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## Therapeutic drug monitoring of novel immunosuppressants

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# THERAPEUTIC DRUG MONITORING OF NOVEL

# IMMUNOSUPPRESSANTS

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# Abstract

Therapeutic drug monitoring is primarily undertaken for narrow therapeutic index drugs, such as immunosuppressants. These drugs have reduced the incidence of acute rejection and improved allograft survival. However, due to their narrow therapeutic index, small variations in blood levels may result in inadequate levels of immunosuppression or toxic drug concentrations. To overcome these problems individual dose regimen based-on pharmacokinetic monitoring has been proposed in the past years. Indeed, several studies have previously documented a significant association between cyclosporine whole blood levels and patient's clinical outcome, expressed as rejection episodes as well as drug-related adverse events.

Novel immunosuppressive agents have been recently introduced on the market (such as rapamycins and mycophenolic acid-releasing formulations). However, data on their pharmacokinetics, which in turn could be useful for the definition of therapeutic ranges, are scanty. To address this issue we have used a chromatographic method for the measurement of mycophenolic acid levels in kidney transplant recipients. Although this immunosuppressant is usually given in a fixed daily dose regimen, we found a positive correlation between drug levels, but not dose, and renal function. As additional analysis, significant pharmacokinetic interactions between mycophenolic acid and concomitant immunosuppressive regimens have been identified. Similarly, novel chromatographic methods for the analysis of rapamycins in the whole blood have been developed in our laboratory, and applied to identify pharmacokinetic interactions between these and other immunosuppressive agents. In the last part of my research project, I have also presented preliminary data on the application of pharmacogenetics analysis in patients given cyclosporine as part of their immunosuppressive regimen.

In conclusion, it can be reasonably speculated that TDM based on pharmacokinetic, as well as novel pharmacogenetic approaches, can be considered as reliable tools to guide drug dosing in organ transplantation setting, ultimately resulting in a significant improvement of long term graft and patient survival.

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*Chapter 1*


**INTRODUCTION**

Organ transplantation as a treatment modality for patients with end-stage organ diseases of the kidney, heart, liver, pancreas and small bowel has achieved impressive results in the past two decades, thanks to a better understanding of basic immunobiology, more advanced measures for medical and surgical management, and new pharmacological treatments [1]. Indeed, although immunosuppressive therapies to overcome host reaction to allografts have been employed since the early days of clinical transplantation, immunosuppressive agents and treatment protocols are constantly evolving [2].

*Historical perspective*

The pharmacological era of immunosuppression began in 1916, when Hektoen [3] showed that benzene treatment reduced antibody production in experimental animals. Later, Schwartz et al observed that treatment with 6-mercaptopurine, a competitive inhibitor of many purine synthetic pathways, reduced antibody production and prolonged skin allograft survival in rabbits [4]. Subsequently, azathioprine (AZA) - an imidazole derivative developed to prevent the susceptibility of the unshielded mercapto-group of 6-mercaptopurine to hydrolysis in the gut - was shown by Calne and colleagues [5] to prolong the survival of human renal transplants. In the same period, steroids, the second stage in the development of therapeutic immunosuppression, showed various effects to reduce inflammatory processes mediated by mononuclear cells [6]. From 1964 to 1978, renal transplants were carried out using combinations of azathioprine and the steroid prednisone, showing about a one-year graft survival rate in 50% of recipients. However, even in successful cases, this approach was complicated by bone-marrow suppression, gastrointestinal disorders or opportunistic infections [7]. Thus, new, potent, selective and less toxic drugs were urgently needed. Since the introduction of cyclosporine (CsA) in 1978 [8], there has been little doubt about its

value as the single most important agent in the armamentarium of maintenance immunosuppression for organ transplantation. In the middle nineties triple-drug therapy with cyclosporine, corticosteroids and azathioprine became the most frequently used regimen for kidney recipients [9].

More recently, better understanding of the basic immune mechanisms and more insights in the pharmacokinetic properties of different molecules have led to the development of several new xenobiotic immunosuppressants (tacrolimus, mycophenolate mofetil, sirolimus, everolimus, FTY720), novel formulations of traditional drugs (cyclosporine Neoral, mycophenolate sodium), as well as polyclonal antilymphocyte agents (antilymphocyte or antithymocyte globulins [ALG, ATG]) or monoclonal antibodies specifically directed against immunological targets (anti-CD3 [OKT3], anti-CD52 [alemtuzumab or campath-1H], anti-CD20 [rituximab]), which have entered clinical trials in the past few years [2]. Altogether, these new drugs contributed to great evolvement of immunosuppressive drug regimens for maintenance of solid organ allografts observed over the past few decades, increasing 1-year graft survival from less than 40% in the early '60 to an actual survival of over 90% [10].

Despite the great results obtained in the short-terms, it should be pointed out that current anti-rejection drugs, however, invariably reduce systemic immunity non-selectively which translates in more risk of infections and cancer. Moreover, these agents present several side effects and, in some instance, can be extremely toxic for the graft, especially for the kidney [11], all factors that can contribute to chronic rejection, ultimately resulting in poor long term graft survival and graft loss [12].

It was soon realized that adverse events associated with pharmacologic immunosuppression were mainly related to daily drug exposure and could be minimized to some extent by carefully titration of the optimal individualized dosage. Several approaches have been used to assess the appropriateness of the dosing regimen for an

immunosuppressive drug. The first involved the assessment of clinical response. This approach, however, has serious limitations, because signs of rejection or toxicity may be difficult to recognize, or the clinical manifestations can appear late, so that patients do not recover also after drug withdrawal [13]. Therefore, it was clear that alternative approaches had to be developed, giving birth to a new important and exciting field of study: *Therapeutic Drug Monitoring* (TDM).

A TDM program [14] for immunosuppressive drugs must include consideration concerning the use of specific and sensitive analytical methods, identification of the optimal biological matrix, and choice of the best parameter to be assessed as surrogate marker of drug activity. Traditional TDM has been performed using pharmacokinetic approaches [14], but also complementary strategies have been developed in the last few years [15,16]. The ultimate goal of traditional as well as innovative TDM studies is to tailor the best immunosuppressive regimen for each patient.


*The pharmacokinetic approach*

Pharmacokinetics involves the measurement of drug concentrations in biological fluids and the subsequent relationship of these levels to drug dosing and ultimately to clinical events. The assumption is that the plasma/blood concentration mirrors the concentration at the site of action. Drug levels could be greatly influenced by environmental factors, such as hepatic or renal dysfunction, as well as hormonal levels and/or pharmacokinetic interactions with other co-administered drugs, all combinations that can vary significantly during the course of treatment [17]. These observations provide support for pharmacokinetics, because periodical evaluations of drug exposure are able to identify external influences, and can predict daily drug exposure and clinical outcome. This approach, also termed as clinical pharmacokinetic monitoring, has been applied to the field of organ transplantation starting from the end of 1960s [17]. Since then, several

studies have shown the predictive impact of pharmacokinetics on decreased mortality, morbidity and efficacy/adverse events from immunosuppressive drug therapy [18,19]. Although pharmacokinetics represents a step forward, as compared to the simply observation of clinical response, it presents some limitations. Pharmacokinetic studies can start only when a given drug is administered to the patients, requiring the assumption of steady state conditions and patient's compliance. Moreover, there is still controversy about which are the best pharmacokinetic parameters that should be considered to monitor drug effect. The area under the time-concentration curve (AUC) represents the golden standard to assess daily drug exposure. Unfortunately, measuring AUC requires the collection and analysis of multiple samples, which is costly and time-consuming for patients and clinical staff. To overcome these problems, alternative approaches have been developed by limited Sampling Strategy (LSS), a technique aimed to estimate a pharmacokinetic parameter, as AUC, using a small number of samples [20]. Several equations have been developed so far, but there is still lack of agreement on the choice of the optimal LSS for each immunosuppressive agent.

All these limitations provide the rationale for searching complementary/additional strategies to manage narrow therapeutic index drugs beyond pharmacokinetics.


*Pharmacodynamic monitoring of immunosuppressive drugs*

Pharmacokinetics has been used for many years to relate immunosuppressants dose to drug exposure in vivo. Although this is the primary method to measure drug absorption, distribution, metabolism, excretion and interactions with other drugs, it does not directly assess the pharmacological effects on immune cells in vivo. As results pharmacokinetics cannot account for the inter-subject variability in the sensitivities to immune suppression by similar blood concentrations of immunosuppressants.

Therefore, the current practice of relying solely on pharmacokinetics for clinical decisions on the dosage and the choice of immunosuppressants is far from ideal.

In contrast to pharmacokinetics, pharmacodynamics of immunosuppressants quantitates drug effects at its target site, after drug exposure to immune cells in vivo. If measurement at the target is not possible, the enzyme activity should be measured in a matrix that provides good surrogate marker for enzyme activity at the target site [15]. Pharmacodynamic assays of orally active immunosuppressants either measure the drug effect on a discrete molecule that is important for lymphocyte activation or measure more complex biological events required for normal immune functions [21]. Indeed, pharmacodynamic assays have been reported for the monitoring of CsA, azathioprine, mycophenolate mofetil, and rapamycin [15,21].

Pharmacodynamic monitoring might provide better understanding of mechanisms of actions of different immunosuppressants, as well as direct assessment of pharmacologic-induced immunosuppression. These approaches have, therefore, the potential to augment pharmacokinetic monitoring to optimise the dosing regime of anti-rejection drugs. However, because many of pharmacodynamic assays are time-consuming and not amenable for use in routine clinical laboratories [15], the future challenge is to develop robust methods to facilitate the further evaluation and application of these approaches.

*Pharmacogenetics as a new tool to tailor immunosuppressive therapy*

The advent of the genomic era has brought several new fields of study, including pharmacogenomics, which seeks to link drug treatment with the individual's genetic make-up. This new science holds many promises for improved treatment of a large variety of medical conditions, including immunosuppression for organ transplantation [22], providing a promising and complementary tool to traditional TDM. The key-point

is to understand whether clinical pharmacogenomics is already applicable today to organ transplantation. Early observations focused simply on drug metabolism [23]. However, today there is great interest in the full spectrum of drug disposition, including absorption, distribution, and pharmacological targets. One of the potential advantages of this type of approach lies on the common metabolic pathways for several immunosuppressive agents. CsA, tacrolimus, sirolimus and the novel rapamycin-analogue everolimus are all transported by P-glycoprotein – a protein encoded by the multidrug resistant gene 1 (MDR1) - and metabolised by the cytochrome 3A4 (CYP3A4) and to a less extent by CYP3A5 [24]. Thus, the study of patient's genotype can provide predictive value for regimens which include the concomitant administration of several drugs, a common condition for organ transplant recipients.

The presence of variants in genes encoding for the drug targets or other genetic polymorphisms with indirect effects on drug response are less known. Thus, they represent an exciting challenge for the future. However, one should consider that also many non-genomic factors, including concomitant drug administration, pharmacological enzyme-induction, and illness may have a significant impact on the above mentioned proteins, and must be taken into account also when a pharmacogenomic approach is planned.

*Therapeutic drug monitoring of novel immunosuppressants: preliminary experience with MPA*

MMF is widely used in kidney or heart transplantation using a fixed-daily dose regimen. However, data are recently emerging that provide the scientific basis for therapeutic drug monitoring of MPA in transplant patients receiving MMF, the parent drug, in combination with other immunosuppressive agents. There is a significant relationship between the dose-interval MPA AUC and risk for acute rejection based on

retrospective investigations in renal and heart transplant patients and on prospective investigations in renal transplant patients [17,19]. The MPA dose-interval AUC varies naturally by more than 10-fold in renal and heart transplant patients. Other significant sources of pharmacokinetic variability for MPA include the effects of concomitant medications – especially when new drugs (i.e sirolimus) are concomitantly used – and the effects of disease states such as renal dysfunction and liver disease on the steady state MPA exposure. MPA is extensively bound to albumin, with a range of protein binding of 97-99% in patients with normal renal and liver function. Factors that alter the protein binding can affect the relationship between total concentration and free concentration which, in turn, is pharmacologically active [114]. For this reason, under circumstances of perturbed binding, the interpretation of total MPA plasma concentration must take into account the altered MPA binding. Further research is, however, needed to draw definite conclusion on this topic.

Individualized MMF dose evaluation, guided by MPA plasma concentrations, is becoming the standard of practice at a growing number of transplant centers worldwide because of these factors and because of the need to closely evaluate the immunosuppressionafforded by MPA when a change in the immunosuppression regimen in stable transplant patients is planned. Investigations of therapeutic drug monitoring strategies with the goal to optimize the use of MMF, focusing in particular on drug-to-drug interactions, are ongoing. Based on statistics from the Analytical Services International Ltd, there are nearly 80 centres participating in the MPA program. Most of them are European, whereas only a few centers in America are currently monitoring MPA. However, given the emerging strong support for the clinical outcome benefit of MPA monitoring, it can be expected that greater demand for MPA monitoring will occur, focusing also on the pharmacokinetics of MPA released from the recently developed enteric-coated formulation [107].

*Therapeutic drug monitoring of novel immunosuppressants: preliminary experience with SRL*

SRL was recently approved in Europe, where the licence specifies its use for prophylaxis of graft rejection in adult kidney transplant recipients, and with the use of blood concentration monitoring. TDM of SRL is still in its infancy but data have accumulated from several clinical trials, some of which were concentration-controlled, showing that there is a good correlation between SRL trough concentration (as surrogate marker of SRL AUC) and clinical outcome, expressed as rejection episodes and drug-related toxicity [17,18]. Therefore, to date, all the monitoring recommendations refer to this sampling. Preliminary evidence is also available that SLR exposure could be significantly influenced by concomitant CsA administration. However, the potential drug-to-drug interactions between SRL and other agents usually administered to organ transplant recipients are unknown. When SRL is used in combination with a calcineurin inhibitors predose concentrations are generally targeted in the range 5-15 ng/mL [17,18], whereas specific SRL ranges in CsA- or TRL-sparing regimen are lacking.

*Therapeutic drug monitoring of immunosuppressive agents in the Centre of Bergamo: single-centre experience with CsA*

CsA is the principal immunosuppressant currently used to prevent graft rejection after organ transplantation. Given the appreciable interindividual variation both in clinical response and in drug exposure despite the same dosage regimen, it was early realized that close monitoring of CsA blood levels was a useful adjunct to its optimal administration. In particular, the AUC, either calculated from the individual complete pharmacokinetic profile or predicted using LSS, was identified as the best predictor of CsA daily exposure and clinical outcome [25]. These approaches are, however, time

consuming and seldom feasible in routine out-patient clinical monitoring. Thus, TDM of trough ($C_0$) blood CsA concentration has been widely adopted to adjust CsA dose in individual subjects since twenty years ago. In the last few years, however, evidence has accumulated that blood sampling at 2 h post-dosing ($C_2$), might provide a better estimate of CsA exposure than the traditional $C_0$-based monitoring [25]. It should be pointed that the definition of the best single-point sampling ($C_0$ or $C_2$) to monitor patient exposure to CsA is still a matter of debate. This concern is mainly related to the limited data available showing the clinical usefulness of using single point strategy to monitor daily CsA exposure. The utility of CsA $C_0$ was initially proposed based upon reports of a correlation between low trough values and increased incidence of acute rejection episodes, as well as high concentrations and nephro- or hepatotoxicity [26]. Nevertheless, this approach was proven as not always reliable in routine clinical practice due to the fact that some patients experienced a worst outcome despite CsA $C_0$ levels within the therapeutic range. In search of more accurate single point sampling markers of CsA drug monitoring, in the last few years $C_2$ has been proposed as a useful surrogate of the maximum drug concentration ($C_{max}$), which in turn is effective in predicting acute rejection [27]. It should be pointed, however, that no studies have formally compared the predictive value of these two samplings in term of clinical outcome. Therefore, we have strictly measured serial $C_0$ and $C_2$ levels in over 330 kidney transplant recipients during the first 6 months post-surgery and found that CsA trough levels early post-transplant (day 2, 9 and 14) were the strongest predictor of acute rejection over 6 month follow-up [28]. In particular, $C_0$ levels within 300 to 440 ng/mL were associated with the lowest risk of rejection, while $C_2$ levels considered alone had no predictive value at all. The large number of pharmacokinetic measurements ($C_0$; N=2236 and $C_2$; N=2128) together with the stringent follow-up protocol, suggests that the predictive value of $C_2$ is overrated, at least in the early phase

post-surgery. To extend our findings, we have retrospectively analyzed full pharmacokinetic profiles of 58 patients at more than one year post surgery [29]. A logistic regression analysis of the interaction between pharmacokinetic parameters and graft function showed that $C_0$ but not $C_2$ significantly predicted long-term clinical outcome, expressed as renal function deterioration. Altogether, these findings suggest that $C_2$ is not a superior surrogate marker of the daily CsA exposure than $C_0$, either in the short and long-term period post-surgery. Additionally, by analyzing full CsA pharmacokinetic profiles, we have shown that not all kidney transplant recipients behave in the same manner as for absorption, distribution, metabolism and elimination of CsA. In particular, interindividual variation in the absorption process may segregate distinct population of patients [29]. Of note, patients with an "atypical" absorption profile are those which benefit less from single point-based limited sampling strategy to monitor daily CsA exposure [29], a condition that may expose these patients to chronic over- or under- cyclosporine exposure with dramatically clinical consequences. This concept was exemplified by our findings that in some patients the use single point-based monitoring may result in inadequate immunosuppression, leading to a significant increased risk to develop long-term complications of chronic CsA overexposure, such as Kaposi's Sarcoma (KS), a skin cancer. Indeed, we have compared the incidence of KS in transplant patients receiving Neoral or Sandimmune as a part of their immunosuppressive therapy [30]. 668 kidney transplant recipient followed at our Nephrology Unit from 1970 to 2003 entered this retrospective analysis. 300 were on CsA Sandimmune-based and 308 on CsA Neoral-based therapy. The primary endpoint was the occurrence of KS. The disease was diagnosed in 20 out of 608 patients given CsA with an incidence rate of 4.7 per 1000 patients per year. No episodes of KS were found in the pre-CsA era. Among patients on CsA, those treated with Neoral had 4 fold higher incidence rates of KS than in the Sandimmune group (10.7 vs 2.3 per 1000

patients per year). Kaplan-Meier analysis shows that patients on Neoral had lower cumulative KS-free probability than those on Sandimmune. Cox's analysis documented that Neoral was a positive predictor of KS development as compared to Sandimmune (hazard ratio: 2.237). Among patients on Neoral those who developed KS had higher daily exposure to the drug assessed by pharmacokinetic studies. Therefore, we concluded that in recipients of kidney transplant CsA Neoral increases the risk of KS as compared to the Sandimmune formulation, possibly due to enhanced drug bioavailability and ultimately patient's daily CsA exposure. To avoid or limit these dramatic consequences, we would suggest that all transplant recipients, beside $C_0$ or $C_2$ routine monitoring, should undergo at least one pharmacokinetic profile in order to be classified according to CsA absorption pattern.

