dosing is directly compared with trough level monitoring, is the only way to determine the impact on the prevention of acute rejection episodes, nephrotoxicity and other known side effects. Once the strategy, to control exposure over time, is optimized, different target levels of AUC could be tested in relationship to clinical outcome.

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Chapter 5

Characterizing the role of enterohepatic recycling in the interactions between mycophenolate mofetil and calcineurin inhibitors in renal transplant patients by pharmacokinetic modelling

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Abstract

Introduction: Controversy remains about the interaction between mycophenolate mofetil (MMF) and the calcineurin inhibitors cyclosporine (CsA) and tacrolimus (Tac). The need to double the dose of MMF in case of CsA co-administration to achieve the same mycophenolic acid (MPA) levels as in Tac co-administration, has been attributed to either inhibition by CsA of the enterohepatic cycle, or an inhibition of glucuronidation to mycophenolate glucuronide (MPAG) by Tac. We explored this interaction clinically in 64 kidney transplant patients.

Methods*:* Plasma MPA/MPAG curves were determined during the first year post transplantation. Using nonlinear mixed effect modeling, MPA/MPAG data were fitted to a 4-compartment model, in which a rate constant describing transfer from the fourth to the first compartment (k41), and therefore enterohepatic recycling, could be introduced.

Results: MPA and MPAG plasma concentrations were adequately described by a 4-compartment model, which was significantly improved by introduction of k41 in case of Tac co-administration (minimum value of the objective function decreased by 181 points, $p < 0.0001$). Using this model, no statistically significant difference was observed in clearance of MPA between CsA and Tac co-administration (11.9 and 14.1 l/h respectively). Total clearance of MPAG was lower in case of CsA co-administration (1.45 and 0.92 l/h respectively), while there was no difference in renal clearance of MPAG(1.09 and 0.92 l/h respectively).

Conclusions: Our study supplies supportive clinical evidence that inhibition of the enterohepatic cycle in case of CsA co-administration explains some of the differences observed in PK of MMF when co-administered with either Tac or CsA. This finding may have clinical consequences for the immunosuppressive management of kidney transplant patients.

Introduction

Kidney transplant recipients usually receive multiple immunosuppressive drugs. Today, the majority of these patients receives the glucocorticosteroid prednisolon, the inosine monophosphate dehydrogenase (IMDPH) inhibitor mycophenolate mofetil (MMF), and one of the calcineurin inhibitors cyclosporine microemulsion (CsA) or tacrolimus (Tac). These combinations have been shown to be successful in the prevention of acute rejection episodes [1-3].

During absorption, the prodrug MMF is hydrolyzed completely into the active metabolite mycopenolic acid (MPA), which undergoes further metabolism [4]. Quantitatively, the most important metabolite is the pharmacologically inactive mycophenolate glucuronide (MPAG), which is excreted either into urine or into the bile [5]. Biliary excreted MPAG undergoes substantial enterohepatic cycling, and is back-transformed to the pharmacologically active MPA during re-absorption [5].

Earlier studies have shown that for patients receiving CsA, roughly twice the dose of MMF is needed to achieve the same systemic exposure to MPA when compared to patients receiving Tac [6,7]. This has been attributed to either an inhibition by CsA of the enterohepatic cycle [8], or an inhibition of glucuronidation by Tac [6]. Where the latter has been shown only in *in-vitro* studies [9], the former has also been shown *in-vivo* in rodents [10].

We further explored this interaction clinically using both MPA and MPAG plasma concentration data derived from a study with 64 kidney transplant patients who were on controlled systemic exposure to either CsA or Tac during the first year after transplantation. MPA/MPAG concentration time curves were taken at regular time points during this year. Using nonlinear mixed effect modeling (NONMEM) we developed an integrated metabolite pharmacokinetic model for both MPA and its glucuronide, in which an enterohepatic cycle was incorporated.

Patients and methods

Sixty-four patients, transplanted at the Leiden University Medical Center between 2000 and 2002, entered the study that primarily investigated the clinical, biochemical and pathological effects of triple therapy with prednisolone, MMF and either CsA

(n = 33) or Tac (n = 31) Details of this study have been described elsewhere [11]. Patient characteristics are listed in Table 1. No statistical difference (Student's t-test, $P > 0.05$) was shown between both groups for the patient's age, weight, length and renal function. Comedication known to influence PK of MMF, CsA or Tac were avoided. After kidney transplantation, CsA and Tac doses were adjusted to reach a predefined AUC (area under the blood concentration-time curves) as described earlier [12,13]. AUCs were determined around the morning dose at week 2, 4, 6, 8, 10, 17, 21, 26, 39 and 52. During the first 6 weeks after Tx, the target-$AUCs_{0\text{-}12h}$ were 5400 and 210 h*µg/L for CsA and Tac, respectively. Thereafter, the target-$AUCs_{0\text{-}12h}$ were 3250 and 125 h*ug/L, respectively. Mean accuracy and precision for reaching the predefined $AUCs_{0\text{-}12h}$ were 7 and 18% fro CsA and 11 and 22% respectively for Tac (data from 12 weeks – 1 year post Tx). MMF doses were 1000 mg bid and 500 mg bid for the CsA and Tac respectively. All patients gave oral and written informed consent before study entry. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center.

Table 1: Demographic and baseline characteristics

Characteristic	Cyclosporine (n = 33)	Tacrolimus (n = 31)
Recipient age (yr) ± SD	48.3 ± 12.5	44.9 ± 12.5
Recipient gender n male (%)	26 (78.8)	23 (76.4)
Recipient weight (kg) ± SD	77.0 ± 16.9	75.3 ± 11.9
Recipient length (m) ± SD	1.74 ± 0.12	1.74 ± 0.09
Primary disease, n (%)		
primary glomerular disease	12 (36.4)	12 (38.7)
diabetic nephropathy	2 (6.1)	1 (3.2)
hypertension	5 (15.2)	4 (12.9)
hereditary disease	4 (12.1)	6 (19.4)
congenital dysplasia/reflux	3 (9.1)	1 (3.2)
etiology uncertain, other	7 (21.1)	7 (22.6)
Donor age (yr) ±SD	44.0 ± 14.6	46.8 ± 13.3
Donor gender n male (%)	13 (39.4)	19 (61.3)
Procedure, n (%)		
Cadaveric, heart beating	16 (48.6)	13 (41.9)
Cadaveric, non heart beating	7 (21.1)	4 (12.9)
Living related	6 (18.2)	9 (29.0)
Living unrelated	4 (12.1)	5 (16.2)
Delayed Graft Function n (%)	9 (27.3)	6 (19.3)
GFR (ml/min) ± SD *	62.5 ± 13.2	65.1 ± 4.0

(* = Nankivell formula)

At week 2, 6, 12, 26, 39 and 52, blood samples were taken at 0, 1, 2, 3, 4 and 6h after drug administration. At week 4, 8, 10, 17 and 21, blood samples were taken at 0, 2 and 3h after drug administration. Blood samples were taken for both CsA/Tac and MPA/MPAG determination. Whole blood concentrations of CsA and Tac were determined by fluorescence polarization immunoassay (FPIA) (Axsym; Abbott Diagnostics, Abbott Park, IL, USA) and micro particle enzyme immunoassay (MEIA; Abbott Diagnostics, Abbott Park, IL, USA), respectively. MPA and MPAG plasma concentrations were determined using a validated high performance liquid chromography (HPLC) method with ultraviolet detection at 305 nm. The method was linear for MPA and MPAG from 0.2 to 60 and 10 to 400 mg/L, respectively. Lower limits of quantification were 0.2 and 10 mg/L, respectively. Accuracy and precision were 97 and 7% and 98 and 3% for MPA at 0.54 and 10.8 mg/L. Accuracy and precision were 104 and 7% for MPAG at 160 mg/L.
Two data sets were analyzed; all available data from 2 weeks post Tx (2748 MPA and 2648 MPAG concentration measurements) and a subset where only measurements obtained starting 12 weeks post Tx were included (1563 MPA and 1514 MPAG concentration measurements). Analysis of the full data set indicated that up to 12 weeks, pharmacokinetic parameters tended to change possibly due to residual effects of the transplantation, changing exposure to CsA or Tac, and variations in co-medication. Therefore the restricted data set (12 weeks and later) was used for the final parameter estimation, while the full data set was used for illustrative purposes only.

Pharmacokinetics of MPA and MPAG was investigated using Nonlinear Mixed Effect Modeling (NONMEM Version V, GloboMax LLC, Hannover, MD), using the first order method and a constant coefficient of variation interindividual and residual error models. Estimation of intraindividual variability was not attempted and all occasions were analyzed as if originating from different subjects. More accurate estimation methods were attempted (FOCE and FOCE with interaction) but these did not lead to model convergence due to numerical instability. As the main trust of this study was in describing combined PK of MPA and MPAG and the influence of CsA co-administration, modeling interoccasion variability was not regarded essential. To develop a metabolite PK model, MMF doses and MPA and MPAG plasma concentrations were transformed into molar equivalents. Pharmacokinetic

parameters therefore apply to molar equivalents. For reasons of recognition by clinicians and pharmacologists, however, in the figures, these molar equivalents have been recalculated to MPA and MPAG concentrations.

A wide range of models was investigated and compared using minimum value of objective function (MVOF) and visual inspection of all individual plasma concentration curves. Likelihood ratios tests to compare models were performed by comparing differences in MVOF between models to chi-square distributions with degrees of freedom equal to the difference in the number of parameters, where $P < 0.01$ was required for significance.

Visual inspection of the curves revealed that MPA was best described by a two-compartment model, while MPAG could be described by a one-compartment model. Probably due to the relatively sparse nature of the data, multiple peaks commonly associated with enterohepatic recycling were not observed. This means that the data do not contain information on discrete biliary excretion episodes and therefore enterohepatic recycling was modeled as a continuous process. As a result, combined MPA/MPAG PK after oral administration of MMF was described by a four-compartment model. The second and fourth compartment represented MPA and MPAG plasma concentrations, respectively, while the third compartment accounted for the peripheral distribution of MPA. The enterohepatic recycling step was modeled by introducing a rate constant describing transfer from the fourth (MPAG) to the first (MMF dosing) compartment assuming complete transformation of MPAG into MPA.

We primarily investigated whether PK of both MPA and MPAG after MMF administration could be described simultaneously by a four-compartment model, and whether introduction of an enterohepatic cycle in case of Tac and not in case of CsA co-administration improved description of the data. Within the overall population analysis, separate population estimates were obtained for Tac and CsA co-administration. The population average parameters describing CsA were parameterized as the difference between population average Tac parameter and the population average CsA parameter. This means that population estimates for CsA co-administration can be calculated (by simple addition) but standard errors are not available for CsA parameters. It would also have been possible to estimate absolute population average values for both co-administration situations. However estimation of the difference instead allows testing of the significance of the difference between

the two co-administrations for each parameter separately, using confidence intervals based on approximate standard error. For this purpose, the approximate standard errors (SEM) for the difference estimates were used to arrive at approximate 95% confidence limits (difference ± 2*SEM). Figure 1 gives a schematic representation of the final PK model used. The detailed NONMEM syntax of this model is available from the authors on request.

Influence of covariates on pharmacokinetic variability was investigated using Pearson's correlation coefficients between empirical Bayes estimates of the pharmacokinetic parameters and patient characteristics (age, height, weight, hemoglobin, serum albumin, creatinin clearance estimated using Cockroft and Gault, and creatinin clearance estimated using Nankivell).

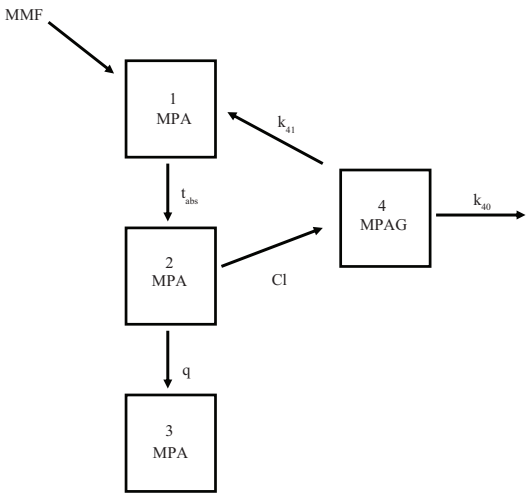

Figure 1: Four-compartment model for the pharmacokinetics of mycophenolate mofetil (MMF). MPA = Mycophenolic acid, MPAG = Mycophenolate glucuronide, t_{abs} = absorption half-life, q = intercompartmental clearance, Cl = clearance of MPA into MPAG, k_{40} = elimination rate constant of MPAG, k_{41} = rate transfer constant describing biliary excretion of MPAG.

Results

Both MPA and MPAG plasma concentrations after MMF administration starting from 12 weeks after Tx were adequately described by a four-compartment model. Absence of systematic model deviation can be seen in Figure 2, illustrating that the mean predicted curves are in close agreement with the mean of the observations. Standard goodness of the fit plot (e.g. observed vs. predicted concentrations) is not shown because the high number of data points result in uninformative graphs. Introduction of k41 into the model in case of Tac co-administration, significantly improved the description of the data (MVOF decreased by 181 points, $p < 0.0001$).
Table 2 summarizes all PK parameters of the final model for both the CsA and Tac group. Figure 2 shows values of predicted and observed MPA and MPAG data, illustrating altered description of the data after incorporating enterohepatic recycling, while Figure 3 shows the values of relevant PK parameters during one year post transplantation for the two groups with and without incorporating enterohepatic recycling (Cl MPA, Cl MPAG, k40) using the full data set. As can be seen, PK is relatively stable from 12 weeks on, in both study groups.
Using the model incorporating enterohepatic recycling, no difference was observed in clearance of MPA between CsA and Tac co-administration (difference 2.2 l/h; 95% CI: – 1 to 6 l/h). This clearance represents almost entirely the glucuronidation of MPA. In case of CsA co-administration, a somewhat higher clearance from the central to the peripheral compartment, and a higher peripheral volume was observed. No significant differences were observed for either absorption half-life or central volume.
No statistically significant difference was observed in the volume of distribution of MPAG, although the average was 40% higher in case of Tac co-administration. Consequently, total clearance of MPAG calculated as (k40 + k41)*V4/F using empirical Bayes estimates was 40% lower in case of CsA co-administration. This difference, however, was caused by the difference in volume of distribution of MPAG as the sum of the rate constants for Tac co-administration (k41 + k40) equalled the rate constant for CsA co-administration (k40).
The strongest relationships between patient characteristics and pharmacokinetic parameters were shown for creatinine clearance and k40, with correlation coefficients

of 0.51 for the Nankivell estimate (Clnk) and 0.45 for the Cockcroft and Gault (Clcr) estimate ($p < 0.0001$, $n = 340$, data from weeks 12 and later). All other correlations were less than 0.39.

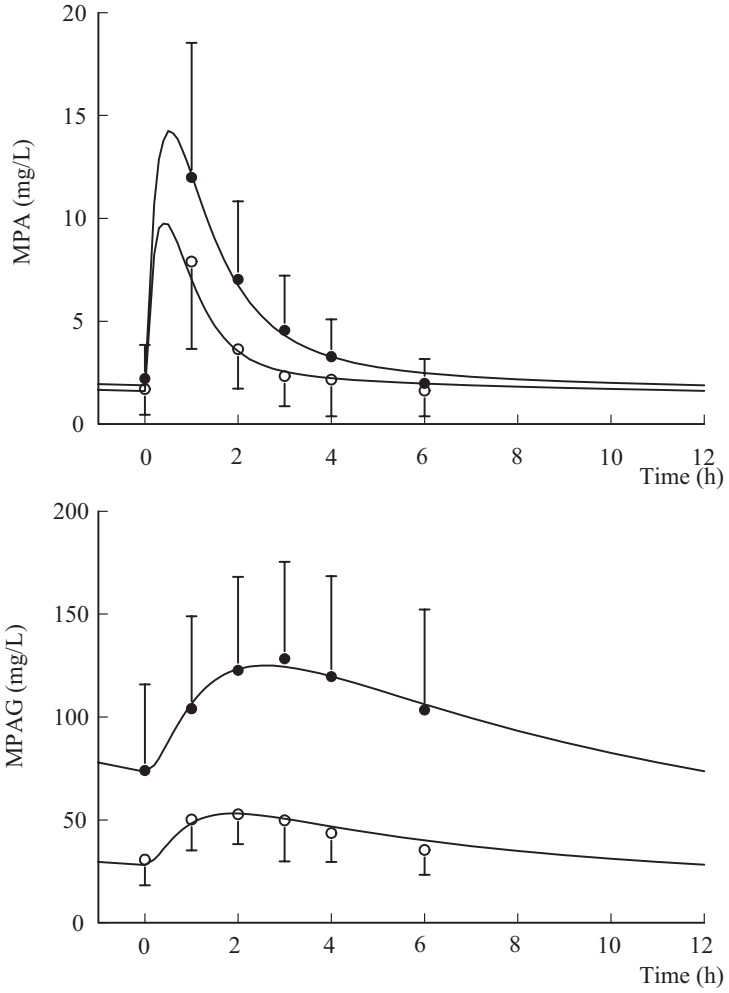

Figure 2: Average (± SD) graph of observed data (black markers: CsA, white markers: Tac) with average predicted curves from weeks 12, 26 and 52 combined. Top graph: MPA, bottom graph: MPAG.

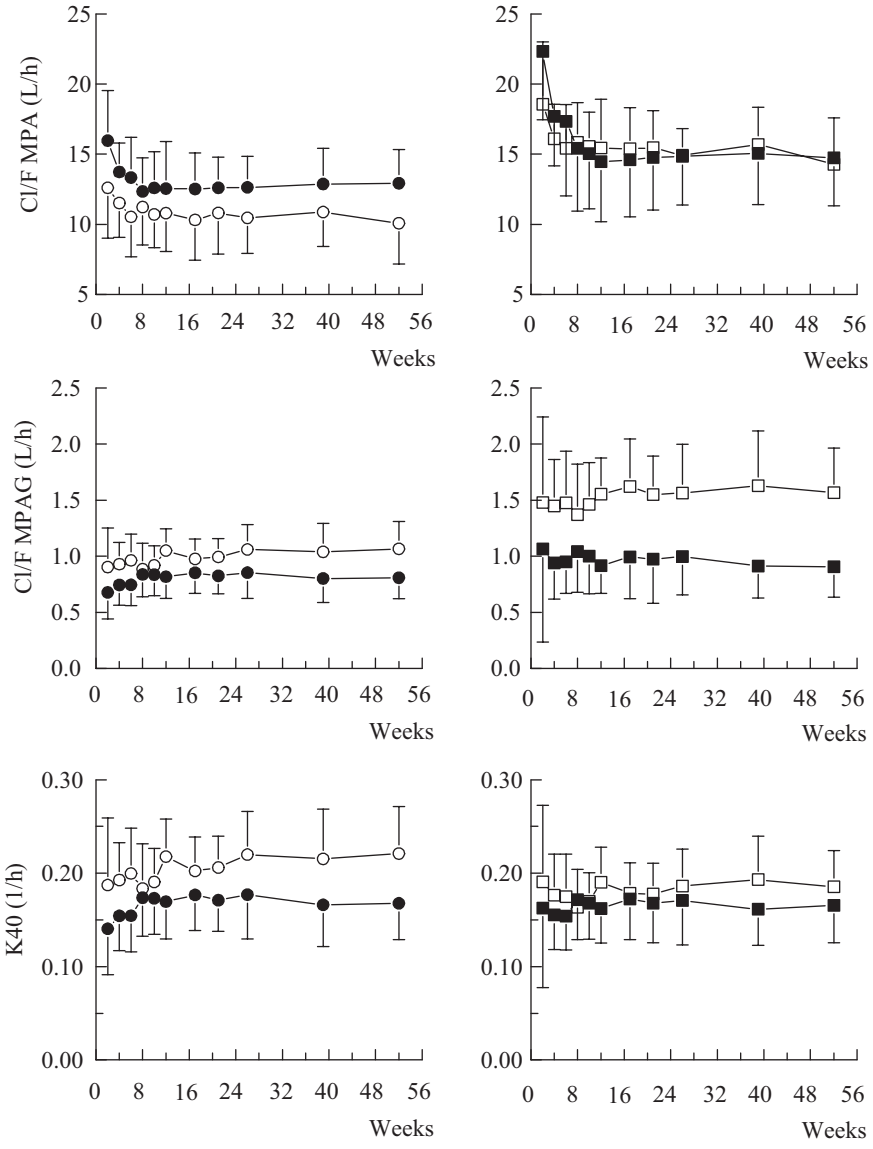

Figure 3: Average (+ SD) empirical Bayes estimates of clearance of MPA, clearance of MPAG and k40 during 1 year post transplantation calculated using the four-compartment model without (left side of the figure) and with the use of k41 in case of Tac co-administration (right side of the figure). White markers represent Tac and black markers CsA co-medication.

Table 2: Final NONMEM parameters for the full model using data from 12 weeks and later.

Parameter	Mean Tac	Mean CsA	Delta (95%CI)	CV (%)
Cl/F (L/h)	11.9	14.1	2.2 (–1 / 6)	30
T1/2a (h)	0.567	0.690	0.123 (–0.18 / 0.42)	57
Q/F (L/h)	11.2	20.1	8.9 (0 / 18)	51
Vc/F (L)	10.3	11.7	1.4 (–3 / 6)	74
Vp/F (L)	183	465	282 (90 / 480)	330
V4/F (MPAG) (L)	8.91	5.60	–3.31 (–7.9 / 1.2)	20
K40 (MPAG) (h-1)	0.122	0.165	0.043 (–0.03 / 0.12)	28
K41 (MPAG) (h-1)	0.0410	NA	NA	78
Error MPA (%)	35			
Error MPAG (%)	14			

Discussion

Earlier pharmacokinetic studies have demonstrated an interaction between calcineurin inhibitors and MMF. However, controversy remains regarding the exact mechanism through which this interaction takes place [14]. Zucker *et al.* reported significantly increased MPA trough plasma concentrations and MPA AUCs in patients treated with Tac, compared with patients treated with MMF and CsA [6], leading to the hypothesis that increased MPA levels are the result of inhibition of glucuronidation of MPA by Tac, which is supported by an *in-vitro* study showing inhibition of uridine-diphosphate-glucuronyl-transferase (UGT) formation of MPAG by Tac but not CsA [9]. Smak Gregoor *et al.*, however, reported significantly decreased MPA plasma trough concentrations in kidney transplant recipients in case of CsA co-administration when compared to no calcineurin inhibitor as co-medication, leading to the hypothesis that CsA inhibits the enterohepatic recirculation of MPAG [15]. This hypothesis was later confirmed by *in-vivo* studies in rodents showing no effect of Tac on MPA concentrations when compared to placebo, in contrast to the known effect of CsA on MPA concentrations [10]. This interaction may occur at the level of the biliary excretion of MPAG, where MPAG can be expected to be a substrate for the active transport by ATP-binding cassette transporters such as multidrug resistance-associated protein 2 (MRP-2) [8], which was recently confirmed by an *in-vivo* study in rodents [16].

Pharmacokinetic (PK) modeling may capture pharmacological information about a drug, especially in complex systems such as the metabolism and biodistribution of a

drug such as MMF. It has become clear, however, that PK modeling based solely on MPA concentrations is difficult after oral administration of MMF [17,18]. In the present study, we modeled both MPA and MPAG plasma concentrations simultaneously, and it seems that this enhanced data set is more useful for the description of MMF PK. Moreover, our data were derived from transplant patients who were stable and on well controlled systemic exposure to the calcineurin inhibitors [12,13], thereby eliminating some potential factors influencing MMF PK.

The present study clearly shows three major findings. First, MPA and MPAG PK in stable renal transplant patients, or at least those data between 0 and 6 h after drug administration, can be described adequately by a compartmental metabolite PK model. Such a model may be of use for therapeutic drug monitoring purposes in case both MPA and MPAG concentrations are determined on a routine basis. Moreover, the model may be of help describing PK in other patient populations and describing PK of other formulations with MPA as active compound. Second, this study strongly suggests that the inhibition of the enterohepatic cycle in case of CsA co-administration explains some of the differences observed in PK of MMF when co-administered with either Tac or CsA. This conclusion is based mainly on the significant improvement of the description of the data by introduction of an enterohepatic cycle into the model in case of Tac co-administration. The finding may explain the less frequent gastrointestinal side-effects caused by MMF in case of CsA co-administration, since biliary excretion of MPAG is believed to play a role in this adverse event [16].

The present study, however, did assume an extreme situation, namely complete inhibition of biliary excretion in case of CsA co-administration, where it is known from animal studies [16] that inhibition is not complete. The reason for not estimating k41 in the presence of CsA, however has to do with identifiability. There is a trade-off between all parameters, where an increase in one will lead to a decrease in another with (almost) the same curve as a result. The presence of k41 effectively increases the amount available for take-up in the gut. Incorporation of k41 in the CsA group would probably lead to nonzero estimates for this parameter compensated by an increase in distribution volume. Posing an extreme situation (presence/absence of k41) enables to see if all other parameters are essentially similar while there is a substantial difference in (dose-normalized) concentrations.

According to the model, no difference exists in glucuronidation between Tac and CsA. However, this finding should not be extrapolated easily to the conclusion that there is no effect on glucuronidation by Tac, especially since we did not investigate specifically whether inhibition or glucuronidation by Tac significantly improves description of the data. To address these issues more appropriately a third patient group consisting of patients only receiving MMF should be studied. However such a group was not available.

In the present study we only determined MPA and MPAG plasma concentrations during 6 h after MMF administration, thereby possibly missing part of the second plasma peak, which MPA sometimes shows, and which has been attributed to the enterohepatic cycle. However, as in our final data set all patients had received MMF for at least three months, substantial contribution of the enterohepatic cycle had also resulted in general accumulation of both MPA and MPAG and thereby an increase in basal levels. In the present study, we also did not investigate possible other metabolites of MMF such as the pharmacologically active acyl-glucuronide of MPA (Acyl-MPAG) [4,19]. However, these metabolites, although pharmacologically active, are quantitatively negligible in the biodistribution and metabolism of MMF. Nevertheless, in the future, it would be interesting to expand the present model with such metabolites.

The present study shows that MPA and MPAG PK in stable renal transplant patients can be described adequately by a compartmental metabolite PK model. Moreover, it shows the ability of modeling techniques to help unravel complex pharmacological systems, supplying supportive clinical evidence that inhibition of the enterohepatic cycle in case of CsA co-administration explains some of the difference observed in PK of MMF when co-administered with either CsA or Tac. This may have clinical consequences for the immunosuppressive management of kidney transplant patients.

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Chapter 6

No difference in the degree of interstitial sirius red stained area in serial biopsies from AUC-guided cyclosporine versus tacrolimus treated renal transplant recipients at one year

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Abstract

Introduction: Interstitial fibrosis is the main characteristic of chronic allograft nephropathy and long-term graft failure. Cyclosporine (CsA) is thought to be more fibrogenic than tacrolimus (Tac).

Material and Methods: In a prospective, randomized, multicenter trial using a calcineurin-sparing regimen renal interstitial volume was compared in CsA and Tac treated renal transplant recipients by image analysis of Sirius red (SR) stained cortical areas in protocol biopsies obtained at 6 months (n = 94) and 12 months (n = 97) after transplantation. Immunosuppression consisted of CsA or Tac, CD25 mAb, mycophenolate mofetil and prednisolone.

Results: CsA therapy increased the 6-month risk of subclinical rejection. The prevalence of subclinical rejection was 38.8% in the CsA and 15.2% in the Tac treated patient group ($p = 0.012$). Strikingly, no difference in the degree of interstitial Sirius red stained area was detectable between the two treatment groups. In particular, prior subclinical rejection episodes did not influence the degree of interstitial volume. Also, no difference in GFR occurred at 1 year when the mean GFR mounted 63 ml/min.

Conclusion: No significant differences in the degree of interstitial Sirius red stained area could be observed at 6 and 12 months between CsA- and Tac-treated renal transplant recipients. Although CsA treated patients developed significantly more subclinical rejections at 6 months, this did not influence the degree of Sirius red staining or the change in renal function at 1 year.

Introduction

Chronic allograft nephropathy (CAN) is the leading cause of long-term graft failure. Its prevalence has been estimated as 50 to 94% at 1 year [1,2]. In concept, clinical CAN refers to kidney transplant disease with a slow decline rate in renal function that histologically can be characterized by interstitial fibrosis, tubular atrophy, fibrointimal hyperplasia of the vessels and glomerulosclerosis. Because multifactorial processes are involved in the pathogenesis of CAN, a clear histopathologic distinction between different causes, such as chronic rejection, toxic effects of calcineurin inhibitors, hypertensive vascular disease and infection is not always possible in one single case [3].

Recently, the histopathological dynamics in the development of CAN were evaluated in retrospective studies of serial biopsies from kidney-pancreas transplant recipients using the Banff semiquantitative grading system [1,4,5]. Two distinctive phases of injury were recognized. The early phase was characterized by tubulointerstitial damage that occurred during the first year and correlated with ischemia–reperfusion injury, immunologic factors such as severe acute rejection and persistent subclinical rejection. The later phase consisted of chronic damage characterized by progressive arteriolar hyalinosis, ischemic glomerulosclerosis and further interstitial fibrosis that was associated among others with long-term calcineurin-inhibitor nephrotoxicity. Regardless of the nature and dynamics of the underlying disease, the extent of fibrosis has been reported to correlate with deterioration of renal function both in CAN and in other primary renal diseases [6-8]. Reduction in creatinine clearance is a late sign of injury due to compensatory mechanisms within the kidney. Accordingly, functional studies underestimate the extent of allograft disease as supported by longitudinal studies of protocol renal biopsies showing histological features of CAN in well-functioning grafts [1,5,9,10]. Therefore, early histological detection of CAN has been suggested to be helpful in predicting the risk for subsequent loss of function, time to graft failure and to estimate the efficacy of therapeutic measures [11,12].

Semiquantitative grading systems like Banff 97 have a wide interobserver variation that makes comparison across centers inaccurate [13-16]. In contrast, computerized image analysis of fractional interstitial fibrosis of Sirius red (SR) stained biopsies has been shown to be a valid and reproducible method to measure the degree of fibrosis [17,18]. Under polarized light, SR-dye is specific for collagen type I and III which

represent 80% and 20% respectively of total collagen synthesized by fibroblasts and thereby important components of renal matrix. Recently, quantification of renal interstitial volume assessed by Sirius red non-polarized technology has been validated: it correlated significantly with glomerular filtration rate as measured by iothalamate clearance in cases of established CAN [19].

On the one hand, the impact of calcineurin inhibitors on improvement of 1-yr cadaveric graft survival has been revolutionary. On the other hand, dosages that are needed to prevent immunologic graft failure are known to cause progressive nephrotoxicity and long term failure [5]. Although structurally not related, tacrolimus (Tac) and cyclosporine (CsA) induce similar histologic lesions, including toxic tubulopathy, de novo arteriolar hyalinosis, striped cortical fibrosis, tubular microcalcification and diffuse interstitial fibrosis [5,20,21]. The area under the concentration-over-time curves (AUC) of both CsA and Tac are reported to predict clinical parameters and nephrotoxicity better than trough levels [22-24]. Up to now, data on relating AUC-guided dosing of calcineurin inhibitors and quantitative measurements of interstitial Sirius red stained area are lacking.

Conflicting results have been reported by randomized trials comparing the effect of CsA and Tac on allograft survival [25-28]. Sparse data are available on differential effects of CsA and Tac on the development of renal fibrosis as measured by histomorphometric methods [2,29-31]. Moreover, these studies are difficult to compare because of different immunosuppressive regimens and different histomorphometric analysis methods as well as the use of trough levels aiming at different target values.

In this study, using a calcineurin inhibitor sparing regimen, we attempt to establish the differences in fibrogenicity between low target-AUC guided dosing of the calcineurin inhibitors CsA and Tac at 6 months and 1 year after transplantation.

Material and methods

Patients

This open–label randomized, controlled trial was conducted in Amsterdam and Leiden, the Netherlands. The study was approved by the medical ethic committees of both centers. Each patient had given written informed consent. 126 renal transplant

recipients (> 18 yr of age) of a first or second graft, except for highly immunized patients with panel reactive antibodies > 50%, were prospectively randomized between October 2000 and October 2002 to either a Tac-based (n = 63) or CsA-based (n = 63) immunosuppressive regimen. Renal function at 6 and 12 months was measured according to the Nankivell formula [32]. Baseline demographic and transplant-related characteristics, such as gender, age, the number of HLA-mismatches, percentage of panel reactive antibodies, underlying disease, type of procedure, warm and cold ischemia time and the occurrence of delayed graft function were not significantly different between the two groups. In the CsA treated group, more patients received a second transplant (previous transplant in CsA versus Tac group: 12.7% vs. 1.6%; p = 0.03).

Renal allograft biopsies

Protocol biopsies were obtained at the time of transplantation and at 6 and 12 months thereafter. At 6 and 12 months, biopsies were obtained in 83% and 88% of the evaluable patients in the CsA group and in 82% and 84% of the evaluable patients in the Tac group. Reasons for missing were primarily inadequate renal biopsy for proper Banff classification scoring, increased bleeding risk and secondary refusal by patients to undergo a protocol biopsy. The prevalence of inadequate samples defined as less than seven glomeruli and/or no artery was 6%. The biopsies were evaluated by two independent nephropathologists who were not aware of any clinical information. The Banff scheme was applied, in which the severity of acute and chronic changes of the glomeruli, vessels, tubules, and interstitium of a renal allograft is assigned a value of 0 to 3 [33]. For gaining insight into the variability of scores between observers, each fifth biopsy was scored by every pathologist independently and the differences were discussed.

Inter-observer agreement

The inter-observer agreement was good for the presence of acute features like tubulitis, glomerulitis, interstitial infiltrate and arteritis with a weighted kappa ranging from 0.6 to 0.8. However, the agreement between observers was poor for chronic features (weighted kappa range: 0.12 − 0.49].

Definitions

Histological CAN was defined as the presence of interstitial fibrosis and tubular atrophy with or without fibro-intimal vascular thickening. CAN grade 0 referred to < 6% affected cortical area, upgrading to grade I with 6 to 25% cortical fibrosis and grade II with 26 − 50% cortical fibrosis. In CAN grade III > 50% of interstitial cortical area was fibrotic. Clinical rejection was defined as an inflammatory process with varying degrees of tubulitis and endothelitis, leading to a decline in renal function. Subclinical rejection is a clinically silent state of the occurrence of histopathologic signs of rejection without deterioration of renal function. Using the Banff scheme, subclinical rejection is classified as acute (Banff grade 1A or higher) or as borderline with tubulitis score of 1 and mononuclear cell infiltration score of 1,2 or 3, or with tubulitis score of 2 or 3 with only mononuclear cell infiltration score of 0 or 1 without arteritis. Calcineurin-inhibitor toxicity was defined as the presence of nodular arteriolar hyalinosis and/or striped fibrosis and tubular microcalcification.

Study medication and AUC-guided dosing of calcineurin inhibitors

Patients were prospectively randomized 1:1 and treated with either a standard CsA-based or with a Tac-based regimen. In the first week after implantation CsA or Tac were given twice daily at 12 hours intervals, starting before surgery (starting dose 4 mg/kg b.i.d. for CsA and 0.1 mg/kg for Tac). The initial target 12 hours trough level (C = 0) in the first week was aimed at 225 ng/ml (range 200 − 250) and 12.5 ng/ml (range 10 − 15) for CsA and Tac respectively. CsA and Tac $AUC_{0\text{-}12h}$ were estimated at week 2, 4, 6, 8, 12, 17, 21, 26, 39 and 52 using a population based 2-compartemental pharmacokinetic model combined with Bayesian fitting. Samples were obtained at 0, 1, 2, 3, 4, 6, 8 and 12 hours post dose. Bayesian forecasting is a therapeutic drug monitoring tool, which uses pharmacokinetic estimates such as mean population drug clearance and volume of distribution along with expected associated variability and information about the patient (e.g. body weight, renal function), to predict drug concentrations achieved with specific doses [34,35]. This technique can inform the clinician of the next appropriate dose, to maintain or reach the desired drug concentration. Adjustments were made to achieve the predefined target AUC: CsA $AUC_{0\text{-}12h}$: 5400 ng.h per ml within the first 6 weeks which corresponds with a mean average trough level of 225 ng/ml; after 6 weeks 3250 ng.h per ml which corresponds to a mean average trough level of 125 ng/ml. Tac $AUC_{0\text{-}12h}$: 210 ng.h

per ml within the first 6 weeks which corresponds to a mean average trough level of 12.5 ng/ml; after 6 weeks 125 ng.h per ml which corresponds with a mean average trough level of 7.5 ng/ml. Outpatient visits were monitored by short AUC using samples taken at 0, 2 and 3 hours after dose. Analysis of pharmacokinetic parameters resulting from the AUC-guided dosing strategy for both drugs revealed that the actual measured AUCs of CsA and Tac were kept closely to the target AUCs. CsA was given at an actual mean dose (mg/kg per day) of 7.62, 5.72, 3.06, 2.86 and 2.82 during the periods between 0 and 2, 2 and 6, 6 and 12, 12 and 26 and 26 and 52 weeks after transplantation. Corresponding actual mean doses for Tac were 0.168, 0.176, 0.110, 0.104 and 0.086. Concomitant immunosuppressive drug therapy consisted of prednisolone (100 mg per day 1 to 3, 50 mg per day 4, 20 mg per day 5 to14, 15 mg per day 15 − 21, 10 mg after day 22 in both groups), mycophenolate mofetil (1000 mg b.i.d. and 500 mg b.i.d. in combination with either CsA or Tac respectively) and basiliximab prophylaxis (20 mg on days 0 and 4). Drugs, that are known to alter concentrations of Tac or CsA, were prohibited. Biopsy-proven acute rejection was treated with methylprednisolone 1000 mg for 3 consecutive days. Anti-thymocyte globulin (rATG Merieux) was given for steroid-resistant rejection episodes and for second acute rejection episodes. Only in case of a rejection > Banff grade II A, treatment conversion to the other calcineurin based protocol was done. Subclinical rejection was not treated.