It should be pointed, however, that drug concentration measurements are only a part of the decision tree for dose adjustment. They must be viewed in the context of other complementary fields that include biochemical and clinical analysis, as well as novel therapeutic monitoring approaches. As an example of this concept, we have conducted a randomized trial aimed at investigating whether per-protocol biopsies, in combination with routine pharmacokinetic studies, could be useful tools to implement steroid or CsA sparing regimens in kidney transplant recipients [187]. As main result, we have found that per-protocol biopsy more than one year after transplantation is a safe procedure to guide change of immunosuppressive regimen and to lower the risk of major drug-related side effects. Alternative approaches, beside pharmacokinetics, have been recently proposed to tailor the best immunosuppressive regimen for each patient. For instance, pharmacodynamic monitoring involves measurement of the biological effect of the drug at its target site [15]. Indeed, we have previously developed a method for the assessment of calcineurin, the pharmacological target of CsA, in whole blood. When we applied this assay to monitor kidney transplant recipients we found that CsA levels did

not predict daily calcineurin activity, whereas a single determination of the enzyme at baseline was a useful surrogate for the daily inhibition of the enzyme by CsA [188]. It should be pointed out, however, that pharmacodynamic tests are actually too complex for clinical use and often require radioactive materials. As additional drawbacks, studies aimed at investigating the potential predictivity of pharmacodynamic approaches in terms of rejection and/or drug toxicity are still lacking.

Those involved in TDM are now realizing that individual patient's exposure to immunosuppressive agents can be influenced by the genetic background. Indeed, it has been proposed that interinvidual differences in CsA response may be due, at least in part, to sequence variants in genes encoding drug-metabolizing enzymes, drug transporters, or drug targets. Recently, genetic variants in the CYP3A4 and CYP3A5 genes, responsible for the metabolism of CsA, have been described, and shown to be associated with an impaired enzyme activity [41]. Another factor that might influence drug disposition is the P glycoprotein (P-gp), the product of the MDR1 gene. This protein is an efflux pump, which removes lipophilic drug, like CsA, tacrolimus and sirolimus, from the intracellular space. Several polymorphisms have been found for the MDR1 gene [41]. Of these, three mutant alleles on exons 12, 21 and 26 correlate with expression of MDR1 gene, and function of P-gp. Actually, only few studies have investigated the impact of these polymorphisms on the bioavailability of CsA or tacrolimus. Most of them showed contradictory results [41]. Many factors can explain the great heterogeneity in the preliminary pharmacogenetic analyses, including limited number of subject considered, and weakness of pharmacokinetic parameters studied, which often do not include full AUCs. The major limitation of these approaches, however, is the study of single polymorphism rather then the potential influence of additive factors such as the haplotypes, that generally contain more information than did individual SNP. Therefore, further investigations in this field are needed.

*Aims of the present study*

According to the actual knowledge in the field of therapeutic drug monitoring of immunosuppressive agents, my research activity will be devoted to:

- develop an analytical method useful for the assessment of MPA plasma levels in kidney transplant recipients, with the goal to optimizing MMF dosing in these patients;

- study potential drug-to-drug interactions between MPA and other agents commonly administered to kidney transplant recipients;

- compare the pharmacokinetics of MPA from patients given MMF with that derived from the novel enteric-coated mycophenolate sodium formulation;

- develop an UPLC-UV method applicable for TDM of sirolimus in the routine clinical practice, with the goal to study the effect of concomitant immunosuppressive therapy and to optimize drug dosing;

- to assess the role of SNPs in the MDR1 gene in predicting CsA exposure in a large population of kidney transplant recipients.

*Chapter 2*

# SCOPE OF THE THESIS

The aim of this thesis is to apply TDM studies to kidney transplant recipients to optimise the management of new immunosuppressive agents, with the ultimate goal to improve patient clinical outcome. In particular, I have focused my research on the pharmacokinetics of mycophenolic acid (MPA)-releasing formulations – namely mycophenolate mofetil (MMF) as well as mycophenolate sodium (EC-MPS) – and sirolimus (SRL), with particular emphasis on the potential drug-to-drug interactions between these novel immunosuppressants and other agents commonly administered to kidney transplant recipients. Additionally, in the last part of the thesis, I have also presented preliminary data on pharmacogenetics, a new science which could represent a complementary approach to pharmacokinetics for the individualisation of immunosuppressive therapy for each patient.

*Mycophenolic acid*

MPA is a potent immunosuppressive agent commonly used following organ transplantation [31]. At clinically relevant concentrations, MPA is approximately 97% bound to plasma albumin. It has been shown that the unbound, rather than the total concentration of MPA is a predictor of MPA inhibitory effect on inosine monophosphate dehydrogenase (IMPDH). Therefore, the measurement of the free concentration of MPA, in addition to the total drug concentration, is advisable for pharmacokinetic investigations.

MPA is metabolized to an inactive glucuronide conjugate MPAG, a pharmacologically active acyl glucuronide metabolite and a 7-O-glucoside metabolite. MPA is primarily excreted renally as MPAG and to some extent in the bile. MPAG is excreted in the gut and reabsorbed as MPA, a mechanism commonly known as enterohepatic recirculation (EHC) resulting in the occurrence of a secondary plasma peak 6-12 h after oral MMF administration [31].

As a guide to dosage adjustment, it is advisable to measure the concentration of MPA (total and free) and MPAG in transplant recipients. An enzyme multiplied immunoassay technique is available on the market, however this method overestimates the MPA concentrations due to cross-reactivity with the MPA metabolites [32]. Therefore, high-performance liquid chromatography (HPLC) methods remain the standard for determination of MPA and its major metabolite MPAG. Thus, simple and rapid methods for the quantification of total, free MPA and MPAG, which could be reliably applied for TDM studies, are welcome.

*Sirolimus*

Sirolimus (SRL), a 31-membered triene macrolide lactone with a hemiketal-masked α-β-dioxocarboxamide and a molecular weight of 913.6 Da, is a novel antirejection drug with potent immunosuppressive activity both in vitro and in vivo [33,34]. Studies in humans have shown a relationship between trough blood SRL concentrations and immunosuppressive efficacy and toxicity of the drug [18]. This suggests that monitoring of the SRL concentrations is advisable to optimize drug dosing regimen.

Since the drug has a narrow therapeutic index (NTI), it is important to develop an adequate method to quantify SRL in biological matrices. Several HPLC methods have been developed so far for quantifying SRL in whole blood with extraction steps using a variety of solvents and mixtures, or with liquid/liquid and subsequent solid phase extraction [35]. These HPLC methods used both ultraviolet (UV) and mass spectrometry (MS) detection (HPLC-MS or HPLC/MS/MS). Although these methods meet many or all generally accepted criteria for validated analysis of immunosuppressive drugs, they are often cumbersome, have a very long sample preparation and/or chromatographic time, require glassware preparation (silanized or light protected tubes), use toxic solvents (i.e. 1-chlorobutane) or involve equipments

(HPLC-MS) not commonly available in the clinical laboratory that need great investment. Therefore, given the increasing use of SRL in clinical transplantation, there is now need for more feasible methods to be applied in the routine clinical practice which also would allow simultaneous measurement of large number of samples.

*Development and validation of analytical methods for the measurement of immunosuppressants in plasma or whole blood*

The application of TDM to routine clinical practice requires the measurement of drugs concentrations in biological fluids. On this regard, selective and sensitive methods for the quantitative evaluation of drugs are critical for the successful conduct of pharmacokinetic studies. It is, therefore, mandatory to demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. Thus, before to apply TDM to MPA and SRL monitoring, I have worked for the development of HPLC methods useful for the quantification of these immunosuppressants and, most important, I have assessed whether the performance of the assays was in agreement with the FDA Guidance for Bioanalytical Method Validation [36]. As described in *Chapters 3-5*, I have determined selectivity, accuracy, precision recovery, linearity and stability for each of the analytes assessed in the present thesis (namely, MPA, MPAG, SRL and CsA), using HPLC methods with UV detection developed in our Laboratory. Once proven acceptable performance, these methods have been subsequently applied to TDM of MPA and SRL.

*Therapeutic drug monitoring of MPA*

In the second part of the thesis I have faced with TDM of MPA, the active metabolite of MMF and EC-MPS. MPA is now routinely used in solid organ transplantation in a fixed daily dose regimen. However, no correlation has been shown between drug dose and

clinical outcome [37]. Therefore, alternative approaches to guide MPA dosing have been advocated.

To address this issue, we have taken advantage from the MYcophenolate Steroid Sparing (MY.S.S.) Study [38]. This multicenter, prospective randomised parallel group trial compared clinical outcome in recipients of cadaver-kidney transplant over 6-month treatment with MMF or azathioprine (AZA) along with CsA and steroids (phase A), and over 15 more months without steroids (phase B). Ninety out of the 356 patients in the MY.S.S. study were enrolled from the Centre of Bergamo, and half of these were given MMF. Therefore, we decided to measure MPA plasma levels (trough levels and MPA $AUC_{0-12}$ predicted using a LSS with sampling at: 0, 20, 40, 75 and 120 after MMF administration) in the 46 patients from the centre of Bergamo, and try to correlate MPA pharmacokinetic parameters with clinical outcome during the first 6-9 months (*Chapter 6*). Graft function was expressed as creatinine clearance, and MMF tolerability was evaluated by periodical assessment of blood cell count. At the completion of phase A, patients entered phase B if they had no more than two acute rejection episodes, no episodes of steroid-resistant rejection during phase A and had stable renal function with no proteinuria. Nearly 50% of the patients enrolled in phase A entered phase B [38]. To investigate the impact of steroids withdrawal on MPA pharmacokinetics, we measured MPA plasma trough levels, predicted MPA $AUC_{0-12}$ and MPA free fraction in 26 patients out of 46 who completed phase B and in an additional control group of patients who did not discontinue steroids. This additional analysis, presented in *Chapter 7*, introduced a "hot topic" in the field of organ transplantation, the problem of drug-to-drug interactions. Kidney transplant recipients are chronically given with 3 or more immunosuppressive agents which, sometimes, are substrates or act as inducers or inhibitors for the same transport-metabolic system. This might be dangerous for the patients, especially when they are treated with fixed drug regimens, as usually done

with MMF. Indeed, evidence is available that CsA and tacrolimus exerted a different effect on the entero-hepatic recirculation of MPA from glucuronidated metabolites [39]. However, no data were available on the pharmacokinetics of MPA in patients given MMF in combination with SRL, when compared with that drawn from patients treated with MMF and CsA. Therefore, as a part of a protocol aimed at investigating the efficacy of Campath-1H induction therapy in a steroid-free regimen, we measured MPA plasma levels in patients treated with low-dose SRL or low-dose CsA both in addition to low-dose MMF. To better compare the effects of both drugs on MPA enterohepatic recirculation, full pharmacokinetic profiles (at 0, 20, 40, 75, 120 min and at 3, 4, 5, 6, 7, 8, 10 and 12 h), instead of limited samplings (at 0, 20, 40, 75 and 120 min), were collected. Results of this study are given in *Chapter 8*. Patients given **Campath-1H** in combination with CsA and MMF were also used as reference group in a subsequent study aimed at assessing the pharmacokinetics of MPA from a cohort of patients given CsA and the new enteric-coated formulation of MPA (EC-MPS) as part of an intensified, multi-factorial therapy open trial. As described in *Chapter 9*, MPA exposure after EC-MPS administration was determined using a full sampling (at 0, 20, 40, 75, 120 min and at 3, 4, 5, 6, 7, 8, 10 and 12 h) both at month 6 and 12 post-surgery, and compared with that observed in the control group of patients given MMF and CsA.


*Therapeutic drug monitoring of SRL*

Rapamycins, namely SRL and everolimus, are a new class of immunosuppressive agents characterized by peculiar mechanisms of action, narrow therapeutic index, and strong relation between drug exposure and patients' outcome [40], all conditions that require strict therapeutic drug monitoring. In 2001, after the development of the HPLC method with ultraviolet detection for the assessment of SRL whole blood levels (described in *Chapter 4*), our Laboratory has become a centralized institution in Italy to

measure drug concentrations in blood samples from 40 Italian Transplant Centres. Over 2,600 SRL trough levels were assessed from nearly 500 kidney transplant recipients. All Centres referring to our Laboratory for the assessment of SRL concentrations were asked to provide information on SRL daily dose and concomitant immunosuppressive regimen. Given the large number of SRL measurements available, we sought to investigate the possible effect of concomitant immunosuppressive regimens (such as CsA, tacrolimus, MPA and steroids) on SRL exposure, expressed as dose-adjusted whole trough levels (*Chapter 10*). As additional aim, we analysed patients with serial SRL measurements with the goal to assess intra-and interpatient variability, and to develop an algorithm useful to guide changes of SRL dosing.


*Preliminary applications with pharmacogenetics*

The advent of the genomic era gives birth to pharmacogenetics, a science that studies how the genome may affect the action of a drug. This science is of particular importance for drugs characterized by a narrow therapeutic index, such as the immunosuppressants [41]. Preliminary studies focused on polymorphisms of genes encoding for enzymes actively involved in drug metabolism, drug transport, and pharmacological target. Pharmacogenomics holds promise for improvement in the ability to individualize immunosuppressive therapy based on the patient's genetic profile, and can be viewed as a support to the traditional pharmacokinetic-based therapeutic drug monitoring. Our preliminary experience of pharmacogenetic approaches applied to CsA monitoring was presented in *Chapter 11*. These data referred to 120 out of 356 kidney transplant recipients from the MY.S.S. trial who gave written informed consent for genetic testing. Presence of single nucleotide polymorphisms (SNPs) in the gene encoding for the P-glycoprotein was assessed.

Finally, the findings of all the studies presented above were discussed and put in the perspective of a better improvement of long term patient's outcome after organ transplantation (*Chapter 12*).

*Part I*

*DEVELOPMENT OF ANALYTICAL METHODS*

*FOR THE MEASUREMENT OF MYCOPHENOLIC*

*ACID, SIROLIMUS AND CYCLOSPORINE*

**Bioanalytical method validation**

The assessment of MPA (total and free), MPAG, SRL and CsA was done using HPLC methods. Prior to the application of HPLC methods to TDM of novel immunosuppressants, the performance of these assays was tested in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [36]. Measurement of each analyte in the biological matrix should be validated. Typical method development and establishment for a bioanalytical method include determination of selectivity, accuracy, precision, recovery, and calibration curve, stability of analytes in spiked samples and application of the method to routine drug analysis. Acceptance criteria for each on the above mentioned parameters are described below.

*Reference standards*

Analysis of MPA, SRL and CsA in the biological matrix (plasma or whole blood) was carried out using samples spiked with reference standards and using quality control samples (QCs). To avoid bias in the preparation of the spiking solutions only authenticated analytical reference standards of known identity and purity were used (either certified reference standards or commercially supplied reference standards obtained from a reputable commercial source).

*Selectivity*

The selectivity of each method was evaluated as lack of matrix interference by analysis of human drug-free blood samples from different volunteers (n=15). To investigate potential endogenous interference, blank samples were spiked with high concentrations of the most common immunosuppressive agents and analyzed. In addition, to test potential concomitant medication or xenobiotic interference, blood from different

transplant patients on immunosuppressive therapy and most common antifungal. antihypertensive and hypolipidemic agents were analyzed.