Staining

Biopsies were processed for routine light microscopy. Tissue was embedded in paraffin, cut into 4-µmsections and stained with hematoxylin and eosin, periodic acid-Schiff, and SR. The slides were left in SR for 60 minutes. Two-µm sections were stained with silver methamine.

Image analysis

Image analysis was performed by a technician blinded to the clinical source of the sample using nonpolarized light. The slides were examined with a Zeiss microscope equipped with full-color 3CCD camera. A background image of a blank area of the slide was initially obtained and background correction was performed in real time to adjust for subtle irregularities in the illumination of the microscope field. The images were acquired using the x40 objective. Ten images of the cortex were obtained in a

serpentine manner. Vessels larger than the size of adjacent tubules, the subcapsular cortex with a width of 0.5 mm, and the medulla were excluded. Image analysis was performed using the Image Pro Plus software package (Media Cybernetics, Gleichen, Germany). After the software was set to differentiate the positively stained from negatively stained areas on the first image, the software sequentially opened each image, did the analysis, stored the data, closed the image, and moved onto the next image until the entire file was analyzed. The amount of cortical collagen (SR positive area) then was measured and was finally expressed as the percentage of the total analyzed cortical surface.

Statistical analysis

All analyses were performed according to the intention-to-treat principle. Results are given as mean ± SD for interval and ordinal variables. Frequencies of nominal (categorical) variables are given as numbers and percentages. For comparison between two categories of interval variables independent samples t-test was used. If statistical assumptions for parametric analysis of interval variables were not met and in case of ordinal data, then Mann-Whitney's two-independent samples test was used and exact p-values calculated. For measurement of association between ordinal or ranked data Kendall's tau-b correlation coefficient was calculated. Nominal variables were analyzed with cross-tables and exact significance was calculated. In case of repeated measurements repeated measures analysis with general linear models procedure was used. The incidence of acute rejection and diabetes mellitus was estimated using Kaplan-Meier's product-limit method and the resulting curves were compared with log-rank test. Regarding the measurement of interobserver agreement for ratings according to Banff-classification weighted kappa-statistic was used and p-values calculated. P-values < 0.05 were considered statistically significant. All analyses were performed using SPSS statistical software package (version 10.07; SPSS Inc. Chicago Ill. USA). Inter-observer agreement calculations were made using PEPI Computer Programs for Epidemiologists (Version 4.0, Sagebrush Press, Salt Lake City Utah USA).

Results

Patient survival, graft survival and graft function

At 1 year, patient survival was 97% in the CsA group and 98% in Tac group; graft survival was 95% versus 91% respectively. In the CsA group one nephrectomy was performed because of posttransplantion lymphoproliferative disease in the graft; in the Tac group there were two primary nonfunctioning grafts, two early graft failures after surgical complications and one failure after a hyperacute humoral rejection.

No differences were found in proteinuria and in renal function at 6 and 12 months: mean creatinine clearance ± SD at one year: CsA 64 ± 16 ml/min vs. Tac 65 ± 17 ml/min ($p = 0.67$). Therefore, no differences in clinical CAN were observed. The change in function between six months and one year was not influenced by the choice of calcineurin inhibitor. The mean difference in change between treatment groups was 1.13 ± 1.74 ml/min (confidence interval: −2.13 to 7.58).

Subclinical rejection and acute rejection

CsA treatment was associated with higher grades of tubulitis at 6 months as compared with Tac (mean tubulitis score ± SD: CsA: 0.6 ± 0.8 versus Tac: 0.4 ± 0.6, $p = 0.06$). Accordingly at 6 months, CsA therapy was accompanied by significantly higher rates of subclinical rejection. The total prevalence of subclinical rejections was 38.8% in CsA versus 15.2% in Tac ($p = 0.012$). The prevalence of subclinical borderline rejections was 27% versus 11% and subclinical Banff 1A 12% versus 4 % in the CsA versus Tac group (Figure 1A). However, this difference did not hold at one year (16% in CsA versus. 22% in Tac treated patients). In the total cohort, the prevalence of subclinical rejection was 27% and 19% at 6 and 12 months, respectively. The change in renal function between 6 months and 1 year (ΔGFR) was not influenced by the occurrence of subclinical rejection at 6 months (mean ΔGFR ± SD: No subclinical rejection; −1.89 ± 8.89 ml/min, subclinical rejection present; –0,24 ± 8.35 ml/min, $p = 0.28$, patients with a previous clinical rejection episode excluded).

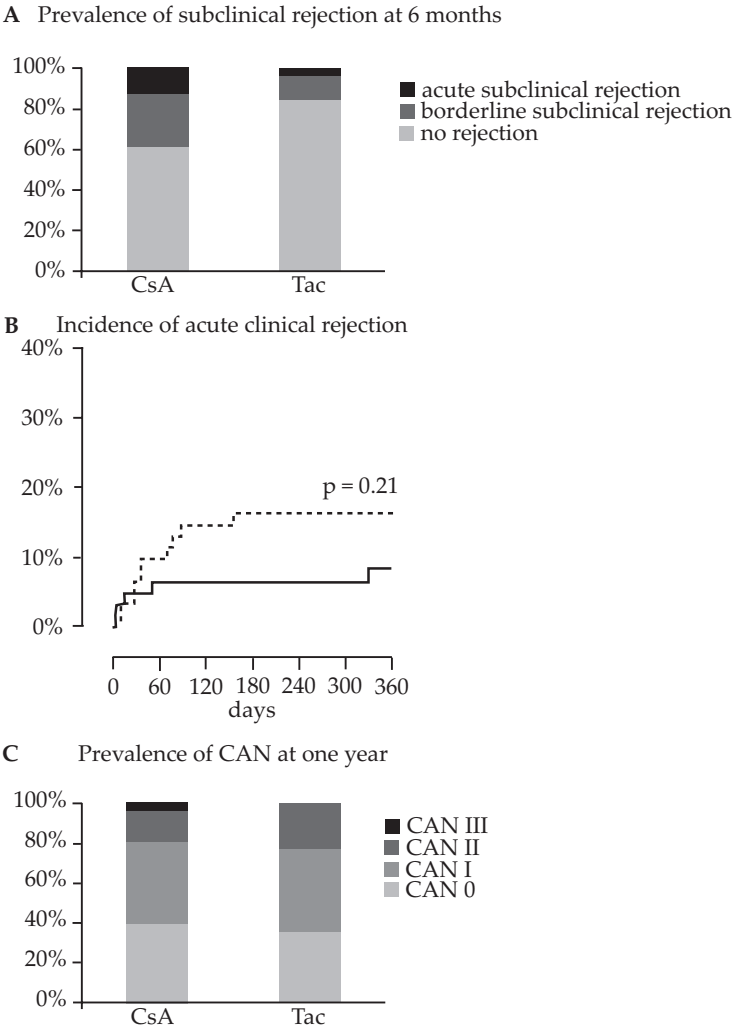

Figure 1: Prevalence of subclinical rejection in sequential biopsies at 6 months after transplantation, and incidence of histologically defined acute clinical rejection in both treatment groups are shown in A and B respectively. The dashed line in B represents incidence of acute clinical rejections in CsA group with the solid line as its equivalent in Tac-treated patients. (C) Prevalence of different grades of CAN according to the Banff classification at 1 year in both groups.

The cumulative incidence of biopsy-proven acute rejections was higher in the CsA as compared with the Tac group (16.2 % versus 6.6 % at 6 months, p = 0.12; Figure 1B). The incidence of vascular rejections and the incidence of steroid-resistance rejections, that required anti-thymocyte globulin therapy, were not different between the two treatment arms. The latter occurred in 6% of the patients in the CsA treated group and 2% of the Tac treated patients. This is compatible with approximately 37% of CsA treated patients who experienced an acute rejection versus 30% of patients who were treated with Tac and experienced an acute rejection (NS). In the Tac group one additional late acute rejection episode (at 330 days post transplantation) due to patient incompliance with undetectable Tac levels at the time of rejection occurred. Patients who experienced acute rejection had a lower creatinine clearance at 6 months (N = 13; 55.2 ± 22.1, mean ± SD, ml/min) than patients without acute rejection (N = 106; 65.2 ± 16.4, mean ± SD, ml/min. P = 0.048). Renal function at 1 year and also the change in renal function from 6 months to 12 months after transplantation was not influenced by the occurrence of acute rejection in our cohort.

Banff assessment

Histopathological characteristics of allografts at implantation, 6 and 12 months after transplantation are given in Table 1. Except the trend towards higher tubulitis scores at 6 months in CsA group, no other differences were found between both treatment groups at different time points. Therefore, no differences in histological CAN could be detected (CAN prevalence: 6 months; CsA 51% versus Tac 57%; one year; CsA 61% versus. Tac 65%, Figure 1C). The Banff degree of fibrosis, tubular atrophy and CAN showed a persistent negative correlation with allograft function at both 6 and 12 months (data not shown).

Calcineurin inhibitor nephrotoxicity

Calcineurin inhibitor nephrotoxicity was found to be present in 15% of the biopsies at 6 months and in 24% of the biopsies at one year with equal distribution between both groups (Table1). Arteriolar hyalinosis was not influenced by the type of calcineurin inhibitor, previous hypertension, dyslipidemia or diabetes mellitus (data not shown).

Table 1: Characteristics of the allograft at the time of transplantation and at 6 or 12 months after transplantation*

Banff characteristics	CsA T0	Tac T0	CsA T6	Tac T6	CsA T12	Tac T12
Tubulitis	0 ± 0.00	0 ± 0.00	**0.61 ± 0.73**	**0.36 ± 0.57**	0.31 ± 0.65	0.49 ± 0.76
Interstitial infiltrate	0.08 ± 0.28	0.06 ± 0.24	0.59 ± 0.70	0.40 ± 0.62	0.61 ± 0.96	0.43 ± 0.69
Glomerulitis	0 ± 0.00	0 ± 0.00	0.16 ± 0.43	0.16 ± 0.47	0.12 ± 0.33	0.15 ± 0.42
Arteritis	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.10 ± 0.50	0.07 ± 0.44
Arterial hyalinosis	0.31 ± 0.67	0.41 ± 0.50	0.41 ± 0.61	0.38 ± 0.68	0.57 ± 0.79	0.60 ± 0.79
Allograft glomerulopathy	0 ± 0.00	0 ± 0.00	0.10 ± 0.37	0.07 ± 0.33	0.02 ± 0.14	0.02 ± 0.15
Interstitial fibrosis	0.36 ± 0.59	0.40 ± 0.60	0.80 ± 0.71	0.71 ± 0.76	0.92 ± 0.89	0.87 ± 0.75
Tubular atrophy	0.56 ± 0.61	0.62 ± 0.55	0.84 ± 0.55	0.98 ± 0.66	1.14 ± 0.75	1.09 ± 0.66
Fibrointimal thickening	0.11 ± 0.31	0.19 ± 0.40	0.49 ± 0.65	0.33 ± 0.52	0.52 ± 0.58	0.46 ± 0.66
Mesangial matrix increase	0.17 ± 0.51	0.03 ± 0.17	0.33 ± 0.63	0.27 ± 0.50	0.37 ± 0.60	0.30 ± 0.47

* Exact P-values between different biopsy time points (6 and 12 months) were not significant for any of the histopathological Banff features (Mann-Whitney test). Banff scores are presented as mean ± SD.

Quantitative SR measurements

The effect of AUC–guided dosing of CsA or Tac on allograft structure was the primary outcome variable. Quantitative assessment of cortical interstitial volume revealed no differences between the CsA and Tac group at 6 months (mean percentage of SR-positive area ± SD, CsA (n = 50) versus Tac (n = 44), 12% ± 5.4% versus 12.3 ± 4.3% p = 0.78). At 1 year, similar results were obtained (mean percentage of SR positive are ± SD, CsA (n = 48) versus Tac (n = 47), 13.7% ± 5.6 versus.13.3% ± 5.9 p = 0.73] (Figure 2A). Increased interstitial SR-stained area corresponded to a higher degree (II and III) of CAN in this cohort (data not shown).

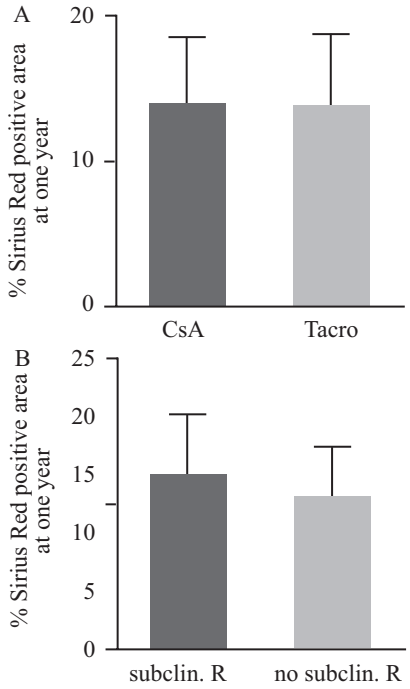

Figure 2: (A) No difference in the degree of interstitial Sirius red stained area in CsA and Tac treated patients at one year. (B) The degree of interstitial Sirius red stained area (y-axis) is not influenced by a prior subclinical rejection (x-axis) in protocol biopsies. Mean ± SD are shown.

The degree of interstitial SR-stained area at 1 year was not influenced by a previous acute rejection episode (p = 0.28). Also, the occurrence of subclinical acute and

borderline rejection episodes did not lead to significantly higher percentages of SR-positive area at 1 year (Figure 2B). No correlations were detectable between donor-related variables (donor age, cold ischemia time, donor type and number of HLA mismatches), delayed graft function and the degree of fibrosis at 1 year. We found a significant correlation between systolic blood pressure and the Banff degree of fibrosis at both 6 and 12 months after transplantation (systolic blood pressure at 6 months and fibrosis at 6 months: p = 0,026, systolic blood pressure at 6 months and fibrosis at 12 months: p = 0,006, systolic blood pressure at 12 months and fibrosis at 12 months: p = 0,031). No correlation was found between diastolic blood pressure and fibrosis at any time point. Renal function at 6 and 12 months was significantly correlated to the degree of interstitial volume as measured by SR (p = 0.03 and p = 0.05 respectively).

Discussion

In this prospective, randomized, multicenter trial using a calcineurin-sparing regimen with tightly controlled systemic exposure, no significant differences in the degree of interstitial SR-stained area were detected at 6 months and 1 year after transplantation between AUC guided dosing CsA and Tac. Patient and graft survival rates were similar between groups, exceeding 96% and 90% respectively. The available 2 year analysis at this moment is congruent with the 1 year results, showing no difference in renal function between CsA- and Tac-treated recipients. The creatinine clearance at 2 year measures 61.1 ± 16 ml/min (mean ± SD, n = 57) in the CsA vs. 62.9 ± 16.3 ml/min (n = 51) in the Tac group. However, at 6 months CsA-treated patients had developed a significantly higher prevalence of subclinical rejections and a higher number of acute clinical rejections than Tac patients without significant consequences for changes in renal function at 1 year.

Although conflicting data have been reported by randomized trials, that have compared the effects of micro-emulsion CsA and Tac on allograft survival, most studies fail to show any difference in patient and graft survival [25-28]. Recently the concept of calcineurin inhibitor (CNI) nephrotoxicity as a late sign of histopathological injury has been challenged by the findings of Nankivell *et al.* [5], who described two phases of CNI related injury by studying the longitudinal development of structural

CNI toxicity using Banff criteria in 99 recipients of combined kidney-pancreas transplants with CsA as the primary calcineurin inhibitor and a median follow-up time of 7 years. Although their findings underscored the persistent and progressing development of CNI nephrotoxicity in time, they described an early phase of acute structural CsA nephrotoxicity within the first year after transplantation, with a 1-year point-prevalence of 12.6%. Chronic CsA nephrotoxicity occurred with a point prevalence of 67.3% by 5 years and 100% by 10 years after transplantation. However, sparse data are available on differential effects of CsA and Tac on the development of allograft fibrosis as measured by histomorphometric methods, showing a stronger fibrogenic effect of CsA as compared to Tac [2,29-31]. The first prospective, randomized comparative study between CsA and Tac using quantitative computerized image analysis of fractional interstitial fibrosis of SR-stained biopsies at 1 year was reported by Murphy *et al.* [29]. Treatment with high-dose CsA (15 mg/kg/day) aimed at mean trough levels of 200 to 300 ng/ml during the first 3 months as compared with Tac (0.1 mg/kg/day) aimed at trough levels of 8 − 15 ng/ml, was associated with significantly higher degrees of fibrosis in CsA-treated patients, whereas no differences in renal function, acute rejection rate and de novo occurrence of diabetes mellitus were observed. During the first 2 weeks, CsA trough levels were as high as 400 − 500 ng/ml. Recently, in a large retrospective study of serial biopsies, Nankivell et al reported that early-onset acute and chronic structural CsA nephrotoxicity with respectively a point prevalence of 13 and 50% at 1 year could be predicted by a trough level > 200 ng/ml at 3 months and by trough levels that exceed the median of 180 ng/ml after 1 year [5]. In general, a CsA threshold dose of 5 mg/kg/day during the first year, which is considered as instrumental for prevention of immunologic graft failure, was inferred from their analysis as contributing to progressive arteriolar injury. CsA-AUC, but not trough levels, predict clinical parameters like acute rejection and acute nephrotoxicity more accurate [22-24]. In our study, analysis of pharmacokinetic parameters resulting from the AUC-guided dosing strategy for both drugs revealed that the actual measured AUCs of CsA and Tac were kept closely to the predefined low target AUCs, which may explain that no difference in the degree of interstitial Sirius red stained area was detected.

CNI-related nephrotoxicity was found to be present in 15% of biopsies at 6 months and in 24% of biopsies at 1 year with equal distribution between two treatment

groups. This incidence is lower than reported by Nankivell et al who retrospectively analyzed longitudinally obtained biopsies from pancreas-kidney transplant recipients where calcineurin-nephrotoxicity was shown to be as high as circa 53% at 1 year [5]. Thus, AUC guided dosing of calcineurin inhibitors may indeed result in less nephrotoxicity with maintenance of efficacy.

In our study, CsA therapy was associated with a significantly higher prevalence of subclinical rejection during the first 6 months and also with a higher number of acute rejections as compared to the Tac group. This is in agreement with previously reported data [1,36]. However, both acute and subclinical rejection did not influence the degree of interstitial SR-stained area, and the change in renal function was similar in both groups. This may be explained by a low cumulative incidence of acute rejections (11.5%) without a significant difference in steroid-resistant rejection rate between the treatment groups, which is congruent with data referring to only severe acute rejections, as defined by the need for antilymphocyte therapy, leading to an increase in Banff scores for CAN [1]. Subclinical rejections have been regarded increasingly as a risk factor for the development of CAN [1,37-39]. Nankivell *et al.* showed moderate CAN in 25.6% of biopsy specimens with previous evidence of subclinical rejection as compared with 7.5% of those without previous subclinical rejection ($P < 0.05$). Shishido *et al.* [37] evaluated protocol biopsies of patients with established CAN. Subclinical rejection was evident in 50%, 32%, 19%, and 16% of cases with CAN at 1, 2, 3, and 5 yr, respectively. However, the long-term effect of subclinical rejection(s) on development of renal allograft function and survival, as well as their impact on induction of quantitatively measurable renal fibrosis has yet to be determined in a prospective design. Future studies are needed to clarify the pathogenesis. Hyperexpression of the specific granzyme B inhibitor proteinase 9 by tubular cells has been described as a potential mechanism for clinical silence of these infiltrates in subclinical rejection [40].