*Linearity*

The linearity of each method was tested by constructing standard curves from the lower limit of quantification (LLOQ) to the upper limit of quantification, plotting the peak height ratios of the drug to internal standard (IS) versus the nominal drug concentration. and applying a linear least squares regression analysis without weighing Each method was considered linear if the coefficients of regression ($r^2$), calculated as mean of 10 curves was equal or better than 0.99.

*Imprecision and inaccuracy*

The within- and between-day coefficient of variation (CV%) and the inaccuracy of each method were assessed by calculating daily and overall CVs and bias values for QC (usually five replicates at each concentration per analytical run) that were assayed in five separate analytical runs. The methods were considered acceptable if imprecision at each concentration was less than 15% for both within and between day variability. The inaccuracy should be between ± 15%.

*Lower limit of quantification*

The lowest standard on the calibration curve was accepted as LLOQ if the analyte response at the LLOQ was at least 5 times the response compared to blank response. In addition the analyte peak should be identifiable. discrete. and reproducible with an imprecision $\leq 20\%$ and inaccuracy between $\pm 20\%$.

*Recovery*

To determine the extraction efficiency, the peak-height ratios of spiked blood samples (plasma for MPA, MPAG) were compared to those obtained from direct injections of the same amount of the analyte. The assay was accepted if recovery exceeded 60%.

*Dilution integrity*

The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by inaccuracy and imprecision parameters in the validation. To establish dilution stability, samples at high concentrations were diluted two folds and five folds using the adequate drug-free biological matrix. Deviation from the nominal value was determined on three replicates.

*Stability studies*

Stability procedures evaluate the stability of the analyte during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature), storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments reflect situations likely to be encountered during actual sampling handling and analysis. The procedure should also include an evaluation of analyte stability in stock solutions.

Each analyte stability (MPA, SRL and CsA) was determined after three freeze-thaw cycles using the appropriate biological matrix. Six aliquots, each at two concentrations (one in the low and one in the high range) were prepared. Three aliquots were analyzed promptly prepared, the other three were stored at $-20^{o}C$ thawed at room temperature and refrozen under the same conditions. The cycle was repeated for two more times and analyzed on the third cycle. Comparison between mean results was performed calculating the percentage difference.

Short term stability of the analytes, simulating work bench conditions, was evaluated on six aliquots, each at two concentrations stored at -20°C. Three aliquots were thawed at room temperature over a time period of 15 hours. After 15 hours the other three aliquots were thawed and both sets were extracted and processed. Means of the response were compared and percentage difference calculated.

Long term stability has been established storing eighteen aliquots of two concentrations at −20°C and measuring the concentration over a period of 28 days. Mean concentrations obtained have been compared to the mean of back-calculated values for the standards at the same concentrations from the first day of long term stability.

Stability of MPA, SRL and CsA in the autosampler was evaluated for 24 hours. Ten sets of quality control were extract and placed in the autosampler at room temperature (at +4°C for SRL). Five sets of samples were analyzed immediately, the other five sets 24 hours later. Long term stability of standard working solutions was evaluated at two temperatures (+4 and −20°C) and over 28 days.

*Application of validated method to routine drug analysis*

Each calibration curve, generated in each analytical run, covered the expected unknown sample concentration. Samples below LLOQ or above the highest standard were not considered (samples with higher concentrations were diluted and reassayed). During each analytical run a number of QC samples prepared separately were analyzed (usually one at the start and one at the end of the analytical run). If QC (matrix spiked with the analyte) fall out the 15% of their respective nominal values, the run was rejected. When available, samples from the MPA, SRL and CsA International Proficiency Testing Scheme (IPTS), were also processed [42,43].

# DETERMINATION OF TOTAL, FREE MYCOPHENOLIC ACID AND ITS GLUCURONIDE METABOLITE USING HPLC WITH UV DETECTION

Total and free MPA plasma concentrations were measured by HPLC, introducing some modifications to already published methods [44,45,46]. MPAG was estimated as MPA after hydrolysis mediated by β-glucuronidase (see below).


### Total MPA

#### Human plasma, chemicals and materials

Calibration standards and QCs were prepared using pools of plasma samples from 15 healthy volunteers and from 20 kidney and liver transplant recipients not given MMF or EC-MPS (used to test potential concomitant medications).

Standards of MPA and MPAG were initially donated by Roche Pharmaceuticals (Palo Alto, CA). After 2002, MPA was bought from Sigma (St Louis, MO), together with p-toluic acid (PTA, used as internal standard). All the batches of MPA have a purity > 98% and were provided with the certificate of analysis. Acetonitrile, methanol were HPLC grade and were purchased by BDH (UK), all other chemicals were from Sigma. HPLC quality deionized water was prepared using Milli Q50 (Millipore, Bedford, MA). Bond-Elut C18, 200 mg, 3 ml cartridges were obtained from Varian (Leini, Italy).


#### Stock solutions, calibrators, and quality control standards

Stock solutions, containing 10, 100 mg/L of MPA and 50 mg/L PTA were prepared in methanol and stored at 4°C until use. Aliquots of the stock MPA solutions were diluted with drug free plasma to give 6 calibrators (0.1, 1, 5, 10, 20 and 40 MPA mg/L). Two in-house QCs were prepared in drug-free plasma with a final concentration of 2 and 20 mg/L MPA. Calibrators and QCs were stored at -20°C until use.

*Sample preparation*

Over 95% of MPA is bound to albumin, whereas only a limited amount of the drug is distributed within blood cells [39]. Therefore, plasma is the matrix of choice for the assessment of MPA levels in the blood.

Five hundred microliters of plasma was mixed with 1.5 mL of water, 50 µL of internal standard and 750 µL of 0.1 N HCl. The mixture was applied to a C18 solid phase extraction column pre-conditioned with 2 mL of methanol followed by 2 mL of water. The column was dried and then eluted with 1 mL of methanol/0.1 N acetate buffer (80:20 v/v) pH 4. Samples were collected in HPLC vials.

*HPLC apparatus and conditions*

A System Gold HPLC equipped with a model 166 UV detector set at 254 nm and a model 507 autosampler (Beckman, Fullerton, CA) were used. The autosampler was kept at room temperature, and a 50 µL aliquot sample was injected. The separation was carried out at room temperature using a C18 column, 250 x 4.6 mm, 5 µm (Hypersil BDS, Hewelett Packard, Ge). A guard column (LiChrosper 100 RP-18, 5 µm) was placed just before the column. The mobile phase for elution of the column was 45% acetonitrile and 55% aqueous phosphoric acid (0.05%), at flow rate of 0.8 mL/min. Data were collected and processed using a 32 Karact software for HPLC system (Beckman, Fullerton, CA).

*Assay validation*

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [36].

# MPAG

MPAG was enzymatically hydrolyzed to MPA using β-glucuronidase (100,000 U/mL, Sigma, St Louis, MO). This enzyme must be stored at 4°C under darkling conditions, to avoid degradation. β-glucuronidase solution was diluted with phosphate buffer (0.1 M, pH 6) to reach a concentration of 100 U/mL, and 950 μL of the final solution was added to 50 μL of plasma for each sample. The mixture was incubated for 1 h at 37°C and then processed as for total MPA determination. Final MPA concentration was the sum of the contribution of MPA present in each sample, and MPA derived from MPAG. Therefore, MPAG concentration was estimated by subtracting the MPA concentration (previously analyzed without β-glucuronidase) from total drug concentration, measured after addition of β-glucuronidase, and normalizing for the molecular weight of both compounds.

# Free MPA

## Materials

The Centifree Micropartition System (Amicon, Beverly, MA) with a molecular weight cut-off of 30 KD was used to obtain an ultrafiltrate for free MPA determination, and the devices were centrifuged in a Beckman centrifuge with fixed angle rotor (JA21). Because some batches of filters were found to contain impurities that interfered with the chromatograms, all filters were routinely sonicated and washed with methanol/water (1:1) before use.

## Stock solutions, calibrators, and quality control standards

Working solutions of MPA (1 mg/L) and PTA (2.5 mg/L) were prepared in methanol. The MPA working solution was used to prepare 6 calibrators in ultrafiltrate of drug free

plasma (0.005, 0.02, 0.04, 0.1, 0.5 and 1 mg/L). Two set of QCs were prepared in methanol (at concentration of 0.01 and 0.7 mg/L) and stored at -20°C until use.

## Sample preparation

For ultrafiltration procedure, 800 µL of plasma was added to the sample reservoir and the tube centrifuged at 5500 rpm (at 20°C) for 40 minutes, yielding approximately 400 µL of ultrafiltrate. Three hundred-fifty µL of ultrafiltrate was mixed with IS (50 µL) and with a phosphate buffer (10 µL, pH 2), and transferred in a polypropylene vial.

## HPLC apparatus and conditions

HPLC system and column were the same used for the assessment of total MPA. Injection volume was 100 µL. The mobile phase consisted of solution A (250 ml acetonitrile and 300 mL of 20 mM phosphate buffer pH 3.0) and solution B (700 ml of acetonitrile and 300 mL of 20 mM phosphate buffer pH 6.5), eluted at flow rate of 1.2 mL/min with a gradient from 3% B to 100% B as follows:

| Time (min) | %B | Duration (min) |
|---|---|---|
| 4.5 | 25 | 0.5 |
| 12.0 | 100 | 0.5 |
| 14.5 | 3 | 0.5 |
| 30.0 | inject | 0.1 |

Compounds were quantified by absorbance at 215 nm. Data were collected using a recorder Shimadzu and processed with Microsoft Excel.

## Assay validation

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [36], as previously described.

## Total MPA

### Chromatographic separation

As shown in Figure 1, MPA (retention time 6.3 min) and IS (retention time 4.6 min) were well resolved and no interference was observed from plasma peaks at the elution times of these analytes. In addition, analysis of plasma samples from transplant recipients receiving immunosuppressants other that MMF revealed no interfering peaks from endogenous and exogenous compounds. The chromatographic run required 8 min per sample.

### Linearity

The assay was linear in the concentration range from 0.1 to 100 mg/L with a mean regression coefficient value of 0.999 of 10 replicated curves (0.1, 0.5, 1, 5, 10, 20, 40, 60, 80, and 100 mg/L). For routine assessment of MPA plasma levels we used a 6-point equation (0.1, 0.5, 1, 5, 10, 20 and 40 MPA mg/L).

### Imprecision, inaccuracy and LLOQ

Imprecision and inaccuracy were tested, both as intra-and inter-day evaluations, at 5 concentrations (0.1, 1, 10, 20 and 40 mg/L). As shown in Table 1, the method was accurate and precise, with inaccuracy and imprecision less than 10%. LLOQ was set at 0.1 mg/L.

### Recovery

The solid-phase extraction procedure had an average recovery of 77% for MPA and 72% for PTA, verified at 2 concentrations (2 and 20 mg/L).

*Stability studies*

Stability studies were performed using plasma samples spiked with MPA at 2 and 20 mg/L. MPA concentration values were not affected by freeze-thaw cycles as well as by short-term (at room temperature for 10h h) and long-term stability tests (stored at -20°C for 1 month). Moreover, both drug concentrations were stable also in the autosampler, at room temperature, for at least 24 h. Working solutions (MPA 10 and 100 mg/L) were stable at 4°C for at least 1 month (see Table 2).

*Application of the validated method to routine MPA analysis*

The present method has been used to routinely measure MPA plasma trough levels as well as complete (from 0 to 12 h) and predicted (from 0 to 2 h) MPA $AUC_{0-12}$ in kidney transplant recipients given MMF or EC-MPS as part of their immunosuppressive regimen. Two in-house QCs were used during each analytical run (MPA at 2 and 20 mg/L). The temporal distribution of the inaccuracy of our HPLC method is presented in Figure 2. From June 2005, the method is enrolled in the MPA International Proficiency Testing Scheme, with a mean inaccuracy of 6%.

## MPAG

To test the feasibility of the proposed procedure, we added fresh β glucuronidase to 9 samples spiked with know amount of MPAG (namely, 5, 10, 20, 40, 60, 80, 160, 320 and 500 mg/L) and the mixture was incubated for 1 h at 37°C. Subsequently, the samples were processed as previously described for MPA. In these experimental conditions, more than 99% of the glucuronide was converted to MPA.

Beta glucuronidase, stored at °4C and protected from the light, was stable for at least 6 months. When exposed to the light, these enzymes gradually lose its activity (tested periodically using known amount of MPAG).

## Free MPA

### Chromatographic separation

Figure 3 shows the first part of the chromatograms from ultrafiltrate drug-free samples spiked with different MPA solutions (we did not collect data during the equilibrating period). The retention time of IS and MPA were 7.9 and 12.4 min. The total chromatographic run required 30 min per sample.

### Linearity and LLOQ

The method was linear in the concentration range from 0.005 to 1 mg/L of MPA ($r^2$=0.992), and the LLOQ was 0.005 mg/L.

### Imprecision and inaccuracy

The performance of the HPLC method for the assessment of free MPA was worse than that used for the measurement of total MPA. However, inaccuracy and imprecision were still in agreement with the FDA Guidelines (see Table 3).

### Stability

The concentrations of MPA (0.01 and 0.5 mg/L) prepared in drug free ultrafiltrate remained unchanged after three cycles of freeze and thaw, and were not affected by storage of the samples at room temperature for 12 h or by storage of ultrafiltrate samples in the autosampler for at least 24 h. In every instance the percentage difference from the nominal values was below 15%.

**Legend to Figures**

*Figure 1*. Typical chromatograms of mycophenolic acid (MPA) and para-toluic acid (PTA, internal standard) from human plasma spiked with MPA (0.5 mg/L) and from a patient given mycophenolate mofetil (MMF) as part of the immunosuppressive regimen (11.3 mg/L). For comparison drug-free plasma taken from healthy volunteer is also given (Bk).

*Figure 2*. Temporal distribution of the inaccuracy of the HPLC method used for the assessment of MPA plasma concentrations, calculated from two in-house quality controls (at 2 and 20 mg/L).