Because the superiority of new agents such as rapamycin has not been validated, optimizing calcineurin inhibitor therapy as the backbone of immunosuppression in solid organ transplantation is warranted. Calcineurin inhibitors act like a double-edged sword with proven immunologic control leading to revolutionary improvement in short term graft survival on the one side, and induction of chronic nephrotoxicity and persistent structural damage on the other side. We conclude that this dilemma can be appropriately approached by a tight controlled systemic exposure of these

drugs based on AUC guided dosing, in combination with quadruple prophylactic immunosuppression.

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Chapter 7

Untreated rejection in 6-month protocol biopsies is not associated with fibrosis in serial biopsies or with loss of graft function

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Abstract

Background: Donor age, Calcineurin inhibitor (CNI)-nephrotoxicity and acute rejection are the most significant predictors for chronic allograft nephropathy (CAN). Protocol biopsies, both in deceased and living donor renal grafts, have shown that cortical tubulo-interstitial fibrosis (TIF) correlates with graft survival and function. The impact of not treating subclinical acute rejection (SAR) is less clear.

Methods: 126 *de novo* renal transplant recipients were randomized to receive AUC-controlled exposure of either a cyclosporine or tacrolimus-based immunosuppressive regimen including steroids, MMF and basiliximab induction. Protocol biopsies were taken before, 6 and 12 months after transplantation. The prevalence of SAR was determined retrospectively. Fibrosis was evaluated by quantitative digital analysis of Sirius red staining in serial biopsies.

Results: Donor age correlated significantly with TIF in pre-transplant biopsies and inferior graft function at month 6 ($r_\tau = -0.26$; $p = 0.033$). Acute rejection incidence was 11.5% and no clinical late rejection occurred. The prevalence of SAR at 6 months was 30.8%, but not associated with differences in serial quantitative in Sirius red staining at 6 or 12 months, proteinuria or progressive loss of GFR up to 2 years. No differences were found in donor variables, histocompatibility, rejection history or exposure of immunosuppressants.

Conclusion: Controlled individualized CNI-exposure and subsequent tapering resulted in a low early acute rejection rate and prevented late acute rejection. Since, by design, we did not treat SAR, these results provide evidence that asymptomatic infiltrates in 6-month surveillance biopsies may not be deleterious in the intermediate term. There is need for reliable biomarkers to prove that not all cell infiltrates are equivalent or that infiltrates may change with time.

Introduction

Chronic allograft nephropathy (CAN) is the most prevalent cause of renal graft failure in studies that censored for patient death. The histopathology is not specific and consists of graft atherosclerosis, multilayering of the peritubular capillaries, transplant glomerulopathy and glomerulosclerosis, interstitial fibrosis and tubular atrophy [1]. At present it remains unknown to what extend these lesions result from an alloimmune response or from other types of injury, including ischemia/reperfusion, earlier (subclinical) acute rejection episodes, hypertension, hyperlipidemia, diabetes mellitus and drug-related nephrotoxicity [2].

The calcineurin inhibitors (CNI), cyclosporine microemulsion (CsA) and tacrolimus (Tac), play a controversial role in this process, because their immunosuppressive efficacy is counterweighted by their nephrotoxic potential and unfavorable cardiovascular profile, including hypertension, hyperlipidemia and now-onset diabetes [3-5]. Most studies that compared CsA- with Tac-based regimen, have reported an advantage for Tac in the prevention of acute rejection [6,7] as well as subclinical acute rejection (SAR) [8]. Despite the improvements seen with contemporary immunosuppressive regimen, SAR still has a high prevalence largely depending on the time-point after transplantation at which a biopsy is taken [9-12].

Protocol biopsies that were obtained two years after transplantation identified donor age, acute rejection and CNI-nephrotoxicity as the most significant predictors for CAN [13]. Studies with surveillance biopsies, both in deceased and living donor renal grafts, agree that interstitial fibrosis correlates with renal graft survival and with graft function at 2 year [14,15]. Sirius red, a specific staining for collagen types I and III, is considered a precise and reliable estimate of renal cortical extracellular matrix accumulation and represents the best parameter to predict the development of progressive renal failure [15,16].

We developed a population-based model with limited sampling and Bayesian estimation to standardize systemic drug exposure for CsA and Tac after renal transplantation [17,18]. We applied these Bayesian estimators, to compare an exposure-controlled dosing strategy to prevent structural changes, quantified by morphometric analysis of non-polarized Sirius Red staining of 6 and 12 months protocol biopsies. In a previous study, we found no difference in the profibrogenic effect as measured by Sirius red staining of either one of the currently available CNIs

in biopsies obtained 6 and 12 months after renal transplantation[19]. At the molecular level, no differences were found in the extent of protein deposition of TGF-β and interstitial collagens, as well as cortical mRNA levels of TGF-β, collagens α1(I) and α1(III) [20].

The histologic features of CAN, although most often mild, may occur early after renal transplantation, with a reported prevalence up to 40% in surveillance biopsies performed as early as 3 months after transplantation [21,22]. Treatment of SAR in 1-, 2- or 3-month protocol biopsies support the clinical relevance of early detection and treatment of SAR [9]. However, there is no doubt that extra boluses of steroids or recycling of the steroid taper add significantly to post-transplant morbidity. The aim of the present study was to evaluate the impact of not treating SAR at 6 months in the context of a prospective study with AUC-controlled systemic exposure on chronic rejection, quantified by histomorphometric analysis of non-polarized Sirius red staining, in serial protocol biopsies at 6- and 12-months after transplantation.

Materials and Methods

Patients

Eligible patients were renal transplant recipients ≥ 18 yr of age, scheduled to receive a first or second graft, from a deceased (non-heart beating included) or living (non HLA-identical) donor. We excluded sensitised patients (panel reactive antibodies > 50%), and patients receiving a dual organ transplant. Because it has been estimated that at least 23 biopsies per treatment modality are required to demonstrate a 10% difference in renal allograft fibrosis as quantified by non-polarized Sirius Red staining [23], we decided to include at least 120 patients. Between October 2000 and October 2002 126 renal transplant recipients were randomly assigned to receive either a cyclosporine- or tacrolimus-based immunosuppressive regimen. This open-label, prospective randomized trial was conducted in Leiden and Amsterdam, the Netherlands. Formal approval from the institutional ethics committee was obtained at the participating sites, and written informed consent was given before enrollment in the trial.

Monitoring of calcineurin-inhibitors

Cyclosporine microemulsion, Neoral® (CsA) and tacrolimus, Prograf® (Tac) were started orally 3 hours before surgery (initial dose 4 mg/kg b.i.d. for CsA and 0,1 mg/kg b.i.d. for Tac). CsA and Tac $AUC_{0\text{-}12h}$ were estimated at week 2, 4, 6, 8, 12, 17, 21, 26, 39 and 52 using a population based 2-compartmental pharmacokinetic model combined with limited sampling and Bayesian fitting [17,18]. After each AUC-assessment dose adjustments were made to reach predefined target AUCs: CsA-$AUC_{0\text{-}12h}$: 5400 ng.h/ml within the first 6 weeks and after 6 weeks 3250 ng.h/ml. Tac-$AUC_{0\text{-}12h}$ was aimed at 210 ng.h/ml, within the first 6 weeks, and after 6 weeks 125 ng.h/ml. Immunosuppressive co-medication existed of prednisolon (in both groups 100 mg at days 1 − 3, tapered to 10 mg after day 22), mycophenolate mofetil (MMF 1000 mg b.i.d. in the CsA group and 500 mg b.i.d. in the Tac group) and basiliximab prophylaxis (20 mg days 0 and 4).

Protocol biopsies

Biopsies obtained at 6 and 12 months were taken as part of the study protocol with a 14-gauge needle. Renal biopsies, blinded for the treatment group, were scored by two independent pathologists according to the Banff '97 working classification [24]. In order to minimize the inter-observer variability, every fifth biopsy was scored by both pathologists, and the findings were discussed. Presence of SAR was based on the absence of functional deterioration and histologic findings indicative of rejection based on the tubulitis ("t") and mononuclear-cell-infiltration ("i") scores. Abnormal findings were divided into two categories: borderline SAR (bSAR) including "t"-score = 1 and "i"-score >0 and SAR defined by "t"-score = 2, and "i"-score ≥ 0. Chronic allograft nephropathy (CAN) was divided into three grades, based on the Banff scores for interstitial fibrosis (ci) and tubular atrophy (ct). Morphometry of Sirius Red stained specimens was performed using a Zeiss microscope equipped with full colour 3CCD camera and KS-400 image analysis software from Zeiss-Kontron. Computerized digital analysis was used to quantify the area of staining in each slide. An average of ten microscopic sections was examined from each slide. Using the X20 objective, an average of 63,2% ± 19.5 % of the total cortex was analysed in each biopsy.

Treatment of acute rejection episodes

A clinically suspected acute rejection episode was confirmed by a percutaneous graft biopsy and graded according to the Banff-'97 classification [24] by an experienced local renal pathologist. An acute rejection episode was treated with methylprednisolone (Solu-Medrol) 1000 mg per day for three consecutive days. Rabbit anti Thymocyte Globulin (r-ATG, Merieux) was given in case of a steroid-resistant rejection episodes and for a second acute rejection episodes. Rejection was considered steroid-resistant if no stabilization or improvement within 20% of baseline creatinine occurred within 7 days after treatment with Solu-Medrol. By design surveillance biopsies were scored retrospectively and SAR was not treated.

Efficacy parameters and side effects

Renal function (creatinine clearance) was calculated by the Nankivell formula [25] and determined at 6 weeks, 3, 6, 12 and 24 months post-transplantation. Other secondary efficacy parameters included the following: patient and graft survival, incidence and severity of acute rejection episodes, blood pressure, lipids and HbA1c. The incidence of new-onset diabetes mellitus was defined by the need for any antidiabetic drug (insulin and/or oral antidiabetic drugs), in the absence of diabetes at baseline. Prescription of lipid lowering drugs (statins) and the number of antihypertensive drugs were recorded during the study.

Statistical analyses

All analyses were performed according to the intention-to-treat principle. Results are given as mean ± standard deviation for interval and ordinal variables. Frequencies of nominal (categorical) variables are given as numbers and percentages. For comparison between two categories of interval variables Student's independent samples t-test was used. If statistical assumptions for parametric analysis of interval variables were not met and in case of ordinal data Mann-Whitney's two-independent samples test was used and exact p-values calculated. Nominal variables were analyzed with cross-tables and exact significance was calculated. The incidence of acute rejection and diabetes mellitus was estimated using Kaplan-Meier's product-limit method and the resulting curves were compared with log-rank test. All analyses were performed using SPSS statistical software package (version 10.07; SPSS Inc. Chicago Ill.).

Results

Patient characteristics

A total of 126 patients were randomly assigned for treatment with either CsA or Tac. The treatment groups were well matched for demographic and transplant characteristics (Table 1). In the CsA treated group more patients received a second transplant (eight patients versus one in the Tac group). Only one of these patients experienced an acute rejection episode and in the analyses this inequality in distribution did not confound the comparison between the treatment groups.

AUC-guided dosing

Figure 1 shows to what extent the actual measured AUCs of cyclosporine and tacrolimus were kept near the target AUCs. MMF was given in a fixed dose in both groups, being 1000 mg b.i.d. in the CsA group and 500 mg b.i.d. in the Tac group. The mean MPA $AUC_{(0\text{-}12)}$ at 2, 6, 12, 26 and 52 weeks were 23.4, 39.7, 46.9, 44.7 and 43.1 ng.h/ml in the CsA patients; and 20.6, 28.4, 29.5, 28.0 and 30.4 ng.h /ml in the Tac patients, respectively. Based on the known interaction between CsA and MMF [26], we hypothesized that this choice would result in a comparable mean exposure to MPA in both groups. Beyond week 6, however, the MPA-AUCs in the CsA treated patients were consistently and significantly higher, most likely as a result of the defined reduction in the target AUC for CsA beyond six weeks.

Patient and graft survival

One-year patient survival was 96.8 vs 98.4%, and one-year graft survival was 95.2 vs 90.5% in CsA and Tac treated patients, respectively. In the CsA group there were two deaths and one patient underwent transplantectomy at day 320 after transplantation because of post-transplantation lymphoproliferative disorder, localized in the graft. In the Tac group one patient died, two patients experienced primary non function after non-heart beating donor procedures, two patients with early graft loss after surgical complications and one additional patient experienced graft loss due to acute humoral rejection.

Table 1: Demographic and transplantation characteristics at baseline

Characteristic	**Cyclosporine** (n = 63)	**Tacrolimus** (n = 63)	**p-value**
Recipient age (yrs)	46.5 ± 14.1	48.5 ± 12.6	0.40
Recipient sex (% male)	71.4	68.3	0.85
Race (% Caucasian)	81.0	85.7	0.44
Diabetes Mellitus at baseline, (%)	11.1	3.2	0.16
Primary disease, %			
glomerulonephritis	14 (22.2)	11 (17.5)	0.46
systemic autoimmune disease	3 (4.8)	1 (1.6)	(overall)
focal sclerosis	4 (6.3)	4 (6.3)	
diabetic nephropathy	2 (3.2)	1 (1.6)	
hypertension	8 (12.7)	11 (17.5)	
hereditary disease	11 (17.5)	12 (19.0)	
congenital dysplasia/reflux	8 (12.7)	2 (3.2)	
interstitial disease	1 (1.6)	2 (3.2)	
etiology uncertain	12 (19.0)	19 (30.2)	
Warm ischemia (min)	39.1 ± 14.1	35.9 ± 12.4	0.18
Cold ischemia (hrs)	14.1 ± 9.8	13.8 ± 10.7	0.88
Donor age (yrs)	47.8 ± 15.8	47.4 ± 15.7	0.87
Donor sex (% male)	49.2	52.4	0.86
Procedure, (%)			
Deceased Donor, Heart Beating	36.5	34.9	0.73
Deceased Donor, Non-Heart Beating	27.0	20.6	(overall)
Living Donor, related	22.2	23.8	
Living Donor, unrelated	14.3	20.6	
HLA mismatches	2.51 ± 1.44	2.67 ± 1.78	0.58
Class I mismatches	1.73 ± 1.12	1.78 ± 1.30	0.83
Class II mismatches	0.78 ± 0.61	0.89 ± 0.74	0.36
Previous transplant, (%)	12.7	1.6	0.03
Delayed graft function, (%) (living donor excluded)	42.5	40.0	0.68

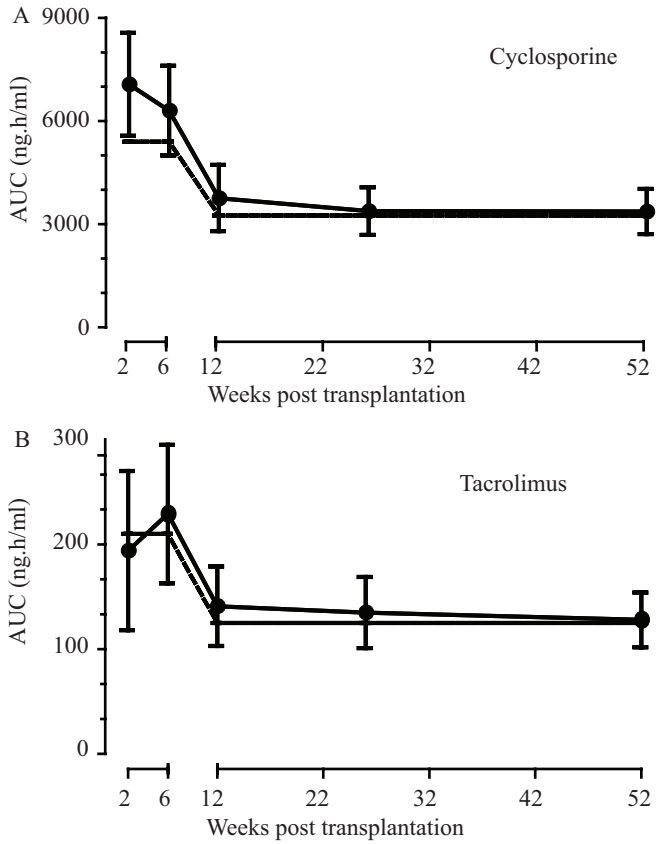

Figure 1: Area-under-the-concentration-over-time curves (AUC ± SD; ng.h/ml) using the Bayesian estimator to guide dosing of Cyclosporine (A) or Tacrolimus (B) in de novo renal transplant recipients (dotted lines indicate the predefined AUC targets).

Acute rejection

The cumulative incidence of acute rejection episodes in this study at 6-months was 11.5% (14/126), no late clinical acute rejection occurred (Figure 2). The incidence of biopsy proven acute rejection episodes was higher in patients randomized to CsA (16.2%), as compared to patients receiving Tac (6.6%). Although clinically relevant, this difference did not reach statistical significance ($p = 0.12$) in this relatively small cohort of patients. Steroid-resistance, requiring ATG therapy, occurred in 6 and 2% of CsA and Tac treated patients, respectively.

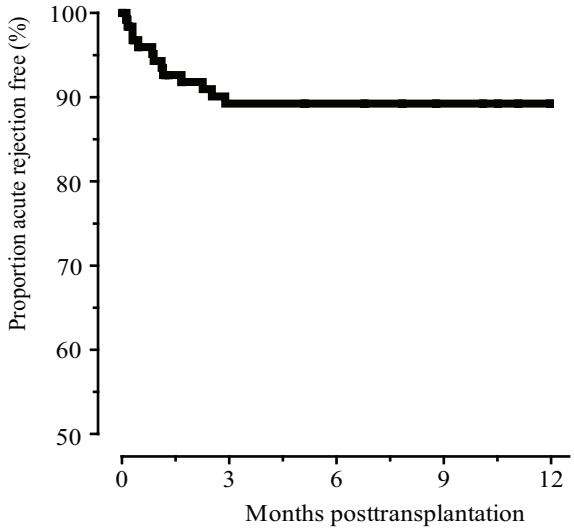

Figure 2: Proportion acute rejection free renal transplant recipients randomly assigned to receive controlled systemic exposure and tapering of cyclosporine or tacrolimus.

Protocol biopsies

At 6 months, biopsies were available in 83% and 82% of the patients randomized to CsA or Tac, respectively. At 12 months these percentages were 88% for the CsA group and 84% for the Tac group. Reasons for missing were primarily "not enough specimen for proper Banff classification scoring", increased bleeding risk and secondary refusal by patients to undergo a protocol biopsy. No quantitative differences in the degree of renal fibrosis were observed between CsA and Tac at 6 months (mean% Sirius red positive area ± SD; CsA: 12.5 ± 3.7% vs. Tac: 12.4 ± 5.4%, p = 0.78) or at one year (CsA: 13.7 ± 5.6 % vs. Tac: 13.3 ± 5.9%; p = 0.73).

Subclinical acute rejection (SAR)

The prevalence of SAR at 6 months was 30.8% (bSAR 23.4% and SAR 7.4%). The prevalence was significantly (p = 0.012) higher in patients who received CsA therapy (38.8%: bSAR 24.5 and SAR 14.3%), as compared to Tac (15.5%: bSAR 11.1 and SAR 4.4%). Prevalence of SAR at 12 months was 19% (CsA 16%, Tac 22%, ns). We analyzed transplant- and outcome characteristics in patients according to the

presence or absence of SAR in the 6 months biopsies (Table 2). No differences in donor age, degree of histocompatibility for HLA antigens or delayed graft function were found. The percentage of patients with previous episodes of acute rejection was also comparable (10.3 versus 10.8% with or without SAR, respectively). SAR in the 6-month biopsy was found more frequent with CAN-grade ≥ 2 in the same biopsy, but was not associated with differences in Sirius Red stained cortical area in 6 and 12 months serial biopsies, proteinuria or progressive loss of GFR (Figure 3). No difference in systemic exposure of immunosuppressants, either in the CsA or Tac treated patients or MMF was found. These results are summarized in Table 3.

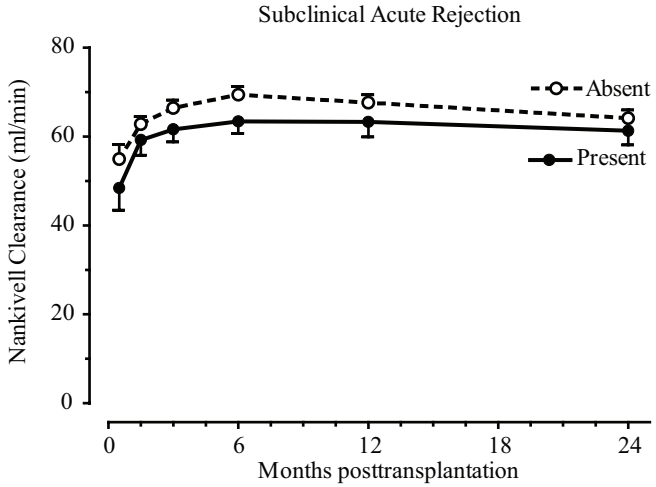

Figure 3: Renal allograft function over time according to the presence of Subclinical Acute Rejection in 6-months protocol biopsies in renal transplant recipients randomly assigned to receive controlled systemic exposure and tapering of cyclosporine or tacrolimus.

Chronic allograft nephropathy (CAN-score)

The prevalence of CAN in the 6 months protocol biopsies was 53% (CsA 51%, Tac 57%) and 63% at one year (CsA 61%, Tac 65%). At 6 months CAN grade 1 was found in the majority of cases (76%) and grade 2 or 3 in 20% and 4%, respectively. The analyses according to Banff CAN-grade at 6 months are summarized in Table 4. CAN-grade ≥ 2 was associated with kidneys from elderly deceased donors, in particular over 60 years of age. Donor age correlated significantly (r_τ = 0.34;

p = 0.003) with cortical tubulo-interstitial fibrosis in pre-transplant biopsies. Fibrosis in the pre-transplant biopsy correlated with inferior graft function at week 6 (r_τ= −0.32; p = 0.007), month 3 (r_τ = –0.26; p =0.029) and month 6 (r_τ = –0.26; p = 0.033). The CAN-score correlated significantly with the percentage of Sirius red positive cortical area (p < 0.001). Inferior function was found in biopsies with CAN-grade ≥ 2 (Figure 4), but not with concomitant SAR, and correlated with a significantly higher proportion of kidneys from deceased and older donors (Table 5). The prevalence of SAR in the 6-month biopsies showing CAN ≥ 2 was significantly higher (58.3 vs 26.8%; p < 0.05), but no differences in Sirius red stained cortical area in 12 month biopsies (p = 0.93), degree of proteinuria or progressive loss of renal function was found (Table 5). There were no differences with respect to matching, delayed graft function or acute rejection history. No correlation between CAN and initial or maintenance systemic exposure for CsA, Tac or MMF was found (Table 4).

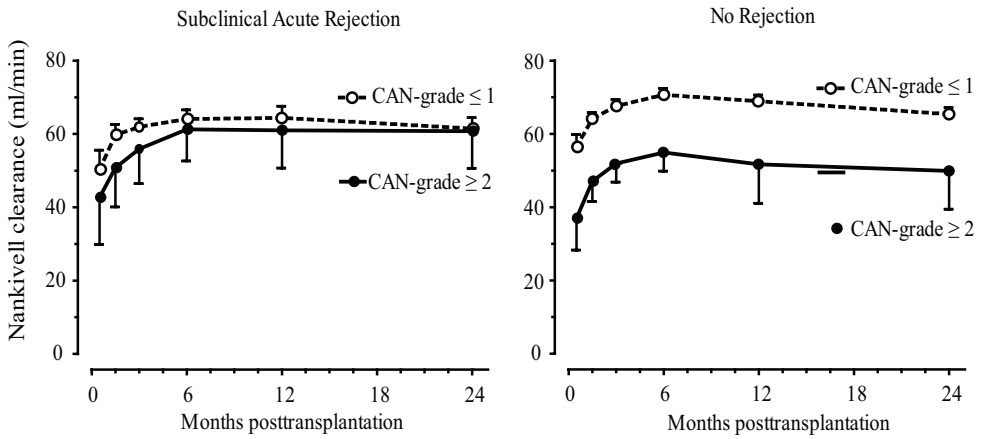

Figure 4: Renal allograft function over time according to Banff-criteria acute rejection and CAN-grade in 6-months protocol biopsies in renal transplant recipients randomly assigned to receive controlled systemic exposure and tapering of cyclosporine or tacrolimus.

Antihypertensive drugs, lipid lowering therapy, new-onset diabetes

No significant differences in systolic or diastolic blood pressure were observed between the two groups, but Tac treated patients tended to require less antihypertensive drugs (Table 6). Cholesterol levels at 6 and 12 months were significantly higher

in the CsA group, despite a slightly higher proportion of patients receiving lipid lowering drugs. New-onset diabetes mellitus occurred significantly (p = 0.007) more frequent in patients randomized to Tac as compared to CsA (Figure 5). No patients were reported in whom all antidiabetic drugs could be discontinued. Within the Tac group an AUC over 210 ng.h/ml at week 2 was significantly associated with a higher probability to develop diabetes (Odds ratio 4.00, p = 0.05).

Table 2: Characteristics according to the presence of subclinical acute rejection in 6-month protocol biopsies

Characteristic		**Present** (n = 26)	**Absent** (n = 68)	*p-value*
Transplant characteristics				
Recipient age (yr)		45.1 ± 12.5	48.1 ± 13.7	*0.32*
Recipient sex (%male)		79.3	76.9	*0.80*
Recipient race non Caucasian %		10.3	4.6	*0.37*
Donor age (yrs)		45.7 ± 16.6	47.1 ± 14.7	*0.68*
Donor > 60 yr (%)		27.6	21.5	*0.60*
HLA mismatches		2.48 ± 1.35	2.71 ± 1.76	*0.62*
Class I mismatches		1.72 ± 1.03	1.83 ± 1.32	*0.70*
Class II mismatches		0.76 ± 0.69	0.88 ± 0.67	*0.44*
Delayed graft function (%)		27.6	21.5	*0.60*
Previous acute rejection episodes (%)		10.3	10.8	*0.95*
Protocol biopsies:				
CAN grade ≥ 2 (%)	− 6 mo	24.1	7.7	*< 0.05*
	− 12 mo	28.0	19.3	*0.39*
Sirius red stained cortical area (%)	− 6 mo	12.3 ± 4.3	12.1 ± 4.7	*0.84*
	− 12 mo	13.2 ± 5.2	13.8 ± 5.1	*0.58*
Clinical parameters				
GFR (ml/min)	− 6 mo	63.4 ± 14.6	9.4 ± 14.5	*< 0.05*
	− 12 mo	63.3 ± 18.2	67.6 ± 14.7	*< 0.05*
	− 24 mo	61.3 ± 15.1	64.1 ± 16.1	*0.43*
Proteinuria (g/day)	− 6 mo	0.37 ± 0.32	0.30 ± 0.23	*0.16*
	− 12 mo	0.34 ± 0.34	0.32 ± 0.49	*0.16*

Table 3: Banff scores in 6-month protocol biopsies and systemic exposure of calcineurin inhibitors and MMF

			CAN-grade			SAR-score		
			≥ 1	0		SAR	noSAR	
	$AUC_{(0-12)}$	*Dosing scheme*			*p-value*			*p-value*
Week 2	CsA	*8 mg/kg/day*	7076 ± 1768	7393 ± 1896	*0.55*	6879 ± 1944	7711 ± 1596	*0.15*
	MMF	2 g/day	24 ± 7	23 ± 11	*0.62*	23 ± 8	24 ± 11	*0.87*
	Tac	*0.2 mg/kg/day*	194 ± 90	195 ± 58	*0.43*	168 ± 46	191 ± 59	*0.30*
	MMF	1 g/day	21 ± 11	20 ± 11	*0.85*	21 ± 7	21 ± 11	*0.86*
Week 6	CsA	*BE*[1]	6364 ± 1385	6447 ± 1313	*0.86*	6817 ± 1483	6109 ± 1161	*0.07*
	MMF	2 g/day	40 ± 22	38 ± 18	*0.68*	41 ± 24	38 ± 17	*0.81*
	Tac	*BE*	250 ± 68	237 ± 66	*0.69*	222 ± 48	250 ± 70	*0.27*
	MMF	1 g/day	24 ± 17	30 ± 17	*0.58*	19 ± 9	29 ± 18	*0.31*
Month 6	CsA	*BE*	3409 ± 571	3365 ± 808	*0.60*	3246 ± 614	3478 ± 722	*0.29*
	MMF	2 g/day	50 ± 16	40 ± 14	*0,10*	51 ± 21	46 ± 10	*0.48*
	Tac	*BE*	150 ± 33	140 ± 36	*0.81*	145 ± 18	142 ± 36	*0.81*
	MMF	1 g/day	25 ± 13	30 ± 16	*0.82*	29 ± 8	27 ± 16	*0.78*

[1] BE Bayesian estimation method

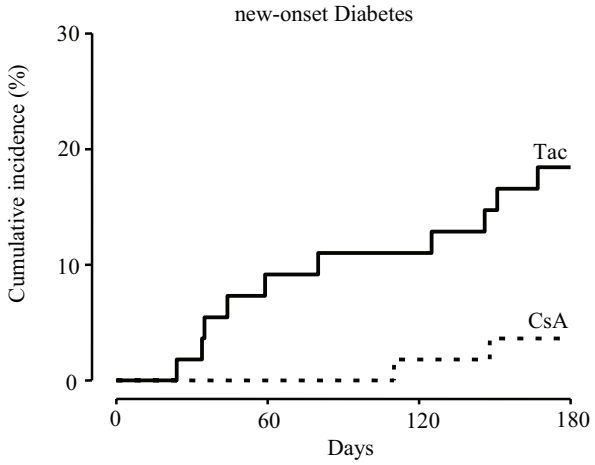

Figure 5: Cumulative incidence of new-onset diabetes mellitus at 6 months in renal transplant recipients randomized to receive controlled systemic exposure and tapering of cyclosporine or tacrolimus.

Table 4: Transplant characteristics, histological and clinical parameters according to CAN-grade in 6-month protocol biopsies

	CAN−grade			**CAN−grade**		
	≥ 1	**0**	*p-value*	**≥ 2**	**≤ 1**	*p-value*
Transplant characteristics						
Recipient age (yr)	45.6 ± 13.6	49.0 ± 13.0	*0.21*	52.6 ± 9.6	46.4 ± 13.7	*0.16*
Donor age (yr)	48.8 ± 14.6	44.3 ± 15.8	*0.13*	51.3 ± 19.4	46.0 ± 14.6	*0.12*
Donor > 60 yrs (%)	30.0	15.9	*0.14*	58.3	18.3	*< 0.01*
L(U)RD (%)	36.0	45.5	*0.40*	16.7	43.9	*0.11*
HLA mismatch:						
− *total*	2.6 ± 1.8	2.7 ± 1.4	*0.54*	2.3 ± 1.5	2.7 ± 1.7	*0.27*
− *class I*	1.7 ± 1.3	1.9 ± 1.4	*0.36*	1.6 ± 1.2	1.8 ± 1.3	*0.53*
− *class II*	0.9 ± 0.7	0.8 ± 0.6	*0.65*	0.7 ± 0.7	0.9 ± 0.7	*0.36*
Delayed graft function (%)	26.0	20.5	*0.63*	41.7	20.7	*0.14*
Previous acute rejection (%)	10.0	11.4	*1.00*	8.3	11.0	*1.00*
Month 6 protocol biopsy						
Sirius red stained cortical area (%)	13.0 ± 4.7	11.0 ± 4.2	*< 0.05*	14.6 ± 3.5	11.8 ± 4.6	*< 0.05*
Subclinical Acute Rejection (%)	34.0	27.3	*0.51*	58.3	26.8	*< 0.05*
Clinical parameters						
GFR (ml/min)						
− *at mo 6*	65.8 ± 14.3	69.6 ± 15.2	*0,14*	58.6 ± 18.5	68.9 ± 13.8	*< 0.05*
− *at mo12*	65.3 ± 17.6	67.4 ± 13.8	*0.38*	56.5 ± 25.2	67.7 ± 13.7	*< 0.05*
− *at mo24*	62.8 ± 16.5	63.9 ± 14.1	*0.44*	55.8 ± 23.6	64.4 ± 13.7	*< 0.05*
Proteinuria (g/day)						
− *at mo 6*	0.32 ± 0.26	0.32 ± 0.26	*0.66*	0.52 ± 0.45	0.29 ± 0.21	*< 0.05*
− *at mo12*	0.32 ± 0.30	0.34 ± 0.58	*0.45*	0.53 ± 0.53	0.30 ± 0.43	*< 0.05*

Table 5: Characteristics according to the presence of SAR and the CAN-grade in 6-month protocol biopsies

	Subclinical Acute Rejection at 6-months			
	Present		*Absent*	
	CAN ≤ 1	CAN ≥ 2	CAN ≤ 1	CAN ≥ 2
Biopsy month 12				
CAN-grade ≥ 2 (%)	17	57	15	60
Arteriolar hyalinosis (0 − 3)	0.55 ± 0.64	1.00 ± 1.00	0.48 ± 0.73	1.00 ± 1.23
Proteinuria (g/day)				
Month 6	0.33 ± 0.20	0.49 ± 0.56	0.28 ± 0.22	0.55 ± 0.27
Month 12	0.29 ± 0.15	0.53 ± 0.65	0.31 ± 0.50	0.54 ± 0.36
Graft function (ml/min)				
Month 6	64 ± 12	61 ± 23	71 ± 14	55 ± 11
Month 12	64 ± 15	60 ± 27	69 ± 13	52 ± 24
Month 24	61 ± 13	60 ± 24	65 ± 14	50 ± 23
Donor-factors				
Deceased donor (%)	68	86	52	80
Donor age (yr;SD)	45 ± 15	47 ± 23	46 ± 15	57 ± 13

Table 6: Cardiovascular side effect profile in renal transplant recipients with controlled systemic exposure of cyclosporine or tacrolimus.

	Cyclosporine		**Tacrolimus**		
	Mean +/-SD	*N*	Mean +/-SD	*N*	*p-value*
Proteinuria (g/day)					
Month 6	0.36 ± 0.35	61	0.28 ± 0.21	58	*0.56*
Month 12	0.35 ± 0.34	60	0.32 ± 0.52	57	*0.10*
Systolic BP (mmHg)					
Baseline	144 ± 20	63	142 ± 21	60	*0.58*
Month 6	141 ± 17	61	139 ± 14	58	*0.51*
Month 12	140 ± 17	61	139 ± 16	56	*0.72*
Diastolic BP (mmHg)					
Baseline	82 ± 12	63	84 ± 11	60	*0.32*
Month 6	82 ± 10	61	82 ± 8	58	*0.99*
Month 12	81 ± 10	61	82 ± 9	56	*0.59*
Antihypertensive drugs (n)					
Baseline	1.5 ± 1.1	62	1.4 ± 1.1	60	*0.68*
Month 6	1.8 ± 1.0	61	1.5 ± 1.0	58	*0.25*
Month 12	1.8 ± 1.1	61	1.6 ± 1.0	56	*0.14*
Total cholesterol					
Baseline	4.85 ± 1.18	60	4.98 ± 1.36	59	*0.58*
Month 6	5.86 ± 1.33	58	5.34 ± 1.26	55	*0.04*
Month 12	5.71 ± 1.22	59	5.26 ± 1.06	56	*0.04*
HDL cholesterol					
Baseline	1.13 ± 0.42	60	1.09 ± 0.30	58	*0.56*
Month 6	1.38 ± 0.46	58	1.34 ± 0.46	55	*0.65*
Month 12	1.39 ± 0.43	59	1.36 ± 0.43	56	*0.70*
Statin use(%)					
Baseline	31	61	26	61	*0.69*
Month 6	31	61	24	57	*0.54*
Month 12	43	60	32	56	*0.25*
Uric acid					
Baseline	0.33 ± 0.10	60	0.37 ± 0.13	55	*0.04*
Month 6	0.44 ± 0.11	60	0.44 ± 0.14	55	*0.83*
Month 12	0.46 ± 0.10	58	0.45 ± 0.12	53	*0.86*

Discussion

This is the first study to evaluate the impact of subclinical acute rejection (SAR) on fibrosis in serial protocol biopsies under controlled systemic exposure of one of the currently available CNIs. Banff-criteria acute rejection was found in 30.8% of protocol biopsies performed 6 months after transplantation, with a significantly higher prevalence in CsA-treated transplant recipients. The mere presence of SAR,

however was not associated with more interstitial fibrosis in serial biopsies, degree of proteinuria or progressive loss of graft function up to two years of follow-up. Because, by protocol, we did not treat SAR, these results provide evidence that the vast majority of asymptomatic infiltrates in 6-month surveillance biopsies may not be deleterious, at least in the intermediate term, and could be adaptive rather than destructive. Sirius red, a specific staining for collagen types I and III, is considered a precise and reliable estimate of renal cortical extracellular matrix accumulation [15,16]. Types I and III collagen represent 80% and 15 to 20%, respectively, of the total collagen synthesized by fibroblasts, and especially the early accumulation of collagen type I, along with collagen III and IV, was found to be a more specific marker for chronic rejection [28].

Comparable to previous observations, donor age and fibrosis in the pre-transplant biopsy was associated with inferior graft function [29,30]. Donor age or quality of the donor organ at implantation was not associated with a higher prevalence of SAR at 6 months. Although SAR was found more frequently in combination with moderate to severe CAN, the presence of these interstitial infiltrates was not associated with differences in serial quantitative Sirius red staining, proteinuria or renal allograft function. Recently a similar incidence and severity of interstitial fibrosis in surveillance biopsies from living and deceased donor kidneys was found, challenging the impact of injury related to brain death and/or prolonged preservation [14]. In addition protocol biopsies of kidneys from a heart beating or non-heart beating donor revealed no significant difference in quantitative Sirius red staining in relation to prolonged periods of warm ischaemia [31].