*Figure 3*. HPLC chromatograms of free MPA and PTA of ultrafiltrate from healthy volunteer (Bk) and from a patient under treatment with MMF (free MPA 0.07 mg/L).
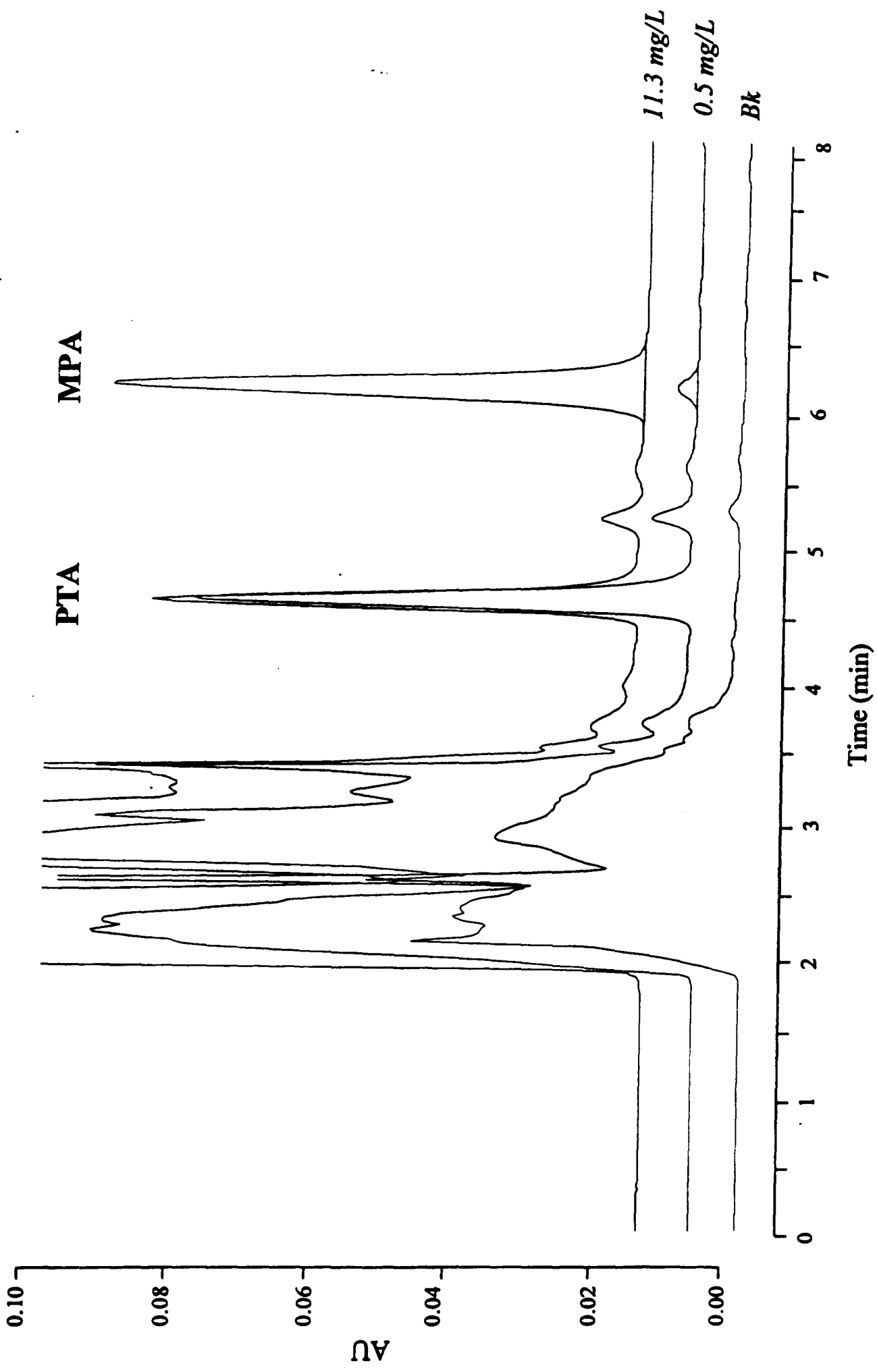
- Figure 1 -
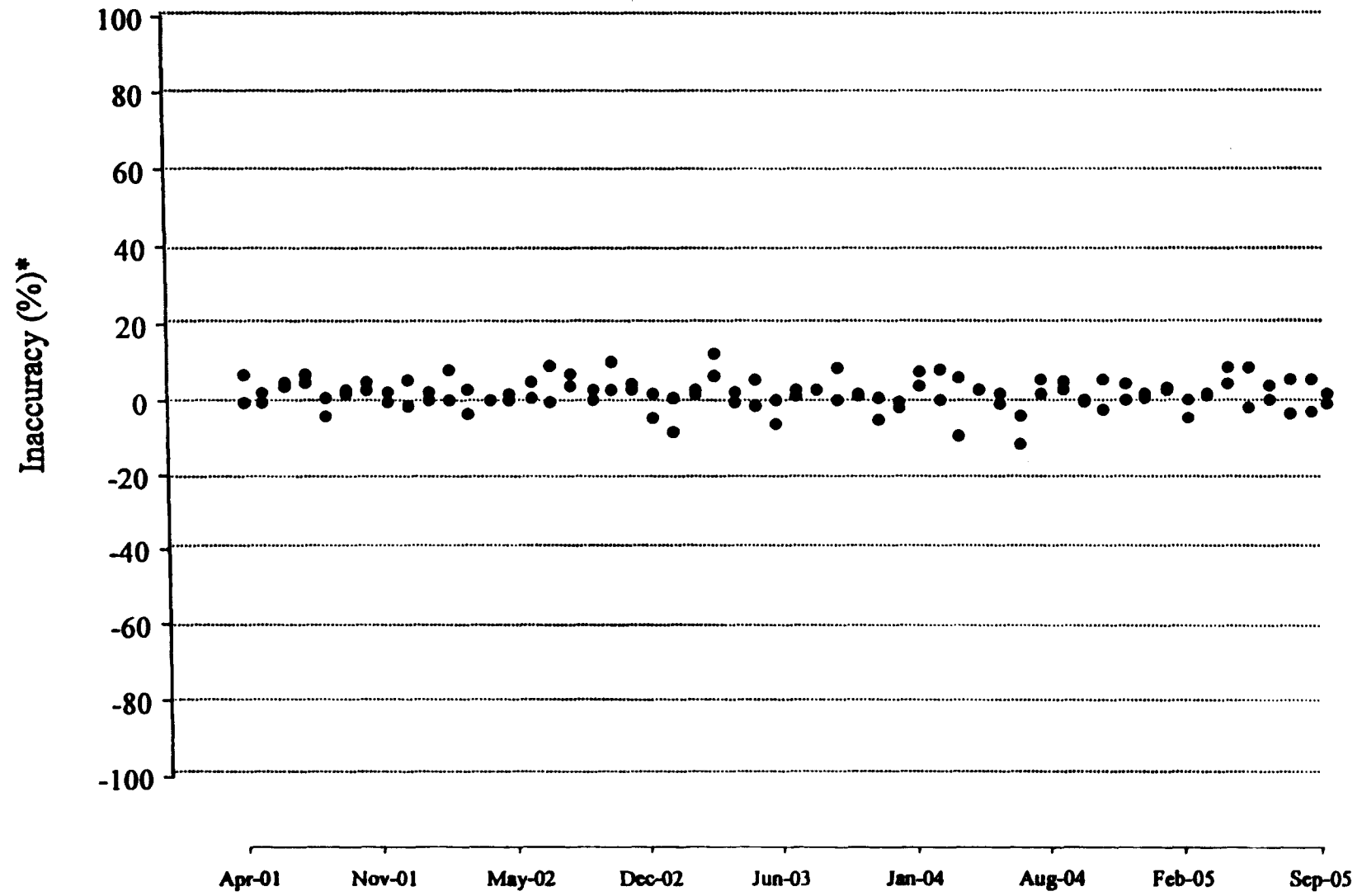
Temporal distribution of MPA QCs

*From in-house quality controls
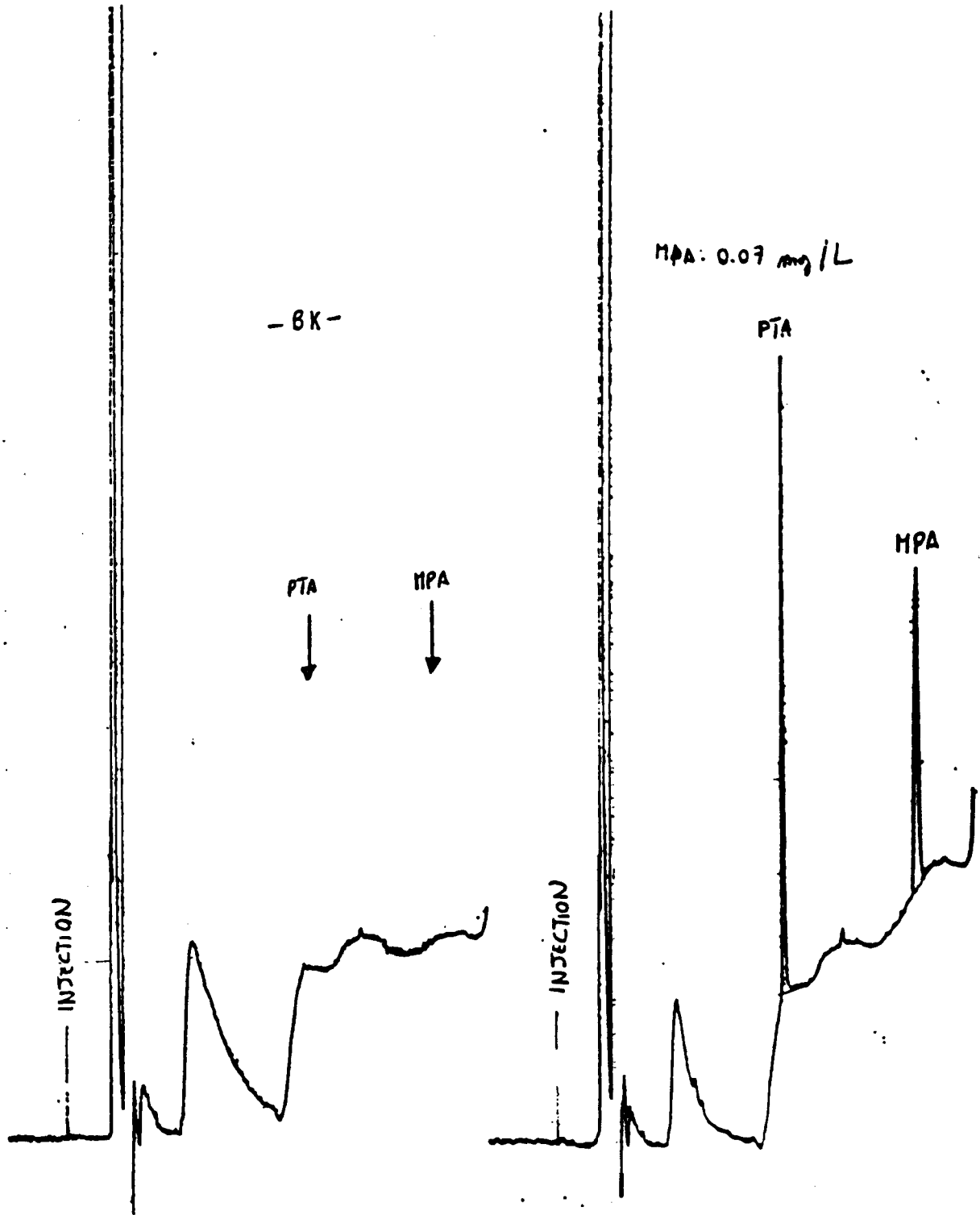
- Figure 2 -

- Figure 3 -

Table 1. Performance of the HPLC method for the determination of plasma MPA concentration

| | Spiked MPA concentration (mg/L) | | | | |
| --- | --- | --- | --- | --- | --- |
| | 0.1 | 1 | 10 | 20 | 40 |
| **Within-day assay** | | | | | |
| Mean ± SD (ng/mL) | 0.11 ± 0.01 | 1.1 ± 0.05 | 10.0 ± 0.1 | 19.9 ± 0.4 | 40.1 ± 0.3 |
| Imprecision (CV%) | 9.1 | 4.5 | 1.0 | 2.1 | 0.7 |
| Inaccuracy (%) | 7.9 | 6.5 | 0 | -4.5 | -0.5 |
| **Between-day assay** | | | | | |
| Mean ± SD (ng/mL) | 0.09 ± 0.01 | 1.1 ± 0.1 | 10.1 ± 0.3 | 19.8 ± 0.5 | 40.4 ± 0.7 |
| Imprecision (CV%) | 11.1 | 9.1 | 3.0 | 2.5 | 1.7 |
| Inaccuracy (%) | -8.9 | 7.7 | 1.0 | -1.0 | 1.0 |

Table 2.   Results of the stability studies using plasma samples spiked with MPA

| Parameters | Results (%)* | |
|---|---|---|
| | 2 mg/L | 20 mg/L |
| Freeze-thaw (3 cycles) | - 2.4 | + 1.7 |
| Short term plasma samples (10 h at room temperature) | + 3.2 | + 2.1 |
| Long-term plasma sample (28 days at –20°C) | - 2.0 | + 3.3 |
| Autosampler (24 h at room temperature) | + 2.0 | - 1.1 |
| Long-term stock solution (28 days at –20°C) | + 3.0 | + 4.4 |
| Long-term stock solution (28 days at +4°C) | + 4.0 | +2.8 |

*comparison between mean results was performed calculating the percentage difference

Table 3. Performance of the HPLC method for the determination of free MPA

| | Spiked free MPA concentration (mg/L) | | |
|---|---|---|---|
| | 0.005 | 0.02 | 1.0 |
| **Within-day assay** | | | |
| Mean ± SD (ng/mL) | 0.006 ± 0.0011 | 0.022 ± 0.003 | 1.12 ± 0.08 |
| Imprecision (CV%) | 18.3 | 13.6 | 7.1 |
| Inaccuracy (%) | 20.0 | 10.0 | 12.0 |
| **Between-day assay** | | | |
| Mean ± SD (ng/mL) | 0.006 ± 0.0011 | 0.019 ± 0.002 | 1.15 ± 0.05 |
| Imprecision (CV%) | 18.3 | 10.5 | 4.3 |
| Inaccuracy (%) | 20.0 | -5.0 | 14.9 |

*Chapter 4*

# ASSESSMENT OF SIROLIMUS CONCENTRATIONS IN WHOLE

# BLOOD BY HPLC WITH UV DETECTION

*Human whole blood samples, chemicals and materials*

For the preparation of in-house QCs and calibration standards, different pools of whole blood samples from 15 healthy volunteers was used. In addition, subsequent to signing of an informed consent form, EDTA anticoagulated whole blood samples were obtained from 30 kidney, heart and liver transplant recipients not given SRL.

Standard samples of SRL (purity ranging from 97 to 98%, according to different batches) and 32-O-desmethoxyrapamycin (internal standard, IS) were generous gifts from Wyeth-Ayerst Research Laboratories (Princeton, NJ) and furnished with adequate information on drug source, lot number, expiration date and certificate of analysis.

Acetonitrile and methanol (BDH, Milan, Italy) were HPLC grade; acetone and hexane HPLC grade were supplied by Fluka (Milan, Italy). Zinc sulphate heptahydrate, analytical grade, was purchased from Fluka and a 5% solution was prepared in distilled water. All other chemicals were analytical grade. Bond-Elut C18, 200 mg, 3 ml cartridges were obtained from Varian (Leinì, Italy).


*Stock solutions, calibrators and quality control standards*

Stock solutions containing 50 and 100 μg/mL were appropriately prepared in methanol for SRL and IS, respectively. SRL working solutions of 100, 500, and 2000 ng/mL were prepared in 50/50 methanol/water, and for IS a working solution of 1000 ng/mL was prepared in methanol. All the solutions were stored at -20 °C.

Taking into account the therapeutic range of SRL trough levels, calibrator samples were prepared mixing appropriate volumes of SRL from stock working solutions to EDTA anticoagulated human whole blood from healthy volunteers to achieve different concentrations from 2.5 to 60 ng/mL (2.5, 5, 10, 15, 20, 40, and 60 ng/mL). Calibrators were prepared by diluting each spiking solution to 10 ml with K3EDTA control human whole blood in 10 ml volumetric flasks. The flasks were stopper and shaken to mix.

Pools are measured into 1 mL aliquots in polypropylene tubes and frozen at −20°C until use. Three in-house QCs, representing the low, medium and high concentrations, were prepared in drug-free whole blood with a final concentration of 3, 10 and 30 ng/mL SRL. Calibration, QCs and reference standards were aliquoted and stored at -20°C until use.

*Sample preparation*

SRL is extensively distributed in red blood cells, independently of concentration and temperature [47], so we decided to use whole blood as the preferred matrix for method validation.

One millilitre volume of whole blood sample was pipetted into disposable polypropylene tubes and supplemented with 50 µL of IS solution (1000 ng/mL). The tubes were vortex-mixed for 40 seconds; 1.5 mL of zinc sulphate solution was first added followed by a 1.5 mL acetone. The tubes were vortex-mixed for a further 50-60 seconds and centrifuged at 3000 g for 5 minutes at room temperature. The clear supernatant was poured into another polypropylene tube, diluted with 2 mL distilled water, mixed and loaded onto a Bond-Elut cartridge (preconditioned with 1 mL acetonitrile followed by 1 mL methanol and finally by 1 mL distilled water) placed on a Vac Elut 20 Manifold (Varian). The Bond-Elut cartridges were washed with 1.5 mL of 70% methanol/30% water. In each step the solvent was allowed to drop out from the cartridge. Then 500 µL hexane was added and the column was allowed to go dry under vacuum. SRL and IS were eluted in polypropylene tubes with 1 mL acetonitrile. In all steps the flow rate did not exceed 1 mL/min. The eluate was taken to dryness either under a gentle nitrogen stream in a water bath at 37 °C or in a model RC 10.09 centrifugal evaporator (Jouan, Saint-Herblain, France) and the residue was dissolved in 0.15 mL of water-methanol-acetonitrile (40/30/30) and transferred in a polypropylene

vial. Internal calibration curves for SRL were prepared for each set of samples. At least 60 samples (including controls and calibration curve) can be extracted in 4 h and processed by HPLC in less than 20 h.

*HPLC apparatus and conditions*

A System Gold HPLC equipped with a model 166 UV detector set at 278 nm and a model 508 autosampler (Beckman, Fullerton, CA) with the sample tray kept at 4 °C, were used. A 90 µL aliquot of sample was injected onto reversed-phase C18, 5 µm, guard column (Alltima, 7.5 x 4.6 mm, Alltech, Sedriano, Milan, Italy) connected to a 75 x 4.6 mm column packed with Ultrasphere C8, 3 µm (Beckman) heated at 50 °C by a Model 880 oven (Spark-Holland, Emmen, The Netherlands) and was eluted by a mixture of distilled water/methanol/acetonitrile (34/30/36) pumped at a rate of 1 ml/min. Due to the high percentage of the organic phase that may dry off, resulting in increased retention time, the mobile phase was prepared every one or two days before analysis, filtered and degassed under vacuum using a polycarbonate 0.4-µm membrane. An in-line filter (0.5 µm) was placed between the autosampler and the column. Data were collected and processed using a 32 Karat software (Beckman, Fullerton, CA).

*Method Validation*

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [36], as described above.

In addition, the present method has been enrolled in the Sirolimus international Proficiency Testing Scheme [42]. For the Proficiency Test initially 78 blinded samples, packaged as 5 batches of samples each, were analyzed. In addition, ongoing proficiency was tested by analyzing 3 blinded samples from the Reference Laboratory every month.