Results from studies that focus on optimal therapeutic index of CNI depend on the drug monitoring strategy that is used and especially the target ranges of drug levels that are accepted. Trough levels of both CsA and Tac are not closely correlated with either drug exposure [17,18,32-34], the risk of allograft rejection [35,36] or their side effects [37,38]. The area-under-the-concentration over time curve ($AUC_{0\text{-}12h}$) and derived parameters, such as absorption profiles, have been shown to correlate more closely with clinical outcome, also in patients who received prophylaxis with basiliximab [39].

One-year post transplant the prevalence of SAR has largely abated, indicating that, at a later stage, progressive histologic alterations are dominated by CNI-related vasculopathy and/or glomerulosclerosis [8]. Most tubular atrophy and fibrosis begin

early after transplantation as a consequence of the summated effects of tissue injury from ischemia-reperfusion, acute rejection [14] and/or, given the initial higher dosing, from early (chronic) CNI-nephrotoxicity [21,27]. This notion is supported by a study that investigated protocol biopsies obtained two years after transplantation [13]. Histopathologic signs of CAN were reported in 62.0% of the patients treated with Tac and in 72.3% of the CsA treated patients, which occurred in the absence of discernible changes in renal function. Renal transplant function is an inadequate measure of ongoing tissue injury either due to ongoing rejection or early interstitial CNI-toxicity, but quantitative histomorphometry using Sirius red at 6 months has been shown to correlate strongly with glomerular filtration rate in patients with established CAN and time to graft failure [15,16]. The semi-quantitative cumulative Banff algorithm, dominated by interstitial fibrosis and tubular atrophy, may also not be precise enough. The assessment of the severity of CAN in serial biopsies and, to a lesser extent, SAR is not only subjected to significant inter-observer variability but also lacks specificity [40-42]. Laminin β2 and TGF-β mRNA levels in the renal cortex, however, allow for distinguishing chronic CNI-toxicity from chronic rejection with high sensitivity and specificity [43]. The nephrotoxic potential of cyclosporine and tacrolimus is indistinguishable either clinically [6,44], histologically [19] or at the renal molecular level [20]. Six months after implantation no significant differences were found in the extent of protein deposition of as well as cortical mRNA levels of TGF-β, but a wide variability within both treatment groups [20]. TGF-β expression in biopsies performed 3-, 6- and 12 months after transplantation was associated with a significant increase in interstitial fibrosis [45].

Under controlled systemic exposure and subsequent tapering graft fibrosis accompanied by subclinical acute rejection in the same biopsy 6 months was not associated with progressive damage or functional impairment. In contrast to previous studies we observed no late clinical acute rejection and the AUC-methodology makes inadequate immunosuppression an unlike cause of ongoing rejection [9,30,37]. The risk profile for inferior function at 2 years included inadequate early immunosuppression with CsA, poorer matching for HLA-class I antigens and late clinical acute rejection [10,30]. The occurrence of late clinical acute rejection in these studies was significantly associated with the development of an increased serum creatinine at 24 months or CAN at 1 year [10,30]. Evaluation of serial protocol biopsies that were taken from the first year in the subgroup of pediatric transplant recipients with established CAN, revealed

that the superimposition of recurrent or persistent SAR, but not borderline changes, was associated with histologic progression and subsequent functional deterioration. Treatment of acute rejection at 1 year with steroid pulses had no beneficial effect on the course of these cases [10]. It remains controversial whether SAR, borderline changes or even inflammation, generally not qualifying for borderline rejection, suggest a different functional prognosis with longer times of follow up [46].

SAR in protocol biopsies that were performed as early as 14 days after living donor transplantation, most likely represents a donor-specific immune response as it correlates with HLA-DR mismatching underscoring that clinical immunosuppression is imperfect [12,47]. Newer drug regimens significantly reduced the incidence of both early and late clinical acute rejection, but the prevalence of subclinical acute rejection remained essentially unchanged [48]. Untreated borderline infiltrates in clinical biopsies performed 2 to 3 months after transplantation tended to persist, but the majority (72%) did not progress to clinical acute rejection within the next 40 days [49]. Of note, cases with evidence of acute CNI-toxicity (58%) in addition to the borderline infiltrates had a low rate of progression to acute rejection despite CNI-dose reduction. We cannot exclude that a significant proportion of patients with SAR in earlier protocol biopsies (≤ 3 months) do benefit from additional treatment or a modification of their immunosuppressive regimen as suggested by the work of Rush *et al.* [9]. This study stratified for donor source, but patients in the control group were less well matched and received kidneys from older donors. Probably more important, however, treatment of early SAR up to month 3 resulted in a 3-fold decreased incidence of late (beyond month 6) clinical acute rejection [9]. The strong correlation between late acute rejection and chronic rejection as well as late graft loss has been reported consistently [37,50]. In the present study, under controlled systemic exposure and tapering of either CsA or Tac, early acute rejection rates were low and no late acute rejection occurred, indicating that individualized CNI-exposure may be at least as effective in the prevention of late clinical acute rejection as treating early subclinical acute rejection with high-dose steroids.

We clearly lack a reliable metric at the time of biopsy to decide whether additional treatment is beneficial, since there is no doubt extra boluses of steroids or recycling of the steroid taper add significantly to post-transplant morbidity. Minimization or preferentially elimination of CNI-nephrotoxicity, an important determinant for late

or progressive deterioration of graft function in a substantial proportion of renal and non-renal allograft recipients, appears to be a key decision to potentially modify long-term outcome [51,52]. At the present we lack a reliable metric to decide whether asymptomatic interstitial infiltrates represent ongoing rejection or the exponent of a regulatory response, self limiting, not harmful to the graft and with no need for drug adjustments We suggest delaying drug withdrawal or minimization until the risk of subclinical acute rejection is diminished or preferably ruled out by reliable biomarkers either in a surveillance biopsy [53] or in biological fluids. The relevance of AUC-guided monitoring may than be found in the control over drug exposure for the remaining drug or the drug that is considered for conversion [54].

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Chapter 8

Summary and general discussion

Kidney transplantation is the treatment of choice for most patients with end-stage renal failure. In recent years the graft and patient survival rates early after renal transplantation have improved progressively. This is probably the result of better general patient care, improving surgical methods and the introduction of newer immunosuppressive therapies. Further improvement is limited by the fact that about 40% of grafted kidneys are affected by "chronic transplant dysfunction" [1]. This clinical syndrome is described by progressive loss of function, occurring after the first three months post transplantation, in combination with hypertension and/or proteinuria. Histologically these kidneys show cortical tubulointerstial fibrosis, tubular atrophy, fibrous intimal thickening of arteries, and glomerulosclerosis, which are the pathological characteristics for "chronic allograft nephropathy" (CAN) [2,3], in itself a non-specific pathological, descriptive diagnosis. The exact mechanism underlying this clinical and histological entity is not known, but it most likely represents the end-result of a complex interplay between donor and recipient factors, of which initial tissue quality, ischemia reperfusion injury, acute and chronic rejection, infections and drug toxicity are the most important determinants [4]. This thesis focused on the options to influence or prevent this condition in renal transplants by refining the drug therapy prescribed to the patients.

Optimal function of the renal allograft is obtained by maintaining a balance between under-immunosuppression with acute rejection on the one hand, and over-immunosuppression resulting in drug-induced toxicities on the other. To minimize side effects while maintaining efficacy, immunosuppressive drugs are commonly used in combination therapy. Pharmacokinetic and pharmacodynamic interactions

between these agents can affect graft survival and function. The evidence supporting the role of therapeutic drug monitoring as applied to commonly used immunosuppressants in transplantation and the increasing role of therapeutic drug monitoring in the optimization of graft- and patient survival rates in the modern era of renal transplantation is the central theme of this thesis.

In **chapter 2** we reviewed the evidence from the literature that drug therapy may be of value to prevent or treat chronic allograft nephropathy. We reviewed immunosuppressive and non-immune therapies in use to treat risk factors associated with chronic allograft nephropathy and focused on their efficacy with respect to long-term outcome or their effect on surrogate markers of long-term survival. In the second part, we indicated potential benefits of novel approaches that have been explored, but most of these data were obtained either in *in-vitro* systems or in experimental animal models. We concluded that a lot of promising drugs and molecules had been studied, but also that evidence for beneficial effects of these therapies on the above mentioned long-term end-points was scarce. As a consequence, no radical change of immunosuppressive possibilities for renal transplant recipients is to be expected in the next decade, and calcineurin-inhibitors (CNIs) will remain the cornerstone drug in standard immunosuppressive regimens. Strategies without the use of these drugs are associated with inferior results, especially with respect to outcome parameters in the first post-operative year. On the other hand, the side effects of the available CNIs including nephrotoxicity, hypertension, hypercholesterolemia and de novo diabetes mellitus, are likely to have a negative impact on long-term results. Rather than developing or introducing newer drugs with unknown side effects and long-term outcome, a more sophisticated use of the "old", well-known drugs may in the intermediate term be a more rewarding method to improve renal allograft survival and quality of life for transplant recipients.

In **chapter 3 and 4** we focused on therapeutic drug monitoring of the current available calcineurin-inhibitors (CNI) cyclosporine-microemulsion (CsA) and tacrolimus (Tac). It has been shown that CsA trough levels do not correlate with clinical parameters such as acute rejection episodes and side effects. The CsA area under the drug concentration over time curve (AUC) is a better measure of systemic drug exposure, but AUC monitoring has not gained wide popularity because of the necessity and inconvenience of drawing multiple, well timed, blood samples.

In **chapter 3** we described the development and validation of a simple limited sampling model (LSM), based on a software package, which uses population based pharmacokinetics in combination with a Bayesian forecasting procedure. A strategy using 3 blood samples, pre-dose (0h) as well as 2 and 3 hours after drug intake, in combination with the model was sufficient to reliably estimate systemic drug exposure, expressed as the CsA AUC(0-12h). The performance of the population-based pharmacokinetic model to estimate the AUC was comparable to the performance of two earlier published LSMs. LSMs that depend on mathematical formulas become useless when one of the defined sampling time points is missed. Our model is however far more flexible since sampling times are not rigid and can be varied as long as dosing and sampling times are recorded accurately. Especially in simultaneous pancreas-kidney transplant (SPKT) patients the performance of our model was markedly better, as compared to the mathematical LSMs. SPKT patients, with their long history of type 1 diabetes mellitus, are known to show unpredictable drug absorption, mainly due to diabetic gastrointestinal dysfunction. When CsA trough levels are used, these patients are at increased risk for under- or overdosing (figure1). For this reason calcineurin-inhibitor absorption profiling is mandatory especially in this group of patients, for which the presented model provide a reliable and simple solution.

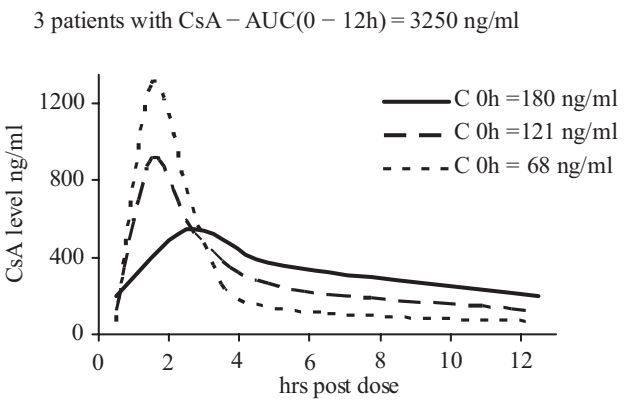


Figure 1: Three patients, on CsA therapy and AUC guided dosing with the PK model; all three reached the desired target AUC(0-12 h) of 3250 ng/ml. Dosing to reach trough levels of 125 nh/ml would lead to high systemic exposure in patient with low trough levels and high peak levels(dotted line) and to underexposure in patients with high trough levels and low peak levels (SPKT patient, solid line).

In **chapter 4** we used the population-based model to develop a strategy for patients on tacrolimus(Tac)-based immunosuppression. In this chapter we showed that also Tac trough levels are not precise enough to predict systemic drug exposure and that the population-based pharmacokinetic model combined with the Bayesian forecasting method and only one additive, but timed, sample is sufficient to reliably estimate the AUC(0-12h) and guide subsequent dosing (table 1). This concept was clinically tested in a prospective study in 15 consecutive *de novo* renal transplant recipients. In this cohort we showed that this approach was feasible in clinical transplantation and very useful to control the AUC within patients over time. Although, in the first postoperative months intra-individual variation in pharmacokinetic parameters made frequent AUC-estimation and dose adjustment necessary. After the first months only minimal intra-individual variation was detected, but with time drug bioavailability increased gradually, a phenomenon during the absorption phase, that is not signalized by trough level monitoring. We concluded that an AUC-monitoring strategy might help to prevent Tac-overdosing in these patients, because the method is sensitive enough to detect this increase in bioavailability.

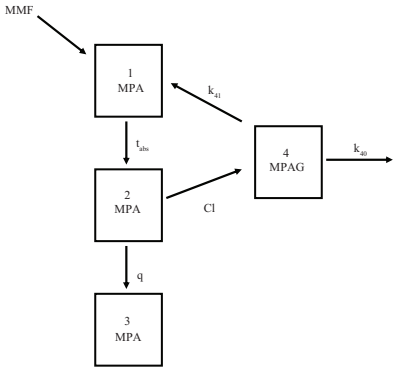
Table 1: Pearson correlation coefficients (r^2) of different strategies to estimate systemic exposure, compared with $AUC_{0\text{-}12h}$ determined by trapezoidal rule in renal transplant recipients treated with tacrolimus.

Sampling strategy	r^2 (All curves)	r^2 < 2wks post tx	r^2 > 2wks post tx
0h	0.79	0.67	0.78
2h	0.77	0.86	0.74
0h + 2h	**0.94**	**0.93**	**0.95**
0h + 3h	**0.96**	**0.97**	**0.96**
0h + 4h	**0.95**	**0.94**	**0.96**

In **chapter 5** we further explored the differences in interaction between CNIs with mycophenolate mofetil (MMF). The need to more or less double the dose of MMF in case of CsA co-administration in order to achieve comparable mycophenolic acid (MPA) levels as compared to tacrolimus, has been attributed to either inhibition by CsA of the enterohepatic cycle, or an inhibition of glucuronidation to mycophenolate glucuronide (MPAG) by tacrolimus. With a 4-compartment model (figure 2), using nonlinear mixed effect modelling we were able to confirm that inhibition of the enterohepatic cycle is the underlying mechanism of the interaction between MMF and CsA. Clearance of MPAG was significantly lower (40%) in case of CsA co-

administration, which could be almost entirely attributed to the biliary excretion of MPAG and reabsorption in case of Tac co-administration.

Figure 2: Four-compartment model for the pharmacokinetics of mycophenolate mofetil (MMF)



In **chapter 6 and 7** we present the results of an open label, prospective, randomized trial in which 126 renal transplant recipients were treated with a quadruple drug regimen consisting of either CsA- or Tac- based AUC-guided therapy combined with steroids, MMF and prophylaxis with chimeric monoclonal anti-IL2 receptor-antibodies. Primary endpoint was the degree of interstitial fibrosis, quantitatively measured by digital image analysis of Sirius red stained surveillance biopsies at 6 and 12 months, a surrogate marker for long term graft function.

In **chapter 6** we compared the serial 6 and 12 months biopsies following either the CsA or Tac based protocol. The quantitative measurements of Sirius red stained areas were equal in the two groups (figure 3). No differences were found in prevalence of chronic allograft nephropathy (CAN: scored according to the Banff 97 classification), nor in prevalence of calcineurin inhibitor nephrotoxicity (defined by the presence of de novo nodular arteriolar hyalinosis and/or striped fibrosis and tubular micro calcification). Calcineurin inhibitor (CNI) related nephrotoxicity was found to be present in 24% of the biopsies at one year. This prevalence is low as compared to results reported by Nankivell, who detected a 53% prevalence of calcineurin inhibitor-related nephrotoxicity at one year [5]. Regarding non-immune toxicity parameters, a marked increased incidence of new-onset diabetes mellitus

after transplantation (NODAT) occurred in the Tac-treated patients (20%). Slightly higher cholesterol values despite the use of more lipid lowering drugs characterized the use of cyclosporine. We concluded that despite the higher incidence of acute rejections episodes in the CsA-treated patients, we did not find any evidence of excess cortical tubulo-interstitial fibrosis in their protocol biopsies. These findings were further elaborated at the renal molecular level [6], when we further quantified the fibrogenic responses in 6 and 12 months protocol biopsies of patients treated with either the CsA- or Tac-based regimen. Cortical TGF-b, collagens a1(I) and a1(III) mRNA steady-state levels were determined with real-time PCR. The extent of protein deposition of TGF-b, α-smooth muscle actin (α-SMA), and interstitial collagens in the renal cortex were quantified with computer-assisted image analysis. The extent of interstitial collagen deposition measured with Sirius red and the accumulation of α-SMA and TGF-b protein after 6 and 12 months were similar for both immunosuppressive regimens. mRNA levels of TGF-b and collagens a1(I) and a1(III) were not significantly different in the treatment groups. These results confirmed that the fibrogenic processes as a result from CNI-related toxicity and/or chronic rejection are similar, with the use of both drugs and the chosen AUC-guided strategies.

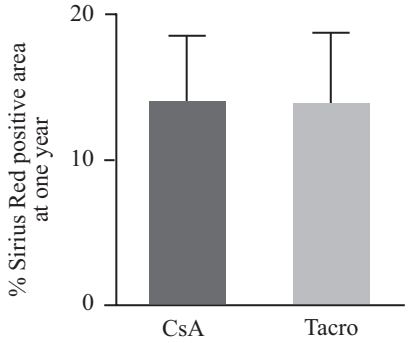


Figure 3

In **chapter 7** we further evaluated the clinical relevance of the presence of subclinical acute rejection (SAR) in the 6-month protocol biopsies. SAR or borderline SAR was present in 30.8% of 6 month-protocol biopsies and as defined by the study protocol not treated. The mere presence of SAR was not associated with differences in Sirius red staining at 6 or 12 months. Importantly neither progressive loss of GFR up to 2 years of follow up (figure 4), nor an increase of proteinuria was detected in patients

with SAR in the 6-month biopsy. In addition, no differences were found in donor or recipient related variables, degree of histocompatibility, prior acute rejection or systemic exposure of immunosuppressants. Donor age and fibrosis in pre-transplant biopsies correlated with inferior graft function, but not with SAR at 6 months. We concluded that we found no evidence that SAR is a progressive, deleterious process that requires treatment in all cases. These results contrast with the study of Rush et al [7], who showed that treatment of SAR in early biopsies with additional steroids was beneficial for long term outcome. It is important to note that in this study the protocol biopsies were taken in the first 3 months post-transplantation, and infiltrates in this early phase are known to correlate with HLA-DR mismatching [8,9] and are more likely to progress to clinical rejection within the next 40 days [10]. It may be that early infiltrates and infiltrates found in 6 months (or 12-month) biopsies are not the resultant from the same processes. In the study of Rush the treatment of SAR in early biopsies proved to result in a 3-fold decrease of late clinical acute rejection [7], an important risk factor for chronic rejection and graft loss [11]. In our study, under controlled systemic exposure early acute rejection rates were low and no late acute rejection occurred, indicating that individualized CNI-exposure may be at least as effective in the prevention of late clinical acute rejection as treating early subclinical acute rejection with high-dose steroids, and in this way preserve renal structure and function.