## Results

### Chromatographic separation

Figure 4 displays the chromatograms of extracts prepared from a blank blood spiked with known amounts of SRL and from blood of a transplant patient given SRL. The retention time of SRL and IS were 13.1 and 14.5 min, respectively, and the chromatographic step required 18 min per sample. Both SRL and IS eluted as symmetrical and relatively sharp peaks. Blank blood samples from healthy volunteers did not show peaks corresponding to SRL and IS retention times. This was confirmed in 30 kidney, heart and liver transplant recipients in a SRL-free regimen. Representative chromatogram of a blank sample is shown in Figure 4. On the other hand, a concentration-dependent increase in the SRL peak height was documented when known amounts of the drug were added to blood samples to achieve a final concentration of 2.5 and 40 ng/mL, respectively. Moreover, a distinct peak of SRL was found in the blood sample collected from a kidney transplant patient given SRL as a part of the immunosuppressive therapy. Furthermore, no chromatographic interference was found between SRL or IS and other immunosuppressants such as CsA, MPA, azathioprine and steroids as shown when blood samples from transplanted patients were added in vitro with SRL, or when blood from healthy subjects was spiked in vitro with SRL and immunosuppressants. Even high concentration of the above mentioned drugs did not affect the SRL chromatographic profiles.

Occasionally a sharp peak occurred in some chromatograms at about 10.5-11 min - which did not affect the assay's performance - probably dependent on the different lot of Bond-Elut cartridges used.

The minimal overlapping between SRL and IS peaks observed when concentrations higher than 40 ng/mL were analyzed did not influence the accuracy of the analysis. This was confirmed by injecting a lower volume (40 µL) of the same sample at two

concentration levels. The complete separation between the analytes was restored and results were similar (40 μL injection: 39.4±1.1 ng/mL; 90 μL injection: 39.8±1.0 ng/mL. 40 μL injection: 80.7±5.5 ng/mL; 90 μL injection: 82.5±4.7 ng/mL).

*Linearity*

Linearity was determined by least-squares linear regression analysis of the peak height of SRL/IS versus SRL concentration. The method demonstrated excellent linearity over the range of 2.5-100 ng/mL ($r^2$, calculated as mean of 10 curves, was 0.998).

Since SRL trough levels from organ transplant recipients were greatly below the upper limit of quantification, we decided to use 7 points ranging from 2.5 to 60 ng/mL for the calibration curves used in the routine clinical practice. Linear regression of the peak height ratio of the drug/IS versus the concentration for calibration curves produced a coefficient of regression greater than 0.998. A typical equation describing the calibration curve was y=0.02358 (SE:0.00015)x + 0.02075 (SE:0.00313).

*Imprecision and inaccuracy*

The within-day and between-day coefficients of variation for SRL in whole blood are reported in Table 4. The within-day imprecision of the assay, as estimated by the coefficient of variation of the measurement from spiked samples, was below 6.6%. The between-day coefficient of variation was still low (8.0%). The precision of our method was good also when replicates from a patient sample were considered (mean 7.7±0.4 nL/ml; CV 5.7%, n=5). The inaccuracy of the method was low and, as expected, data were better for the highest SRL concentration tested (40 ng/mL). The result of inaccuracy obtained with the lowest concentration of the drug (2.5 ng/mL) was still acceptable (8.8%).

The extraction procedure of the samples was tested with different blood volumes (ranging from 300 to 600 μL) at 7.5 ng/mL (mean 7.9±0.4 ng/mL; n=5) and 22.5 ng/mL (mean 22.0±0.7; n=5). In each instances we obtained an acceptable precision (CV less than 6.0%). These results suggest that there is no need of additional changes in the method protocol when small volume of samples is available.

*LLOQ*

The LLOQ was established at 2.5 ng/mL, in agreement with the FDA Guidelines (signal/noise ration > 5, imprecision below 20%, inaccuracy between ±20%). This is a suitable value to detect accurately the expected low SRL trough concentration in transplant patients.

*Recovery*

The overall recovery, calculated by comparing the peak-height ratios of spiked samples with those obtained by direct injections of the same amount of SRL and IS, was checked at 5 and 20 ng/mL. The overall recovery was 64.4 ± 3.0% for SRL and 63.3 ± 1.9% for IS.

*Dilution integrity*

To establish dilution stability, blood samples at concentration of 100 and 200 ng/mL were diluted with SRL-free whole blood, and SRL concentration was determined on three replicates. A two-fold dilution of samples with concentration of 100 ng/mL and 200 ng/mL was associated with an inaccuracy of -2.1 ± 3.6% and 1.4 ± 3.9%, respectively, while a 5-fold dilution of 200 ng/mL revealed an inaccuracy of -5.4 ± 4.3%.

*Stability studies*

Stability studies were performed using whole blood samples spiked with SRL at 5 and 50 ng/mL. As shown in Table 5, SRL concentration values were not significantly affected by freeze-thaw cycles as well as by short-term (at room temperature for 15 h) and long-term stability tests (at least 28 days stored at -20°C). Both concentrations of SRL were stable, also when leaved in the autosampler for at least 24 h.

Working solutions of SRL (methanol/water 50/50) were stable for at least 28 days if kept at -20°C, but not at 4°C.

*Application of validated method to routine drug analysis*

We have applied the validated method for the assessment of SRL daily blood concentration profile after the morning oral drug administration in several kidney transplanted patients. In addition, the present method has been used to routinely monitor SRL trough levels from heart and kidney transplant recipients given the drug as part of their immunosuppressive regimen. An analytical run was usually performed every 7-10 days. Two or three in-house QC (prepared separately from the calibration curves) were used during each analytical run. In addition monthly samples from the SRL IPTS were processed. Temporal distribution of the inaccuracy of our HPLC method (from results of the SRL IPTS) from November 2001 to September 2005 is presented in Figure 5. The median inaccuracy of the present method was -6.0% (95% confidence intervals: -8.9 to -3.1%).

**Legend to Figures**

*Figure 4.* Typical chromatograms of sirolimus (SRL) and internal standard (I.S.) from drug-free blood taken from healthy volunteer (Bk), blood spiked with 2.5, 40 ng/mL SRL, and a trough blood sample (7.1 ng/mL) taken from a kidney transplant recipient immediately before the next oral dose of SRL.

*Figure 5.* Temporal distribution of the inaccuracy of the HPLC method used for the assessment of SRL whole blood concentrations, derived from the results of the Sirolimus International Proficiency Testing Scheme.

SRL   I.S.

40 ng/mL.

7.1 ng/mL.

2.5 ng/mL.

Bk

AU

0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0

0    2    4    6    8    10    12    14    16    18

Time (min)
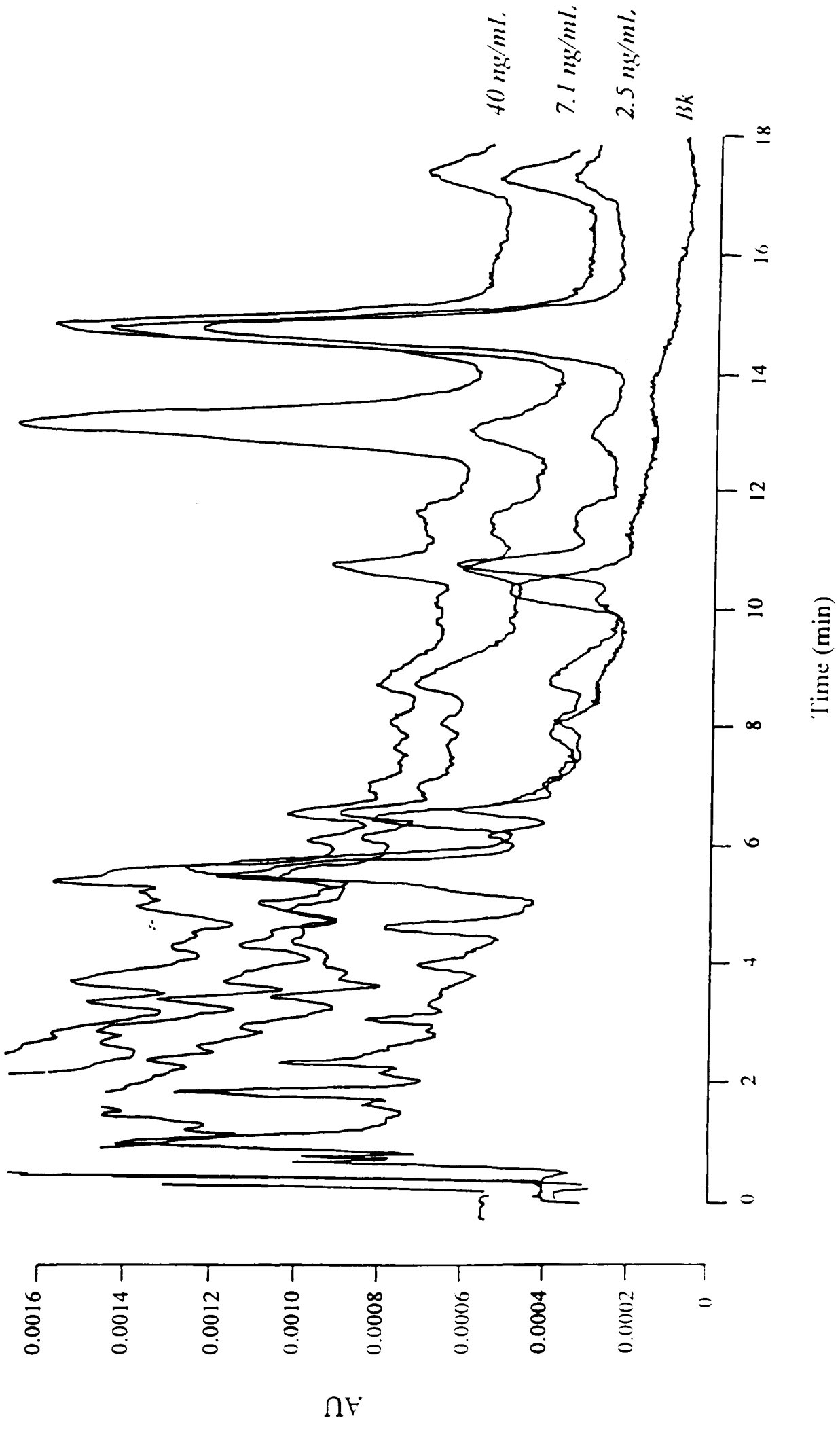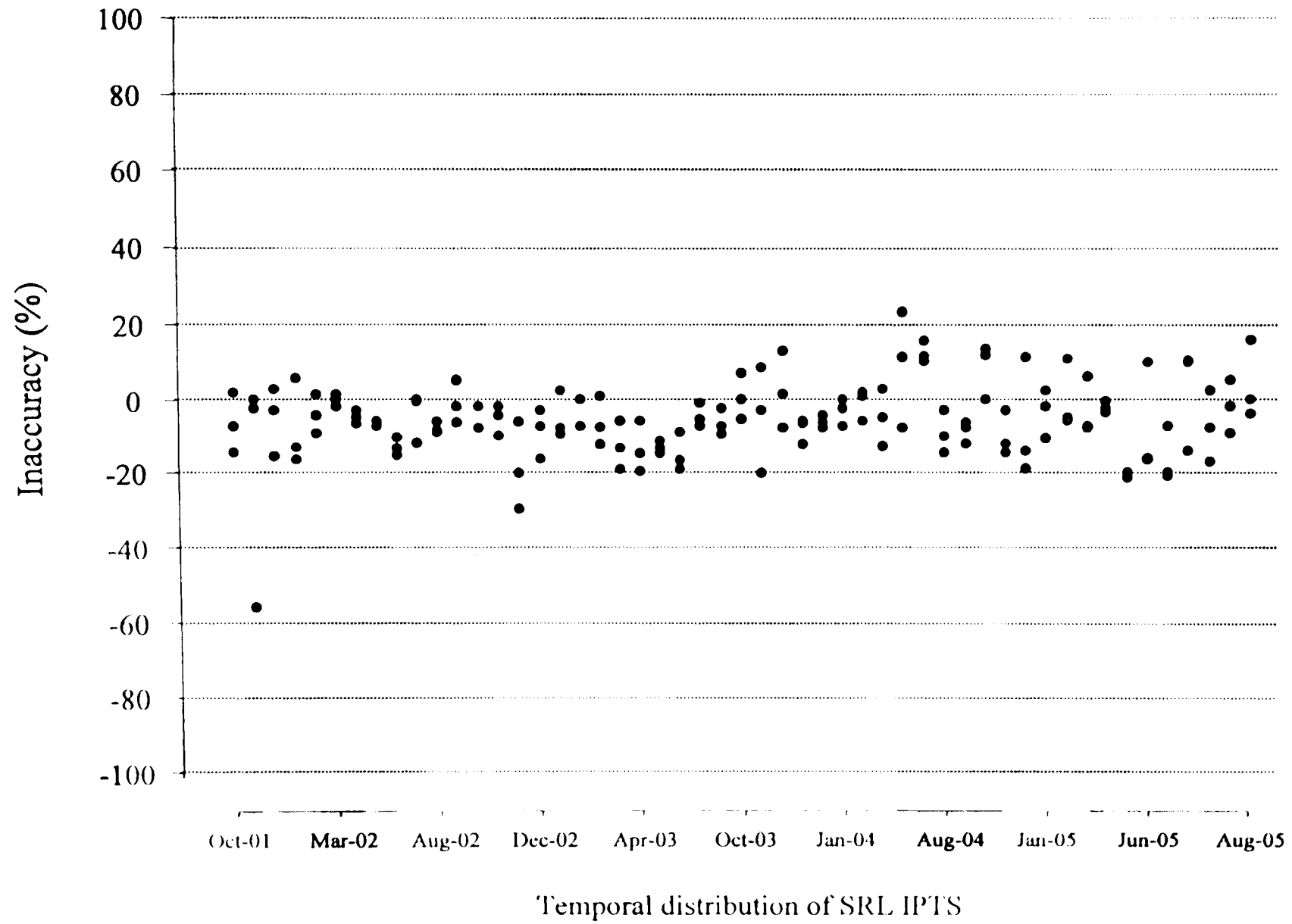
- Figure 4 -

- Figure 5 -

Table 4. Performance of the HPLC method for the determination of SRL concentration in whole blood

| | Spiked SRL concentration (ng/mL) | | | | |
|---|---|---|---|---|---|
| | 2.5 | 5 | 10 | 20 | 40 |
| **Within-day assay** | | | | | |
| Mean ± SD (ng/mL) | 2.7 ± 0.2 | 5.0 ± 0.4 | 10.3 ± 0.2 | 19.1 ± 0.7 | 39.3 ± 0.6 |
| Imprecision (CV%) | 7.4 | 8.0 | 1.9 | 3.7 | 1.5 |
| Inaccuracy (%) | 8.0 | 0.0 | 3.0 | -4.5 | -1.8 |
| **Between-day assay** | | | | | |
| Mean ± SD (ng/mL) | 2.7 ± 0.2 | 5.0 ± 0.2 | 10.3 ± 0.3 | 18.8 ± 0.5 | 42.3 ± 0.8 |
| Imprecision (CV%) | 6.6 | 3.1 | 2.7 | 2.7 | 1.8 |
| Inaccuracy (%) | 8.8 | -0.8 | 3.0 | -5.8 | 5.7 |

Table 5. Results of the stability studies using whole blood samples spiked with SRL

| Parameters | Results (%)* | |
|---|---|---|
| | 5 ng/mL | 50 ng/mL |
| Freeze-thaw (3 cycles) | - 4.1 | + 2.5 |
| Short term blood samples (15 h at room temperature) | + 7.7 | - 1.9 |
| Long-term blood sample (28 days at –20°C) | + 2.4 | + 3.7 |
| Autosampler (24 h at 4°C) | + 4.4 | + 3.1 |
| Long-term stock solution (28 days at –20°C) | + 4.0 | + 6.1 |
| Long-term stock solution (28 days at +4°C) | + 32 | +25 |

*comparison between mean results was performed calculating **the** percentage difference

*Chapter 5*

# MONITORING OF CYCLOSPORINE IN WHOLE BLOOD

# USING A HPLC-UV METHOD

CsA was measured by UPLC with UV detection, using the method from Kahn et al [48], with some modifications, as described below.

## Human whole blood, chemicals and materials

Calibration standards, QCs and blanks were prepared using pools of whole blood samples from healthy volunteers (n=15) and from kidney (n=10) and liver (n=10) transplant recipients not given CsA.

CsA and cyclosporine D (IS) were kindly supplied by Novartis Pharma (Basel, CH), with a declared purity of 100%. Methanol and acetonitrile (BDH, UK) were of HPLC grade. All other solvents were of analytical grade (Sigma, St Louis, MO). Deionized water was prepared using a Milli Q50 system (Millipore, Bedford, MA).

## Stock solutions, calibrators and QCs

Stock solutions containing 100 mg/L of CsA and IS, as well as working solutions (10 mg/L for CsA and 20 mg/L for IS) were prepared in methanol. For calibration of the analytical system appropriate volumes of CsA from stocked working solutions were added to 1 mL EDTA anticoagulated human whole blood to achieve 7 different CsA concentrations (20, 50, 100, 200, 500, 1000 and 2000 ng/mL). QCs were prepared spiking known volumes of CsA from working solutions to drug free whole blood in order to obtain three concentrations (30, 300, 900 ng/mL CsA). CsA solutions, calibrators and QCs were stored at -20°C.