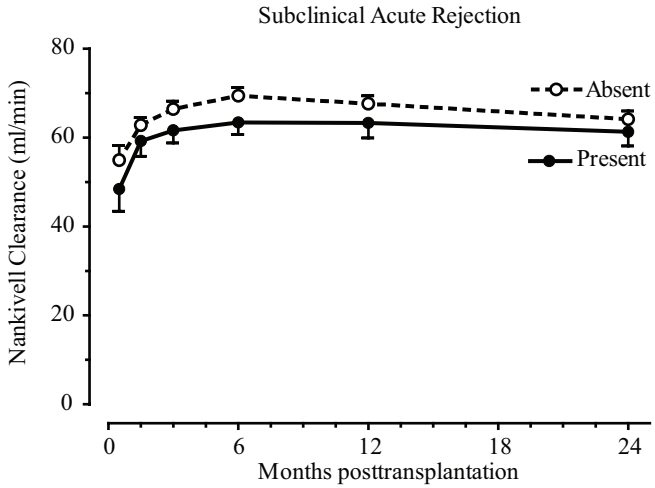


Figure 4

Cyclosporine and Tacrolimus

Most studies, comparing the CsA gel-capsule formulation with Tac based immunosuppression, have reported an advantage for Tac in the prevention of acute rejection episodes [12-14]. At one-year, however, no significant differences in graft survival or renal function parameters were found. As early as two years after transplantation, histopathologic signs of CAN are present in 62.0% of the patients treated with Tac and in 72.3% of the CsA treated patients, which may occur in the absence of discernible changes in renal function [15]. A retrospective analysis of paired kidneys, one recipient treated with CsA micro emulsion and the recipient of the contralateral kidney with Tac, found no difference in 5-year renal allograft survival [16]. Initial renal function was superior in the patients receiving Tac therapy, but the slope of 1/Cr over time did not differ between the agents. Hypertension, hypercholesterolemia, hypertrichosis and gingival hyperplasia are more pronounced with CsA, while post transplant diabetes mellitus occurs more frequently in the Tac treated patients [17-19]. All these comparisons are derived from studies with, at best, CNI trough levels as drug monitoring tool and thus, as discussed in **chapter 3** and **4**, have to deal with unknown systemic exposure to the drugs most critical for all the above mentioned end-points. In this thesis we explored a better standardized dosing strategy for these drugs, to minimize the pharmacokinetic differences between the drugs in a comparative, prospective study between CsA and Tac **(chapter 6 and 7**). The AUC targets defined in this study were derived from our population PK data and aimed at AUC(0-12h) values, corresponding with CsA trough levels of 225 ng/ml and 125 ng/ml or Tac trough levels of 12,5 ng/ml and 7,5 ng/ml. Using these AUC targets, the patients on CsA-based immunosuppression experienced more acute rejections episodes and patients in the Tac arm of the study showed a higher incidence of new onset diabetes mellitus after transplantation (NODAT). The degree of cortical tubulo-interstitial fibrosis at 6 and 12 months and renal function were comparable in the two groups. Studies using different (lower) target AUCs could lead to different results but randomized studies to determine the optimal target AUC for the currently available calcineurin-inhibitors are still lacking.

Under- and overimmunosuppression

It is impossible to make general definitions for the terms “under- and overimmunosuppression” and at various levels different aspects should be taken into

account. Classic examples, to illustrate this problem are case histories, in which discontinuation of all immunosuppressive drugs did not lead to any deterioration of graft function over a prolonged time of follow up [20]. For these patients theoretically every prescribed immunosuppressant should be considered overimmunosuppression. The main point in this discussion comes down to the controversy "standardization versus individualization": Every new way to standardize immunosuppression can possibly result in a better average outcome for a certain patient group. However, subgroups can always be found, for whom the 'new' standardization may in fact be deleterious. In renal transplantation there are at least two reasons for this problem:

1) Potent immunosuppressants have a narrow therapeutic index, and are not cell-selective. Their intrinsic side effects never leave a concentration interval in which they do not (theoretically) harm the patient. Therefore, always the lowest effective level of immunosuppression should be strived to.
2) To date, there is no effective and reliable tool to quantitate the direct effect of immunosuppressants on the relevant cells of the immune system. In addition, not every donor/recipient combination probably requires the same level of suppression of the immune system. Therefore no reliable prediction can be made about the effect of a prescribed immunosuppressive regimen on the individual and his/her allograft.

In the case of CNIs this controversy is even more pronounced, because the long term clinical resultant of over-immunosuppression (CNI-related nephrotoxicity) and under-immunosuppression (chronic rejection) are both clinically (gradual loss of function) and histologically (chronic allograft nephropathy) difficult to differentiate. Although recently better methods at the molecular level have been suggested to separate these two entities [21,22], in individual cases no proper discrimination can be made. This is further complicated by the fact that in most cases structural signs of CNI-related toxicity and chronic rejection are present in the same biopsy [10]. Moreover, structural lesions, presumed to be specific for CNI-related toxicity, have been encountered in biopsies of patients who never had been exposed to a CNI. This means that in studies comparing drug regimens including CNIs in renal transplant patients, the interpretation of functional and histological outcome constitutes an ongoing challenge as long as the standardization of drug dosing is not optimal. In Table 2 a number of possible standardization methods are summarized, that may be useful for CNI-inhibitors.

Table 2

Standardization	Limitation	Example of individualization
Drug prescription	Patient drug adherence	Patient education
Fixed dose drug prescription	Inter- and intraindividual PK variability	Trough level monitoring
Trough level monitoring	Inter- and intraindividual PK variability	Alternative single points Limited sampling models AUC-monitoring
AUC-monitoring	Pharmacodynamic variation	Calcineurin assay
AUC-monitoring	Pharmacogenetic variation	P- glycoprotein, Cyp3A
PK/PD monitoring	Differences in transplant history, HLA-matching, acute rejection episodes etc.	Surveillance biopsies

Patient drug adherence

Several studies have suggested that in adult renal transplant recipients nonadherence to immunosuppressants is relatively common and constitutes a major cause of late graft failure. Results of a meta-analysis of 15 cross-sectional studies indicated that about 22% of recipients may be nonadherent to drug prescription [23]. Meta-analysis of ten cohort studies indicated that nonadherence contributed substantially to graft loss, since a median of 36% of graft losses were associated with prior nonadherence, with a sevenfold increased risk of graft loss in nonadherent patients. Therefore, a significant improvement in graft survival could be expected from interventions that improve drug compliance. Although we did not systematically study non-adherence, it is important to be aware of this problem, while studying the influence of drug pharmacokinetics on long term graft outcome. In our experience, the continuous pharmacokinetic monitoring during our studies, was useful to improve the awareness in patients, that regular drug intake is of critical importance to preserve graft function, and as such it is an important addition to our standard drug education program. Even under the intensively controlled conditions, we identified two patients (1.5%), who presented with undetectable calcineurin-inhibitor blood levels at certain time points. This underlines the magnitude of the problem of non-adherence.

Therapeutic Drug monitoring of CNIs

For more than 20 years the calcineurin inhibitors CsA and Tac have been the cornerstone of immunosuppression. Due to its narrow therapeutic index, potential nephrotoxicity and multiple drug interactions the importance of therapeutic drug monitoring of CsA, has constantly been discussed and consensus has yet to be

achieved. The first proposed use was at fixed doses, but this was soon abandoned, and the predose, trough (C0) blood level concept was introduced as a convenient tool for drug monitoring. No significant correlation could, however, be shown between the various proposed trough level therapeutic windows and major clinical events [24,25]. The microemulsion formula of CsA (Neoral) was introduced, instead of gel caps (Sandimmun), because it produced an increased dose-linearity with the area under the concentration-time curve (AUC). Inter-patient variability during the first 12 postoperative weeks is however not different between Neoral and Sandimmun, and the C0 has an equally poor correlation with the AUC. The inter- and intrapatient variability in CsA pharmacokinetics led Kahan and co-workers to further investigate AUC-guided monitoring [26]. The use of multiple blood samples taken over a dosing interval made it possible to estimate the exposure of a patient to CsA by calculating the AUC during a dosing interval. Area under the curve or average concentration (C_{av}) was found to be a more sensitive predictor of clinical outcome as compared to C_0 [27]. Several studies have documented that systemic exposure to CsA, as measured by AUC, correlates well with occurrence of acute rejection episodes and graft survival at 1 year in renal transplant patients [28,29]. Despite the fact that AUC is a superior method to optimize CsA-dosing, the procedure is inconvenient, time-consuming and results had higher costs due to the analysis of multiple blood samples. The introduction of Neoral demonstrated that the region of most variability in pharmacokinetics and the strongest CNI- inhibition were confined within the first 4 hours post-dose ($AUC_{(0\text{-}4h)}$), introducing the concept of absorption profiling [30]. A further simplification came with studies showing that C2, the single blood concentration measurement 2 hours after Neoral administration, was an accurate estimator of the $AUC_{(0\text{-}4h)}$ [31]. Studies on single C2 sampling were primarily focused on the first months after transplantation but beyond the early transplant period only limited data are available [32]. This recent study showed, that in stable maintenance patients a C2 concentrations between 500 and 600 ng/ml provided effective rejection prophylaxis. Both the C0 and C2 value showed a high intra-patient variability, and the correlation between C2 and the AUC(0-12h) was only marginally better. Neither C0 nor C2 was associated with relevant clinical events. Especially in patients with delayed absorption (such as diabetic enteropathy) C2 levels tended to be low, which may result in significant overdosing, when corrected to achieve C2 target levels [32]. Limited sampling models using a mathematical formula have been designed as a tool

to control drug exposure ($AUC_{(0\text{-}12h)}$) but prospective studies with long-term follow-up are lacking and they have the drawback of the need for the accurate timing of blood sample drawing.

There is a growing consensus that also Tac trough levels do not accurately reflect systemic drug exposure [33-35]. In a study of 15 stable kidney transplant recipients C4 had the best correlation with AUC (0-12h), whereas C0 had the least (r2=0.61) [36]. In our study **(Chapter 4)** Bayesian forecasting with a trough level and a second sample obtained between 2 and 4 hours post-dose, significantly improved the squared correlation with the AUC(0-12h) ($r^2 = 0.94$). Compared with trough level monitoring this approach reduced the 95%-prediction interval by 50 % [37]. Before new monitoring strategies can be generally recommended, further prospective studies to clarify the relationship between both non-trough and abbreviated AUC monitoring and clinical outcome are necessary. Especially studies to determine the optimal target range of AUC (0-12h) are warranted.

Integration of pharmacodynamics and pharmacogenetics in TDM

When optimal pharmacokinetic control of CNIs could be accomplished, the next important question that needs to be addressed is: Does a certain target AUC of either CsA or Tac cause the same level of immunosuppression (or side effects) in all patients?

Pharmacodynamics (PD) is the study of the biochemical and physiological effects of drugs and the mechanisms of drug action. Characterization of pharmacodynamic mechanisms is helpful to explain interindividual differences in the relationship between drug concentration and effect. Calcineurin inhibitors affect the immune response by forming drug/protein complexes with intracellular receptors called immunophillins. These complexes inhibit calcineurin, a calcium dependent serine-threonine phosphatase whose activity results in events leading to T-cell activation. Several studies have looked at the relationship between calcineurin activity and CsA / Tac blood concentrations [38,39]. Maximum inhibition of calcineurin activity occurs from 1 – 2 hours after oral intake of the drug, returning to predose levels within 6 hours [40]. In these studies the overall calcineurin inhibition was expressed as the area under the calcineurin activity time curve from 0 – 6 hours post dose (AUC_{CaN}). No single blood concentrations of CsA / Tac correlated with $AUC_{CaN.}$. Additional factors may regulate calcineurin activity, for example limitation of available immunophillins

at higher drug concentrations [38]. Recently in our center, a spectrophotometric assay for calcineurin activity in leukocytes was developed, requiring only 2.5 ml fresh blood and standard laboratory equipment [41]. Multiple samples can be prepared and assayed for calcineurin activity within 1 working day with high intra- and interassay reproducibility. In both CsA and Tac patients, calcineurin inhibition was observed, given that significantly higher drug concentrations at C2 resulted in significantly lower calcineurin activities, when compared with C0. Interestingly, no total inhibition of calcineurin activity was observed for any patient. In vivo measurement of calcineurin activity in patients who undergo immunosuppressive therapy by CNIs, with successively integration of these results to our population based PK model, could be a next step to further refine TDM of CNI-inhibitors and improve clinical outcome.

Another way to a more individualized immunosuppressive therapy could be to include pharmacogenetic covariates. The variability in CsA and Tac disposition has been attributed to interindividual differences in the expression of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A5 [42,43], and expression of the drug transporter P-glycoprotein (encoded by the ABCB1-gen). Single nucleotide polymorfisms (SNPs) have been shown to significantly influence expression of the CYP3A5 enzyme. Individuals with the wild type allele (CYP3A5*1) have the highest amount of CYP3A5 protein in both their liver as small intestine and exhibit the highest dosage requirements for Tac [44,45]. Patients who express the CYP3A5 *1 not only need more Tac, but are also slower in their capacity to reach a target concentration, despite therapeutic drug monitoring. Screening transplant recipients for CYP3A5 expression has the potential to further optimize Tac therapy. To incorporate pharmacogenetic covariates in our population-based pharmacokinetic model is a logical next step to facilitate validation in prospective studies and to translate these findings into daily practice.

Biological monitoring and surveillance biopsies

Even when the pharmacodynamic level to prevent acute rejection theoretically is titrated and standardized, it is conceivable that not every patient (or every donor/acceptor combination) requires the same level of immunosuppression. This probably also depends on other factors, such as ethnicity, recipient age, co-morbidity, HLA matching and quality of the transplanted tissue; and constantly be influenced by post transplantation events (rejections, infections, transfusions).

As long as the knowledge of the mechanisms causing chronic allograft nephropathy are still largely unknown and tools to control drug dosing not optimal, we will have to rely on measurements in biologic fluids, of which creatinine, creatinine clearance and proteinuria are most extensively studied. These are important predictors of long term transplant outcome, but are dependent on several variables, lack specificity and are not fit as tools to guide immunosuppression.

In many studies transplant biopsies have been used to identify differences in renal histopathology in relation to different drug regimen [46]. To use a surveillance biopsy in the decision making to modify maintenance therapy is an intriguing option, but to date not much systematic evidence has been collected to support such a strategy. As discussed in **chapter 7,** and in other publications [5,8,47], surveillance biopsies of renal transplants with normal and stable function show remarkable high percentages of subclinical acute rejection (SAR), presumed calcineurin inhibitor toxicity and early stages of chronic allograft nephropathy. Data regarding subclinical rejections and their influence on occurrence of chronic damage are inconsistent, but only a few relative small studies support the indication to treat early SAR in order to preserve graft function [7]. Timing of surveillance biopsies and the phenotype of the infiltrate may prove to be of critical importance in this discussion. Several studies have shown an association between early SAR (< 3 months post transplantation) and a higher grade of CAN in subsequent biopsies [5,47,48]. The presence of CAN in stable allografts has consistently been associated with a decrease in renal allograft survival [49-51], while a direct correlation between SAR and graft survival has not been described thus far. In our study SAR at 6 months was not associated with differences in serial quantitative Sirius red staining measurements at 6 or 12 months, nor with proteinuria or progressive loss of GFR up to 2 years. Still, more studies and longer follow-up are needed to confirm these findings.

CNI Withdrawal studies

To date, for most patients receiving a kidney transplant a CNI-containing regimen is still the optimal choice in the early phase post transplant, and data on CNI- free transplantation are still sparse. On the other hand there are accumulating data that for the majority of transplant recipients CNI-withdrawal will not give rise to an acute rejection. As documented in several studies [52-54], withdrawal of CsA from an MMF-containing immunosuppressive regimen, resulted in an improved creatinine

clearance, but also in an increased risk for acute rejection episodes (10 – 22%) and graft loss as a result of rejection during a 5-year follow-up period [54]. Still, there are important arguments to presume that CsA withdrawal is likely to be beneficial in MMF-treated patients, regarding long-term outcome.

1) In long-term (> 15 years) follow-up studies of azathioprine-treated patients after cyclosporine-withdrawal, despite a similar incidence of acute rejection episodes shortly post withdrawal, graft survival was superior in the withdrawal group, as compared to patients who continued on CNI-therapy [55,56].
2) The improved renal function as observed after CsA withdrawal is likely to contribute in itself to reduction of cardiovascular events and the risk of death [57,58].
3) As compared to other side effects of CNIs, with known impact on the increased risk of cardiovascular disease in this population, new onset diabetes mellitus (NODAT) has been identified as the most detrimental, being associated with reduced graft function and patient survival, and increased risk of graft loss [59-64]. NODAT and impaired glucose tolerance [65] are conditions with a direct impact on cardiovascular morbidity and mortality, and generally not easy to control by standard therapy. CNI withdrawal should be considered a serious option to (partially) reverse or prevent this condition.

It is important to note, that, CNI withdrawal imposes increased risk of an acute rejection episode and, in some cases, irreversible effects on graft structure despite adequate anti-rejection therapy. To optimize withdrawal results in future studies pharmacokinetic monitoring of the remaining drug and subsequent tapering may improve safety after CNI (or MMF) withdrawal. Long term dual therapy with steroids and azathioprine has been shown to provide at least comparable long term outcome compared with a maintenance schedule containing steroids and CsA [55]. Modern drugs as MMF and rapamycine are more effective in prevention of renal allograft rejection and both drugs are postulated to prevent atherosclerosis, but conclusive or long term results in transplant recipients are still awaited. Also these drugs show a wide inter- and intraindividual variation in pharmacokinetic parameters [66,67]. In the case of MMF most centers are used to prescribe fixed doses of MMF. In the above mentioned Abramowicz study the doses of both MMF and steroids decreased significantly during a follow-up of 5 year, resulting in a respectable number of patients, who received less than 2 g / d MMF and/or less than 10 mg/d steroids. In this

study this subgroup of patients in the CsA-withdrawal group was at an increased risk for acute rejection and chronic rejection with graft loss. So, especially in the context of withdrawal studies, underdosing of the remaining drugs should be prevented and TDM of drugs like MMF could prove to be essential to prevent excess acute rejection after CNI- withdrawal.

Concluding remarks

Facilitated by a simple population based pharmacokinetic model, limited sampling and Bayesian fitting, long-term AUC-monitoring is feasible in daily clinical practice and helpful to individualize CNI treatment in renal transplant recipients. Incorporating pharmacodynamic and/or pharmacogenetic covariates in this model may further optimize therapeutic drug monitoring. AUC-guided dosing and subsequent tapering to low AUC targets maintained low acute rejection rates and prevented late acute rejection episodes. The currently available CNIs, CsA and Tac, were equally associated with the occurrence of renal cortical interstial fibrosis in surveillance biopsies. To justify surveillance biopsies in general practice evidence is required, that pharmacotherapeutic interventions in relation to defined abnormalities in these biopsies are beneficial and improve renal allograft and patient survival. To date this evidence is scarce and randomized studies are needed to answer these questions. There is also a clear need for more reliable biomarkers that correlate with clinical outcome and markers that can serve as tool to tailor immunosuppressive therapy.