## Sample preparation

To one mL of peripheral vein blood samples we added IS (50 µL), hydrochloric acid 0.2N (1 mL), and heptane (1 mL). The mixture was vortexed for 10 sec to lyse the blood cells. Subsequently we added diethyl ether (8 mL) and each tube was tightly

capped. Extraction of CsA was effected on a reciprocal shaker. The organic phase was clarified by centrifugation for 15 min at 3000 RPM. The ether layer was decanted into a clear glass tube and washed with sodium hydroxide 0.1 N (1mL). Following a second centrifugation for 10 min, the ether layer was transferred into a clean glass tube and evaporated to dryness under a gentle nitrogen stream in a water bath at 37°C. The residue was redissolved in 200 μL of the mobile phase and washed by vortexing for 30 sec with heptane (1 mL). The sample was finally centrifuged (10 min at 3000 RPM) and the lower aqueous layer transferred in a polypropylene vial.

*HPLC apparatus and conditions*

A system Gold HPLC with a UV detector set at 214 nm and a model 580 autosampler (Beckman, Fullerton, CA) was used. A 50 μL of aqueous layer was injected into a C-8 HPLC column (150 x 4.6 mm, 5 μm, Beckman) heated at 72°C by a LC oven 101 (Perkin Helmer, Milan). Isocratic liquid chromatography separation was carried out using a mobile phase of water/methanol/acetonitrile (27/32/41) at a flow rate of 1 mL/min. Data were collected and processed using a 32 Karact software (Beckman).

*Method Validation*

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies [36], as described above.

Since July 2003, this method is enrolled in the Cyclosporine international Proficiency Testing Scheme [43]. Ongoing proficiency is tested by analyzing 3 blinded samples from the Reference Laboratory every month.

## Results

### Chromatographic separation

Figure 6 displays the chromatograms of extracts prepared from a CsA-free sample, a blank blood spiked with known amounts of CsA and from blood of a transplant patient given CsA. The retention time of CsA and IS were 12 and 16.5 min, respectively, and the chromatographic step required 20 min per sample.

### Linearity and LLOQ

Linearity was determined by least-squares linear regression analysis of the peak height of CsA/IS versus CsA concentration. The method demonstrated excellent linearity from 20 to 5000 ng/mL ($r^2$, calculated as mean of 10 curves, was 0.996). The LLOQ was established at 15 ng/mL (inaccuracy: $12.3 \pm 3.3\%$, imprecision: $15.8 \pm 4.9\%$).

### Imprecision and inaccuracy

The within- and between-day performance of this method was assessed by calculating CV% and bias values for the three QCs (30, 300 and 900 ng/mL in five replicates at each concentration per analytical run) that were assayed in 5 separate analytical runs. As shown in Table 6, the performance was satisfactory.

### Recovery

The overall recovery, checked at 50 and 1000 ng/mL of CsA, was $70.4 \pm 3.8\%$ for CsA and $68.3 \pm 4.5\%$ for IS, respectively.

### Dilution integrity

To establish dilution stability, blood samples at concentration of 1000 and 4000 ng/mL were diluted with CsA-free whole blood, and CsA concentration was determined on

three replicates. A two-fold dilution of samples with concentration of 1000 ng/mL and 4000 ng/mL was associated with an inaccuracy of 4.5 ± 2.1% and 2.2 ± 3.9%, respectively, while a 5-fold dilution of 4000 ng/mL revealed an inaccuracy of -3.8 ± 2.8%.

*Stability studies*

Stability studies were performed using whole blood samples spiked with CsA at 50 and 1000 ng/mL. CsA concentration values were not significantly affected by freeze-thaw cycles as well as by short-term (at room temperature for 15 h) and long-term stability tests (at least 28 days stored at -20°C). Both concentrations of CsA were stable, also when leaved in the autosampler for at least 24 h at room temperature. Working solutions of CsA were stable for at least 2 months, kept at 4°C (Table 7).

*Application of validated method to routine drug analysis*

The present method has been used to routinely monitor CsA trough levels and full pharmacokinetic profiles from kidney transplant recipients given the drug as part of their immunosuppressive regimen. An analytical run was usually performed every 2-4 days. Three in-house QC were used during each analytical run in addition to samples from the CsA IPTS (when available). Temporal distribution of the inaccuracy of our HPLC method compared with results from the IPTS is presented in Figure 7. The median inaccuracy of the present method was -7.9% (95% confidence intervals: -9.8 to 2.8%).

**Legend to Figures**

*Figure 6.* Chromatograms of cyclosporine (CsA) and I.S. (cyclosporine D or CsD) from whole blood sample spiked with 25 ng/mL CsA and from kidney transplant recipients given CsA as part of their immunosuppressive regimen (CsA $C_0$: 243 ng/mL, CsA $C_2$: 581 ng/mL).

*Figure 7.* Temporal distribution of the inaccuracy of the HPLC method used for the assessment of CsA whole blood concentrations, derived from the results of the Cyclosporin International Proficiency Testing Scheme.

CsD

CsA

581 ngm/L

243 ng/mL

25 ng/mL

Time (min)

AU

0.007   0.006   0.005   0.004   0.003   0.002   0.001   0.000   -0.001

0   1   2   3   4   5   6   7   8   9   10   11   12   13   14   15   16   17   18   19   20
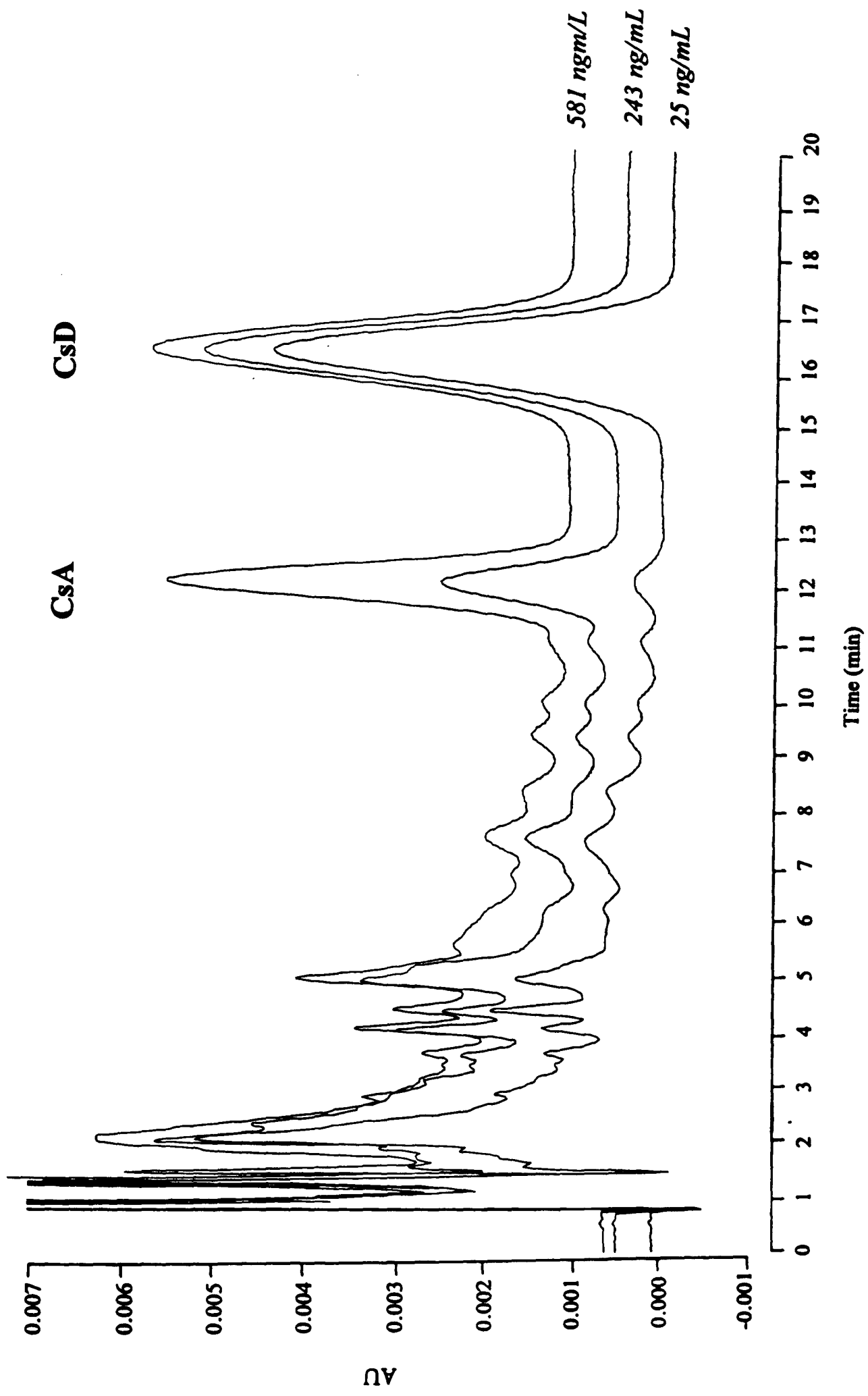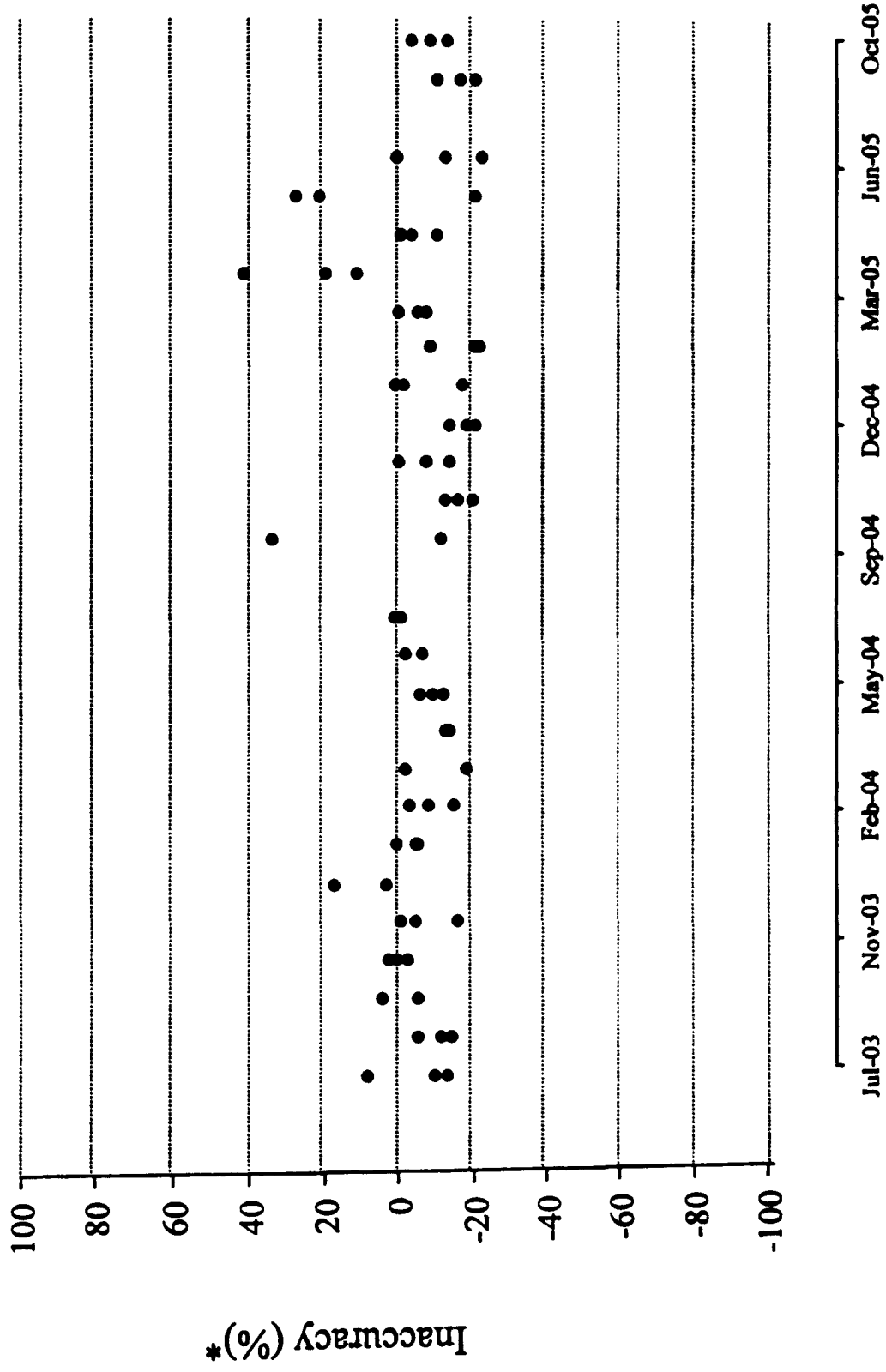
- Figure 6 -

Temporal distribution of CsA IPTS

- Figure 7 -

Table 6. Performance of the HPLC method for the determination of CsA levels

| | Spiked CsA concentration *(ng/mL)* | | |
|---|---|---|---|
| | 30 | 300 | 900 |
| **Within-day assay** | | | |
| Mean ± SD *(ng/mL)* | 32 ± 2 | 297 ± 15 | 888 ± 23 |
| Imprecision *(CV%)* | 6.3 | 5.1 | 2.6 |
| Inaccuracy *(%)* | 6.7 | -1.0 | -1.3 |
| **Between-day assay** | | | |
| Mean ± SD *(ng/mL)* | 28 ± 3 | 299 ± 12 | 890 ± 21 |
| Imprecision *(CV%)* | 10.7 | 4.0 | 2.4 |
| Inaccuracy *(%)* | -6.7 | -0.3 | -1.1 |

Table 7.   Results of the stability studies using whole blood samples spiked with CsA

| Parameters | Results (%)* | |
|---|---|---|
| | 50 ng/mL | 1000 ng/mL |
| Freeze-thaw (3 cycles) | 3.4 | - 2.3 |
| Short term blood samples (15 h at room temperature) | + 6.7 | - 1.7 |
| Long-term blood sample (28 days at –20°C) | + 3.1 | + 1.3 |
| Autosampler (24 h at room temperature) | + 5.0 | - 2.2 |
| Long-term stock solution (28 days at –20°C) | + 2.0 | + 2.1 |
| Long-term stock solution (28 days at +4°C) | + 3.2 | - 1.5 |

*comparison between mean results was performed calculating the percentage difference

*Part II*


# THERAPEUTIC DRUG MONITORING OF

# MYCOPHENOLIC ACID-RELEASING

# FORMULATIONS

*Chapter 6*

# PHARMACOKINETICS HELP OPTIMIZING MYCOPHENOLATE MOFETIL DOSING IN KIDNEY TRANSPLANT RECIPIENTS

# Introduction

Three large double-blind randomized trials in kidney transplant recipients [49,50,51] have shown that addition of MMF, a novel immunosuppressive drug [52], to CsA and prednisone allowed reduction in the rate of acute rejection during the first 6 or 12 months after transplantation as compared to azathioprine- or placebo-treated patients. Since then, MMF has been widely used in a fixed daily dose of 2 g, in two divided administrations, as a part of a combination regimen with CsA and steroids. A fixed dosage of MMF has been proposed also for patients undergoing heart or liver transplantation, based on results of clinical trials [53,54], as well as for the limited number of patients with chronic nephropathies unresponsive to conventional treatment [55].

MMF is the morpholinoethyl ester prodrug form of the active immunosuppressive molecule MPA, toward which is rapidly converted following oral ingestion [56]. MPA is, itself, metabolized to the phenolic glucuronide (MPAG), which is mostly eliminated in the urine [56]. By selectively and reversibly inhibiting IMPDH, a key enzyme in the de novo pathway of purine synthesis, MPA prevents the proliferation of both T and B lymphocytes [52,56].

Thus, as in the case of CsA and tacrolimus [57,58,59], individualizing MMF dose instead of using a fixed dose, might help to optimize immunosuppression and minimize potential toxic effect. This would require routine therapeutic drug monitoring by measuring plasma MPA concentration. Previous studies have shown a correlation between the MPA AUC and the risk of graft rejection [37]. However, contrary to CsA, data showing the usefulness of monitoring MPA concentration or defining a therapeutic window in terms of plasma MPA concentrations are scanty [60].

In the current study we examined 1) the need of optimizing MMF dosing by MPA pharmacokinetic monitoring in stable kidney transplant patients; 2) the possible association between MPA pharmacokinetic parameters and kidney graft function; and 3) whether measurement of free MPA compared to total plasma MPA concentration provides additional information as for MMF-induced change in blood cell count.

# Methods

## Patients

Forty-six consecutive adult renal transplant patients (17 female, 29 male) who had received kidney graft from 6 to 9 months before, were studied (Table 8). All were recipients of first kidney transplant from cadaver donors, aged 15 to 58 years. They were on triple immunosuppressive therapy with CsA Neoral, prednisone and MMF. Patients started with the conventional 2g/day MMF dose, in two divided administrations. Eighteen of them, however, required MMF dose reduction within the first 2 months post-transplant due to adverse events, i.e. leukopenia, thrombocytopenia, diarrhoea and CMV infections. Changes in MMF dosing was performed by the physicians on the basis of clinical parameters suggesting drug-induced toxicity and not based on MPA pharmacokinetics. Thereafter MMF daily dose remained fixed, ranging from 500 mg to 2g/day according to patient needs. At the time of the evaluation all patients had stable graft function.

## Study design

The study was described in detail to all patients before admission and informed consent was obtained in each instance. On the morning of the study, blood samples were collected for routine haematological analysis, and for determination of trough blood level of CsA and plasma total MPA and MPAG. Then each patient was given the morning dose of CsA and MMF and underwent evaluation of simplified pharmacokinetic profiles. Thus for CsA pharmacokinetics blood samples were collected in heparinized tubes at 60 and 180 minutes after dosing [61] and stored at -20 °C until analysis. For MPA and MPAG pharmacokinetics blood samples in EDTA-tubes were drawn at 20, 40, 75 and 120 minutes [37]. Thereafter samples were centrifuged at 3000

g, plasma separated, and stored at -20 °C until analysis for MPA and MPAG concentration. In a subgroup of patients (n=23) the percentage of free MPA concentration at time 0, in addition to total MPA trough level, was determined.

*MPA, MPAG and CsA measurement*

MPA (total and free) and MPAG plasma concentrations as well as CsA whole blood levels were measured by reverse phase HPLC as described in *Chapter 3*.

*Pharmacokinetic parameters*

Trough levels of MPA and MPAG were determined. The MPA $AUC_{0-12}$ was predicted using the equation of Hale et al [37]. AUC from 0 to 2 h ($AUC_{0-2}$) was calculated using the trapezoidal rule. For the 2- to 12 h interval, the concentrations ($C_t$) at 6, 8 and 12 h were estimated by the empiric equation:

$$C_t = 0.14 + 1.25 \times C_0$$

In which $C_0$ is the predose MPA level. Main MPA pharmacokinetic parameters were adjusted for the daily MMF dose and expressed as per g MMF. Free MPA was expressed as percentage rate of unbound / total MPA.

Trough level of blood CsA was determined and the CsA $AUC_{0-12}$ was calculated using a three-point strategy (sampling at 0, 1 and 3 h post CsA dosing) with the following equation:

$$AUC_{0-12} = 5.189 \times C_0 + 1.267 \times C_1 + 4.150 \times C_3 + 135.079 \ [61]$$

## Statistical analysis

Results are reported as means ±SD. Data were analyzed with paired or unpaired t test for normally distributed parameters. For non-normally distributed parameters the Wilcoxon test was used. The statistical significance level was defined as $P<0.05$.

# Results

*Variability of MPA pharmacokinetics*

Table 9 summarizes baseline graft function and MPA pharmacokinetic parameters. Serum creatinine and urea concentrations were widely distributed ranging from normal to moderate renal insufficiency values. In each patient, however, these values were comparable to those measured during the previous 3 months, indicating stable graft function.

Plasma MPA trough levels ranged from 0.24 to 7.04 mg/L, and the estimated MPA $AUC_{0-12}$ values were in parallel from 10.1 to 99.8 mg*h/L. Similar wide range values were found for average MPAG concentration.

Since in these patients the MMF daily dose was not uniform, although fixed for a given patient we normalized MPA pharmacokinetic parameters for the dose regimen. As shown in Figure 8 (panel A), only 37% of patients had similar normalized MPA trough level, the remaining being largely distributed in the low or high range of the frequency profile for a given plasma MPA value. Similarly, a large interindividual variability in MPA $AUC_{0-12h}$ was documented (Figure 8, panel B).

*Association between MPA pharmacokinetics and kidney graft function*

Figure 9 reports the correlation between MPA pharmacokinetics parameters and graft function. Plasma MPA trough levels were positively and significantly correlated with patients' creatinine clearance values (panel A, $r=0.50$, $p<0.01$). A significant correlation was also found between MPA $AUC_{0-12}$ and renal function measured as creatinine clearance (panel B, $r=0.52$, $p<0.01$). Both MPA trough levels and MPA $AUC_{0-12}$, however, did not correlate with the given MMF dose ($r=0.1$).

Table 10 shows demographic characteristics and functional and pharmacokinetic parameters in two subgroups of transplant patients characterized according to MPA $AUC_{0-12}$ > or <40 mg*h/L. The two groups were comparable for gender, body weight, kidney donor age as well as number of rejection episodes. Patients with MPA $AUC_{0-12}$ group >40 mg*h/L were slightly but significantly younger than those of group <40 mg*h/L ($p<0.05$). Patients with MPA $AUC_{0-12h}$ >40 mg*h/L had better renal function, as documented by a significantly lower serum creatinine concentration ($P<0.05$), and higher creatinine clearance ($P<0.01$) values, than those with lower MPA AUC. This finding was confirmed even after normalizing creatinine clearance values for body surface area to limit the potential influence of gender and body weight on renal function estimation. However, no difference in CsA dose, blood CsA trough level as well as CsA-AUC was found between the two groups of patients. Similar findings were achieved when patients were considered according to plasma MPA trough level > or <1.5 mg/L (data not shown).

We also analyzed values of MPA pharmacokinetic parameters in patients arbitrarily subdivided according to tertiles of serum creatinine concentration, namely $\leq 1.3$ mg/dL (mean 1.1 mg/dL normal graft function), >1.3 to $\leq 1.6$ mg/dL (mean 1.5 mg/dL mild renal insufficiency) and >1.6 mg/dL (mean 2.1 mg/dL moderate renal insufficiency). As shown in Figure 10, the highest MPA $AUC_{0-12}$ value was associated with the lowest tertile of serum creatinine concentration. At variance the lowest mean MPA $AUC_{0-12}$ values were found in patients with the highest serum creatinine (moderate renal insufficiency). Similar findings were observed for MPA trough level: the highest MPA trough level ($2.33\pm1.80$ mg/L) was linked to the lowest serum creatinine tertile, whereas the lower MPA trough level ($1.17\pm0.71$ mg/L) was associated with the highest tertile of serum creatinine concentration. The difference between MPA AUC or trough levels and serum creatinine tertiles was statistically significant ($p<0.05$). However, CsA dose (130,

152, 134 mg/day), blood CsA trough level (194.9, 182.5, 168.5 ng/mL) and CsA-AUC (4981, 4825, 5045 ng/mL.h) were comparable between the tertile groups.

*Plasma free MPA fraction and blood cell count*

The percentage of free MPA trough concentration in plasma of patients with anaemia was higher than that in patients with normal red blood cells (RBC) count (RBC <4.2 x$10^6$/μL: 2.31±0.71 %; RBC >4.2 x$10^6$/μL: 1.63±0.88 %, P<0.05). Similar findings were observed with free MPA trough concentration (RBC <4.2 x$10^6$/μL: 0.064±0.025 μg/mL; RBC >4.2 x$10^6$/μL: 0.030±0.022, P<0.05) By contrast total MPA trough concentration was comparable in anaemic and normocytemic patients (RBC <4.2 x$10^6$/μL: 3.32±2.26 μg/mL; RBC >4.2 x$10^6$/μL: 3.53±2.57 mg/L). Moreover, the percentage of free MPA and the free MPA concentration but not total MPA trough values significantly correlated with RBC count (% free MPA: r=0.51, P<0.05; free MPA concentration: r=0.50, P<0.05; total MPA: r=0.13, P=0.54; Figure 11) or hematocrit values (% free MPA: r=0.46, P<0.05; free MPA concentration: r=0.54, P<0.05; total MPA: r=0.27, P=0.21; Figure 11).

A significantly negative correlation was also shown between percent free but not free or total MPA trough level and leukocyte cell count (% free MPA: r=0.45, P<0.05; free MPA concentration: r=0.13 P=0.30; total MPA: r=0.18, P=0.41; Figure 12).

## Discussion

The first finding of the present study is that in kidney transplant recipients with stable graft function given MMF as a part of their antirejection therapy, both plasma MPA trough level and MPA $AUC_{0-12h}$ were highly variable despite patients received a fixed daily dose of the prodrug, even after normalization of values for the different total MMF dosage used by single patients. Difference in MPA pharmacokinetic values can not be attributed to poor reproducibility of the assay, since our coefficient of variation for MPA measurement by HPLC was 3.4%. On the other hand, our results confirm previous observation that, although the bioavailability of MPA is high and approaches 94% in healthy subjects and renal transplant patients [60], the 12-h dose interval MPA AUC showed a >10-fold range for renal transplant patients on a fixed MMF dose of 2 g/day [60,63]. Moreover, it has been reported in healthy subjects and kidney transplant recipients that the plasma MPA concentration-time profile for a single dose of oral MMF, after an overnight fast, rapidly increased and peaked approximately after 1 h, followed by an initial rapid decrease, and then a secondary peak at 6-12 h [64]. This pattern is probably attributable to an enterohepatic pathway involving MPAG passage into the gastrointestinal tract via biliary excretion, conversion to MPA via glucuronidase action in gut flora, and reabsorption of the latter into the systemic circulation [56]. Thus, the interpatient MPA pharmacokinetic variability as well the documentation of an enterohepatic circulation pathway for MPAG/MPA, would suggest that, as currently performed with CsA or tacrolimus, dosage adjustment to achieve an appropriate plasma MPA trough level or MPA AUC target concentration should be advisable.

Somewhat earlier in the clinical development of MMF, a retrospective statistical evaluation of MPA dose-interval AUC data in relation to the incidence of acute rejection was performed in patients enrolled in a MMF Japanese renal transplant clinical

study [65] and given fixed doses of 1, 2, 3, or 4 g MMF daily. A pharmacokinetic/pharmacodynamic correlation between MPA $AUC_{0-12}$ and the risk for acute rejection [60] was observed in that the risk for acute rejection decreased with increasing MPA AUC. A more recent prospective concentration-clinical response study confirmed a strong significant relationship between rejection risk and MPA AUC but not MMF dose [37]. Moreover, similar results were found in heart transplant patients in which the incidence of acute rejection episodes were clearly related to the MMF plasma trough level [66,67], in the presence of therapeutic CsA or tacrolimus blood levels. These studies strongly support the need of therapeutic drug monitoring to optimize MMF dosing and reduce the risk of graft rejection early post-transplant. Here we extended these observations and found a significant correlation between MPA $AUC_{0-12}$ and graft function (as creatinine clearance) in kidney transplant recipients 6 to 9 months post-surgery. Moreover patients with renal function in the highest tertile of serum creatinine concentration had the lowest MPA $AUC_{0-12}$ and trough level, despite no difference in CsA exposure between the tertiles. Certainly these findings do not allow us to conclude for a causal relationship between plasma MPA levels and renal function. However, some considerations may indirectly help to confirm the possible association between these parameters. It is known that MPA levels and probably metabolism are influenced by protein binding, since MPA is avidly and extensively bound to human serum albumin [44]. Many reports describe reduced drug binding in disease associated with hypoalbuminemia, e.g. liver disease, various types of renal dysfunction, burn injuries and malnutrition [68,69]. However, our transplant patients were in good health, with normal serum albumin level, and no clinical and laboratory evidence of liver disease, which could exclude a reduced binding of MPA. Moreover, free MPA concentrations and free fraction of MPA are elevated in many patients with severe renal dysfunction (creatinine clearance <20 mL/min) when on chronic MMF

therapy [70], due to both the uremic state per sè and by competition for albumin binding sites with the renally eliminated metabolite MPAG. This was not the case for the patients we studied, who had normal renal function or mild to moderate renal dysfunction and average plasma MPAG level <100 mg/L. Furthermore, differences in graft function in the two groups of patients with MPA AUC > or <40 mg*h/L were not due to different immunological response or CsA exposure, since the number of rejection episodes early post-surgery and CsA dose and pharmacokinetic parameters were comparable. This would also exclude that difference in plasma MPA levels were due to different blood concentrations of CsA, given the fact that CsA has been previously shown to influence MPA trough level in kidney transplant recipients [71].

On the other hand, attempts to define the pharmacokinetic relationship for MMF-related adverse events have not been always successful and experience suggest that drug dose (not MPA pharmacokinetics) provide the most predictive information for adverse events such as diarrhoea [50,51,72]. Others, however, have documented that increased level of MPA were significantly related to the occurrence of overall side-effects, albeit in a small group of patients [73]. Moreover, haematological side-effects such as the low haemoglobin level but not low leukocyte count were also related to MPA trough concentrations [74]. All these MPA pharmacokinetic data related to total plasma MPA concentrations, i.e. the sum of both protein bound and free MPA, but it is only the free MPA concentration that has pharmacological activity [75]. Thus, post-transplant total MPA plasma concentration may not reflect changes in free MPA level and hence the occurrence of MMF-related side-effects. Here, we evaluated the percentage of free MPA trough concentration as compared to total MPA level in relation to different blood cell counts in a subgroup of the same kidney transplant recipients. Actually, the percentage of free MPA concentration over total MPA better reflects the actual exposure of patients to the drug than the free MPA concentration itself. We found that

the percentage of free plasma MPA but not total MPA significantly correlated with the risk of anaemia, indicating the importance of measuring unbound MPA fraction at least to monitor changes in blood cell count possibly related to MMF administration. Although in most of our patients white blood cell count was in the normal range, a significant correlation between free MPA plasma fraction and total leukocyte count was also observed.

In summary, we have confirmed that in kidney transplant recipients given MMF at a fixed dose regimen, as a part of a triple immunosuppressive therapy including CsA and steroid, MPA pharmacokinetics parameters are highly variable. We also showed 1) an association between MPA $AUC_{0-12}$ or plasma MPA trough level and graft function, and 2) that percentage free but not total plasma MPA trough concentration negatively correlated with RBC as well as leukocyte count.

Altogether these findings suggest that a fixed dose regimen of MMF might no longer be the best approach for the management of transplant patients. MPA pharmacokinetic monitoring might contribute to limit the risk of MMF-related toxicity. Whether optimizing MPA dose by monitoring levels may help to maintain graft function in the long-term is an intriguing possibility raised by the present findings. Prospectively designed clinical trials are required to definitely address this important issue.
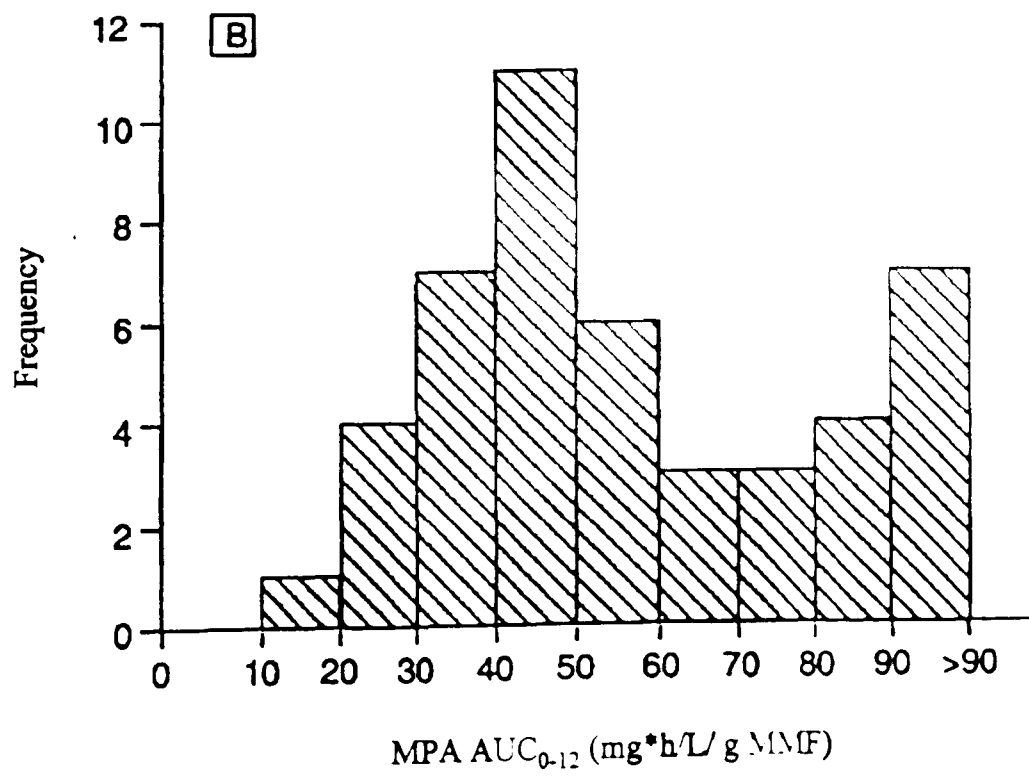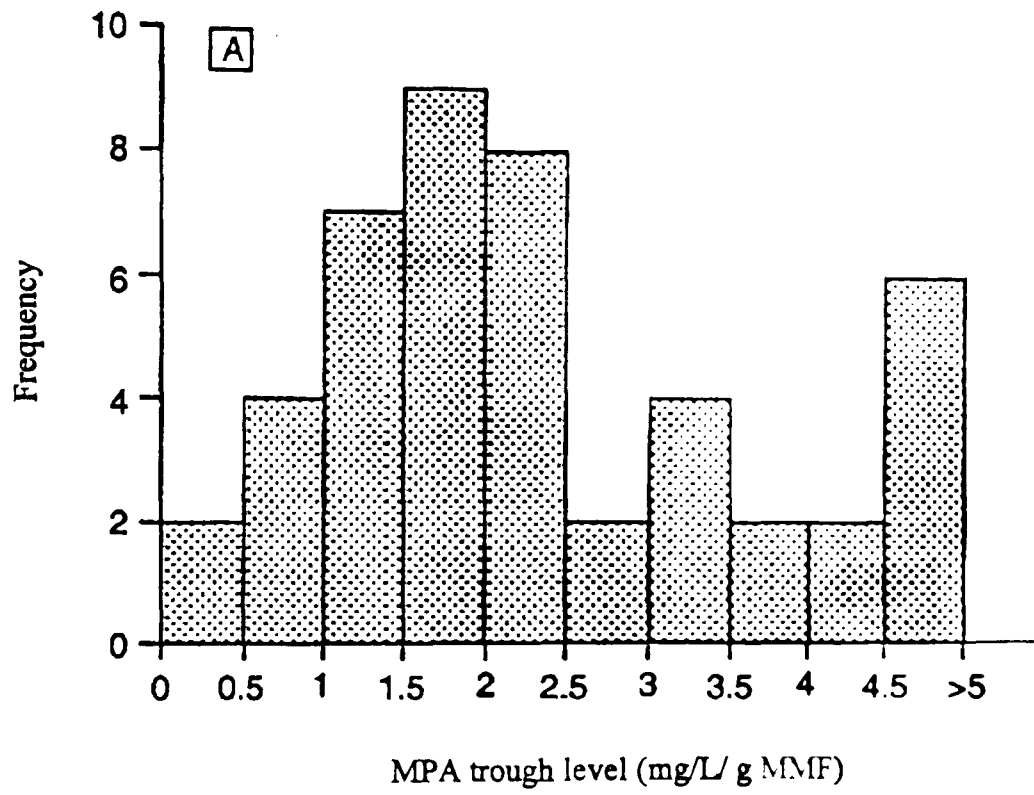
# Legend to Figures