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Towards individualized controlled drug exposure in renal transplantation

Nederlandse samenvatting

Voor iemand met nierfalen is behandeling met een niertransplantatie vaak de beste oplossing. De kwaliteit van leven verbetert aanmerkelijk, natuurlijk deels door wegvallen van de noodzaak tot dialyse, maar ook omdat de algemene conditie van getransplanteerde patiënten meestal snel verbetert. Verder is ook aangetoond dat na een geslaagde transplantatie de kans op langdurige overleving aanzienlijk is toegenomen. Door steeds weer nieuwe ontwikkelingen op het gebied van chirurgische technieken; afweerremmende middelen (immunosuppressiva), en ook de verdere voortschrijdende medische zorg zijn de resultaten van niertransplantatie de laatste decennia beter geworden; de kans op acute afstotingsreacties is kleiner geworden, en de gemiddelde overleving van getransplanteerde organen is sterk verlengd. Toch zijn er op de langere termijn problemen, waarvoor tot op heden geen goed antwoord gevonden is. Zo blijkt bij een aanzienlijk deel van de getransplanteerde nieren de functie toch langzaamaan te verminderen, een proces dat vaak gepaard gaat met verhoogde bloeddruk en eiwitverlies in de urine. Dit syndroom wordt chronische transplant disfunctie (CTD) genoemd. Als bij deze patiënten een nierbiopt wordt verricht, wordt vaak een beeld gezien van diffuse verbindweefseling (fibrose) van nierweefsel, gecombineerd met schade aan bloedvaatjes en verschrompeling van nierfiltertjes (nefronen) en nierbuisjes (tubuli), samengevat onder de term “chronic allograft nephropathy” (CAN). Dit is niet anders dan een beschrijvende diagnose, waarvan de oorzaak heel verschillend kan zijn. In eerdere studies is aannemelijk gemaakt dat de schade soms wordt veroorzaakt door een voortdurende afweerreactie van de ontvanger tegen het transplantaat, ofwel “chronische rejectie”. Maar schade door infecties en acute afstotingsreacties in het verleden, schade door hoge bloeddruk en hoog cholesterol bij de ontvanger en zelfs schade die al bestond bij de nierdonor kan hiervan eigenlijk niet goed onderscheiden worden. En ten slotte blijkt dat een groep

van veel gebruikte immunosuppressiva, de calcineurine-remmers, ook een dergelijk beeld kan veroorzaken. Tot op heden is er geen manier om al deze verschillende factoren in een nierbiopt van elkaar te onderscheiden, wat het onderzoek naar eventuele oplossingen ernstig bemoeilijkt. Er is geen goede behandeling voor CAN en eigenlijk betekent het vaststellen van ernstige CAN in een biopt voor de patiënt, dat de het transplantaat op termijn vaak toch weer verloren zal gaan en dat dan dialyse weer nodig zal zijn.
In **hoofdstuk 2** wordt een overzicht gegeven van mogelijkheden om het ontstaan van CTD en CAN met aanpassing van medicamenteus beleid te voorkomen of te vertragen. Zowel bestaande en recent ontwikkelde immunosuppressiva worden hierin besproken, als ook medicijnen, die een niet afweerremmende werking hebben. Veel van deze opties zijn interessant, maar van geen van deze medicijnen of moleculen is een positieve invloed gerapporteerd op de lange termijn resultaten van niertransplantatie. Een slimmer en meer gecontroleerd gebruik van de bestaande, vertrouwde middelen zal mogelijk sneller tot meetbare verbetering in uitkomsten van niertransplantaties zal kunnen leiden. In dit proefschrift wordt hier nader op ingegaan.

Immunosuppressiva worden vrijwel altijd in combinatieschema's gegeven; vaak drie of vier middelen per patiënt gedurende de eerste maanden na transplantatie, waarna soms op de lange termijn kan worden teruggegaan naar twee middelen.
Een groot deel van dit proefschrift gaat over de calcineurine-remmers, cyclosporine en tacrolimus. Deze groep van immunosuppressiva wordt tot op heden gezien als de basis van de medicamenteuze behandeling na niertransplantatie; omdat zonder deze medicijnen de kans op acute afstootreacties te hoog blijkt te zijn. Naast een sterk afweerremmende werking, hebben deze middelen echter een zeer ongunstig bijwerkingenprofiel, zeker voor nierpatiënten. Ze verhogen de bloeddruk en het cholesterol, en kunnen suikerziekte veroorzaken, stuk voor stuk factoren die de kans op hart- en vaatziekten doen toenemen, terwijl cardiovasculaire sterfte juist bij nierpatiënten al zo een prominent probleem is. En, het gebruik van calcineurine-remmers leidt in een groot deel van de patiënten tot chronische (transplantaat-) nierschade (nefrotoxiciteit).
Hiernaast hebben de calcineurine-remmers een zeer nauwe therapeutische breedte. Dit betekent dat er maar een klein verschil is tussen de dosering die een minimaal

vereiste (afweerremmende) werking bewerkstelligt en de dosering waarbij (ernstige) bijwerkingen optreden. In verband hiermee werden bij elk polikliniekbezoek bloedspiegels bepaald bij de patiënten die deze middelen gebruikten. Aan de hand van de "dalspiegel", de spiegel in het bloed, gemeten vlak voor inname van een nieuwe dosis pillen, werd besloten of een patiënt te veel of te weinig medicijn toegediend kreeg en werd zonodig de dosering aangepast.

De laatste jaren is aangetoond dat deze dalspiegels een zeer matige correlatie hebben, met bloedspiegels die op andere tijdspunten in de dag gemeten worden. Bij sommige patiënten met een dalspiegel van bijvoorbeeld 125 ug/l worden 2 uur na inname van de pillen spiegels van 1200 ug/l gemeten, bij anderen slechts 400 ug/l. Om een betere indruk te krijgen van het concentratie beloop, kan je elk uur de bloedspiegels bepalen en dan berekenen wat de optelsom van bloedspiegels is gedurende een doseringsinterval (= "area under the curve", AUC). Gebleken is, dat dalspiegels niet alleen zeer slecht correleren met de AUC bepalingen, maar ook slecht voorspellen wat het klinische beloop in patiënten zal zijn. Patiënten, bij wie acute afstotingsreacties van het transplantaat worden vastgesteld hebben bijvoorbeeld gemiddeld even hoge dalspiegels als mensen zonder acute afstotingen, en hetzelfde geldt voor patiënten met veel bijwerkingen zoals nefrotoxiciteit. Aan de andere kant zijn er meerdere publicaties waaruit blijkt dat de hoogte van de AUC wel correleert met het klinische beloop, in het bijzonder met het optreden van acute rejectie episoden en met nefrotoxiciteit. Wanneer het dus mogelijk is aan de hand van AUC-bepalingen, de dosering van cyclosporine en tacrolimus bij te sturen, zou dit mogelijk betere resultaten opleveren. Maar dit is nooit gedurende een langere follow-up ten uitvoer gebracht, aangezien het onmogelijk is om bij elke patiënt bij elk polikliniekbezoek gedurende 12 uur spiegels af te nemen.

In **hoofdstuk 3** presenteren we een methode om de bepaling van AUCs bij cyclosporine te vereenvoudigen. Hiervoor hebben we gebruik gemaakt van een eenvoudig farmacokinetisch computerprogramma, dat in elke ziekenhuisapotheek in Nederland wordt gebruikt. Aan de hand van een database van een aantal eerder bepaalde cyclosporine curves, kan hiermee de AUC bij nieuwe patiënten bepaald worden op grond van slechts enkele bepalingen. Uiteindelijk bleek een strategie van drie spiegels, een dalspiegel gecombineerd met cyclosporine spiegels 2 en 3 uur na inname een betrouwbare voorspelling van de AUC op te leveren. Dit ging zelfs op voor patiënten met een nier-pancreas transplantatie, een groep patiënten,

van wie bekend is dat de opname van geneesmiddelen uit het maagdarm kanaal erg onvoorspelbaar is, en dus de variatie in AUC nog extremer. Het voordeel van het model is dat de afname tijden steeds gevarieerd kunnen worden zonder dat de AUC-berekening hierdoor wordt beïnvloed, wat de toepasbaarheid groter maakt in de poliklinische setting: Het maakt bijvoorbeeld niet uit als een spiegel 15 minuten te laat wordt bepaald (door bijvoorbeeld plaatsgebrek in de bloedafname kamer), als de afnametijden maar goed worden genoteerd.

In **hoofdstuk 4** beschrijven we een soortgelijk model, ontwikkeld voor patiënten behandeld met tacrolimus. Voor het monitoren van tacrolimus blijkt een dalspiegel, gecombineerd met een spiegel, afgenomen tussen de 2 en 4 uur na medicijninname, voldoende te gegevens op te leveren om met het model een betrouwbare AUC te berekenen. Bij een cohort van 15 opeenvolgende niertransplantatie patiënten werd de methode gedurende 1 jaar prospectief getest, waarbij het model in praktijk goed bleek te functioneren. De eerste maanden na transplantatie blijken er intra-individueel nog zoveel farmacokinetische veranderingen op te treden dat frequente metingen nodig zijn, om de AUC steeds op het gewenste niveau te handhaven. Na 3 maanden zijn er steeds minder en ook steeds kleinere dosisaanpassingen nodig. Toch blijkt op termijn dat gemiddeld per patiënt steeds een iets lagere dosis tacrolimus nodig om dezelfde AUC te handhaven. Door de nauwkeurigheid van het model wordt dit steeds gecorrigeerd, terwijl dit bij het gebruik van dalspiegels niet zou worden gedetecteerd.

In **hoofdstuk 5** worden de pharmacokinetische interactie van cyclosporine en tacrolimus met mycofenolaat mofetil (MMF) onderzocht. MMF is een immunosuppressivum dat na niertransplantatie vaak als derde middel wordt gegeven, naast prednison en één van de calcineurine-remmers. Meestal wordt MMF in een vaste dosis gegeven, zonder controle van bloedspiegels. Er is bekend dat bij het gebruik van cyclosporine meer MMF nodig is om adequate bloedspiegels te bereiken, in vergelijking met patiënten bij wie tacrolimus en MMF worden gecombineerd, of bij wie geen calcineurine-remmer wordt gegeven. Met behulp van een 4-compartement model en de farmacokinetische gegevens van 64 patiënten kon aannemelijk gemaakt worden, dat deze interactie een gevolg is van een remming van de enterohepatische kringloop van MMF, bij gelijktijdig gebruik van cyclosporine (en niet bij tacrolimus). Hiernaast blijkt dat tussen individuen die een vaste dosering MMF innemen, de gemeten spiegels van de werkzame metaboliet (MPA) enorm

kunnen variëren, zowel de dalspiegels als AUC-bepalingen. Recente publicaties tonen dan ook aan dat betere monitoring strategieën voor MMF nodig zijn om de transplantatie resultaten te verbeteren.

In **hoofdstuk 6** worden de resultaten beschreven van een studie waarin voor zowel cyclosporine en tacrolimus het boven beschreven AUC geleide doseringsmodel wordt toegepast. In een prospectieve studie werden 126 patiënten behandeld met prednison, MMF en gerandomiseerd voor cyclosporine- of tacrolimusbehandeling. Op 6 en 12 maanden na de transplantatie werd een protocollair nierbiopt genomen, en vervolgens werd hierin systematisch de hoeveelheid chronische schade gescoord. Met een zogenaamde Sirius Red kleuring werd de hoeveelheid fibrose gekwantificeerd. Deze laatste meting is een goede voorspeller (surrogaatmarker) voor lange termijn transplantaat overleving. Tevens werden de nierfunctie, acute afstotingsreacties en de bijwerkingen vervolgd. Als belangrijkste uitkomst bleek de hoeveelheid fibrose in beide groepen vrijwel identiek bij gebruik van cyclosporine of tacrolimus, op zowel 6 als 12 maanden. Ook de nierfunctie in de twee groepen was vergelijkbaar. Ondanks het toepassen van de AUC geleide dosering waren bij het gebruik van cyclosporine meer middelen nodig om de bloeddruk te controleren, en ook het cholesterol was vaker verhoogd, bij het gebruik van tacrolimus ontwikkelden patiënten veel vaker post-transplantatie diabetes mellitus (20%). In de met cyclosporine behandelde groep werden, vaker acute afstotingreacties gezien, zonder dat dit in deze studie dus leidde tot verschil in nierschade en nierfunctie tot 2 jaar na transplantatie.

Hoofdstuk 7 gaat over subklinische acute rejectie (SAR). Hier spreekt men van wanneer in een protocol biopt een beeld gezien wordt dat eigenlijk identiek is aan biopten bij acute afstoting, terwijl de functie van de transplantaatnier geheel stabiel blijft. SAR kwam in 30.8% van de protocol biopten op 6 maanden voor, en werd door ons niet behandeld met anti-rejectie therapie. In onze studie kon niet worden aangetoond dat de aanwezigheid van (onbehandelde) SAR geassocieerd was met een achteruitgang van nierfunctie tot 2 jaar en ook niet met toename van fibrose in het biopt op 12 maanden. In een eerdere studie werd juist wel aangetoond dat, als SAR steeds behandeld wordt met hoge doses steroïden, de nierfunctie op langere termijn significant beter zou blijven. Het verschil met genoemde studie is echter dat de biopten in die studie veel korter na transplantatie werden genomen (1 tot 3 maanden). Dit is een periode waarin de kans op een klinische acute afstotingsreactie nog groot is, wat met de in die studie gegeven steroïdenbehandeling potentieel

wordt voorkomen. Onze conclusie is dat bij SAR op 6 maanden geen anti-rejectie behandeling gestart hoeft te worden en dat ernaar andere markers gezocht moet worden, die verslechtering van transplantaat functie voorspellen.

Samenvattend is in dit proefschrift naar een aantal manieren gezocht om onder- en overbehandeling met immunosuppressiva op te sporen en te voorkomen. **Onderbehandeling** kan op vele manieren worden gedefinieerd, maar eigenlijk bij elke patiënt bij wie acute of chronische afstoting wordt vastgesteld, dringt zich de vraag op of dit voorkomen had kunnen worden door een betere immuunsuppressieve therapie. Soms is de oorzaak te vinden bij de patiënt zelf, en worden de pillen gewoon niet trouw en regelmatig ingenomen. Ondanks het feit dat er na niertransplantatie veel aandacht wordt gegeven aan patiënteneducatie over het belang van regelmatige medicatie-inname; blijkt structurele medicatie-"ontrouw" voor te komen bij meer dan 20 % van de patiënten. Continue aandacht voor dit probleem blijft dus essentieel. Zoals beschreven in dit proefschrift kan een farmacokinetisch drug-monitoring programma van belang zijn om chronische onderdosering op het spoor te komen: Ten opzichte van dalspiegel bepalingen is het doseren op geleide van AUC-metingen een sterke verbetering. Maar niet alle patiënten met een trouwe medicatie inname en een voldoende hoge AUC zullen volledig gevrijwaard blijven van afstotingsreacties. Oorzaken hiervoor zijn divers: Steeds duidelijker wordt dat ook het genetische profiel van de patiënt kan bijdragen aan de wisselende effectiviteit van geneesmiddelen. Zo kan het zijn dat mensen jarenlang een geneesmiddel slikken, waar zij niet of nauwelijks op reageren of juist onaanvaardbare bijwerkingen op ontwikkelen. Binnen de farmacogenetica onderzoekt men in hoeverre verschillen in het genetische profiel van mensen een verklaring vormen voor de inter-individuele verschillen in effectiviteit en bijwerkingen van geneesmiddelen. Toenemend inzicht in farmacogenetica en ook farmacodynamica kunnen in de nabije toekomst als hulp dienen voor geïndividualiseerde keuzes voor een bepaald immunosuppressivum of bijvoorbeeld een bepaalde streefconcentratie. In onderzoek naar farmacogenetische en farmacodynamische verschillen tussen patiëntengroepen is vaak simultaan inzicht in de farmacokinetiek nodig en het in hoofdstuk 3 en 4 beschreven model kan hiervoor als "tool"dienen.
Voor het voorkomen van **overbehandeling** zijn voor een groot deel dezelfde aspecten van belang, maar wellicht is de belangrijkste vraag: Is er wel een noodzaak voor het

gebruik van een bepaald immunosuppressivum? Er zijn gevallen bekend waar een niertransplantaat na stoppen van alle medicijnen jarenlang goed bleef functioneren; waarbij dus achteraf zou kunnen worden gezegd dat de immunosuppressiva als overbehandeling kunnen worden beschouwd. Kans op chronische afstoting wordt onder andere beïnvloed door mate van HLA-overeenkomst, immunisatie van de ontvanger (door bijvoorbeeld zwangerschap of bloedtransfusie), leeftijd, ras en eerdere acute afstotingsepisoden. Toch zijn deze gegevens maar beperkt bruikbaar om in een individueel geval een voorspelling te maken of mindering van medicatie tot afstoting zal leiden. Zoals beschreven in **hoofdstuk 7** zou een protocol biopt kunnen bijdragen om te beslissen tot onttrekking van een van de middelen, waarbij de calcineurine-remmers (gezien hun bijwerkingenprofiel en nefrotoxiciteit) de voornaamste kandidaat zijn. Toch zijn er nog te weinig prospectieve studies om een dergelijke strategie te rechtvaardigen. Vooralsnog zal de beslissing tot onttrekking van calcineurine-remmers vooral berusten op de empirie. Adequate "therapeutic drug monitoring" van de resterende medicijnen (bijvoorbeeld MMF) zal in deze situatie kunnen bijdragen om de kans op een late afstotingsreactie te minimaliseren.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 14 april 1966 te Den Haag. In 1984 behaalde hij het diploma gymnasium β aan het Johan van Oldenbarnevelt gymnasium te Amersfoort. In hetzelfde jaar is hij begonnen met de studie Geneeskunde aan de Rijksuniversiteit Groningen. In 1989 behaalde hij zijn doctoraal examen en tijdens het doorlopen van zijn co-assistentschappen verbleef hij in 1991 gedurende 10 maanden in Salt Lake City, USA waar hij in het kader van een onderzoeksstage werkte op het Artificial Heart Laboratory onder begeleiding van Prof. Dr. W.J. Kolff. Na het behalen van het artsexamen in 1992, behoorde hij tot een van de laatste lichtingen dienstplichtige artsen en werd hij gedetacheerd bij de Militaire Bloedbank te Amsterdam. Van 1994 tot 1997 werkte hij als arts-assistent in het kader van de opleiding Interne Geneeskunde in het Rode Kruis ziekenhuis te Den Haag (opleiders Dr. D. Overbosch en Dr. R.M. Valentijn). Van 1997 tot 2000 werd de opleiding voltooid in het LUMC te Leiden (opleider Prof. Dr. A.E. Meinders). Vervolgens doorliep hij de opleiding tot nefroloog bij de vakgroep Nierziekten in het LUMC (opleider Prof. Dr. L.C. Paul) , alwaar ook het onderzoek werd verricht wat geleid heeft tot dit proefschrift. Vanaf mei 2005 is de auteur werkzaam in het Medisch Centrum Haaglanden te Den Haag als internist-nefroloog binnen de maatschap Interne geneeskunde en Gastroenterologie.

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Nawoord

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