*Figure 8.* Frequency distribution plot of plasma MPA trough levels (panel A) and MPA area under the time concentration curve from 0 to 12 h post-MMF dosing ($AUC_{0-12}$) (panel B) in 46 kidney transplant patients.

*Figure 9.* Relationship between plasma MPA trough levels (panel A) or MPA $AUC_{0-12}$ (panel B) and creatinine clearance values in kidney transplant recipients (n=46).

*Figure 10.* Relationship between mean MPA $AUC_{0-12}$ values and tertiles of serum creatinine concentration in 46 kidney transplant recipients. Values are mean $\pm$ SD. *$p<0.05$ versus $\leq 1.3$ mg/dL serum creatinine tertile.

*Figure 11.* Correlation between total MPA trough levels (panel A) or percentage of free MPA (panel B) and red blood cell count (RBC) in 11 kidney transplant recipients. Relationship between total MPA trough levels (panel C) or percentage of free MPA (panel D) and hematocrit in the same patients.

*Figure 12.* Correlation between total MPA trough levels (panel A) or percentage of free MPA (panel B) and leukocyte cell count (WBC) in 11 kidney transplant recipients.

- Figure 8 -

Graph A: Creatinine CL (mL/min) versus MPA trough level (mg/L/ g MMF); r=0.50, p<0.01

Graph B: Creatinine CL (mL/min) versus MPA $AUC_{0-12}$ (mg*h/L/ g MMF); r=0.52, p<0.01
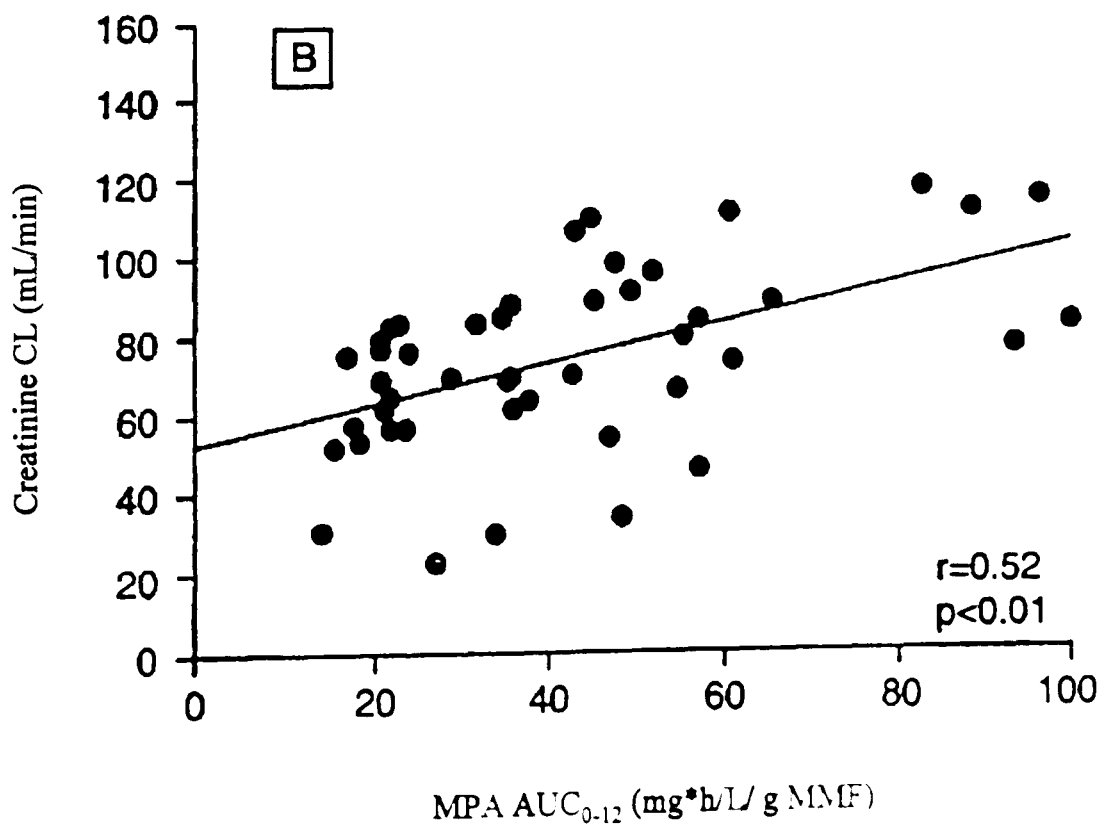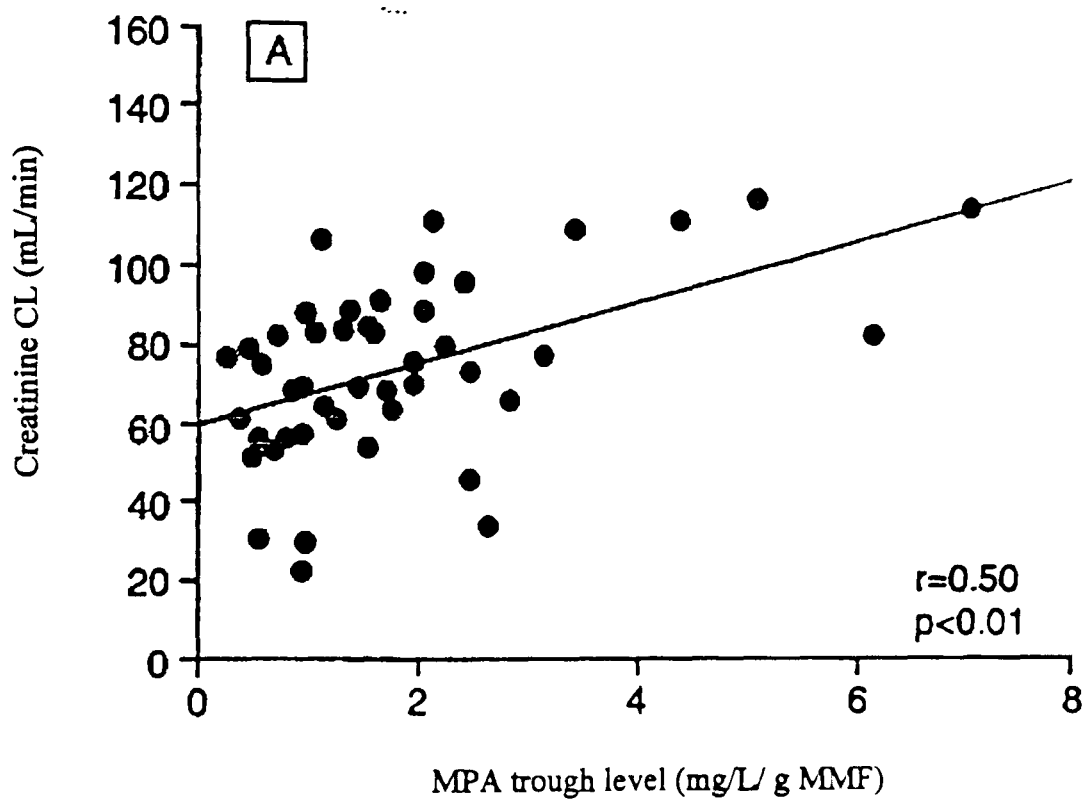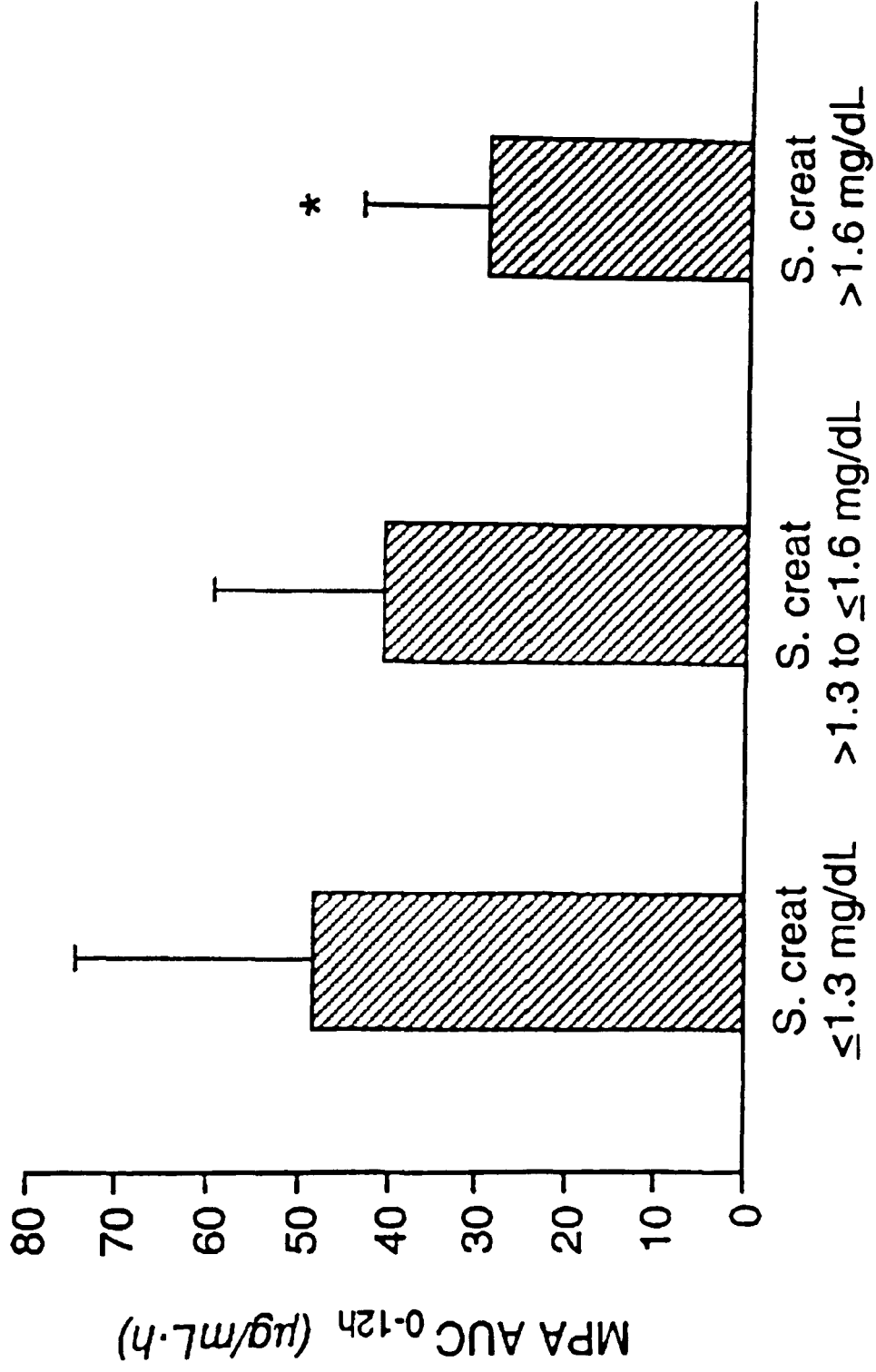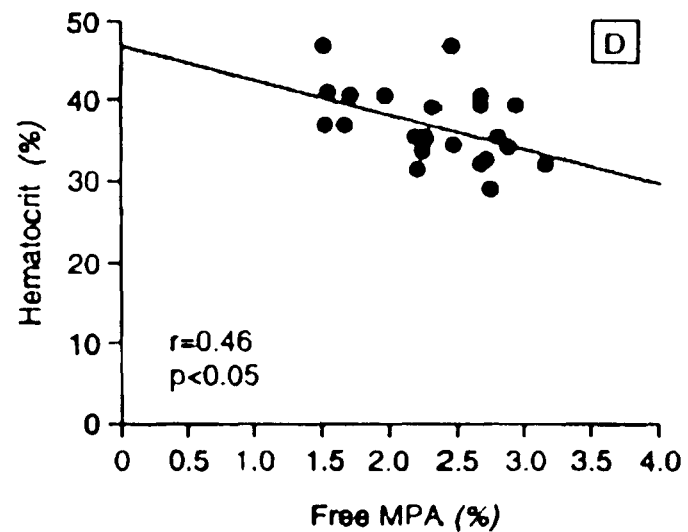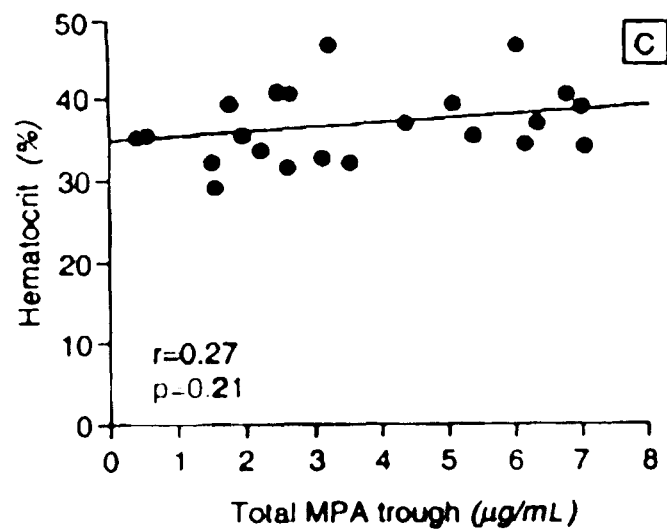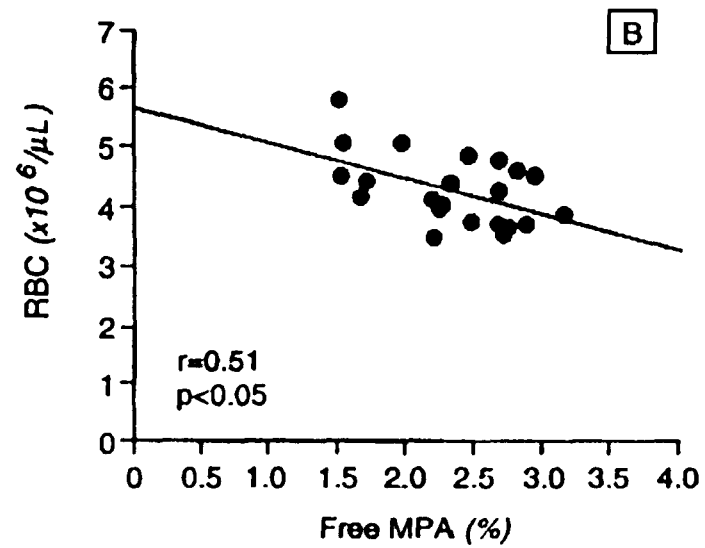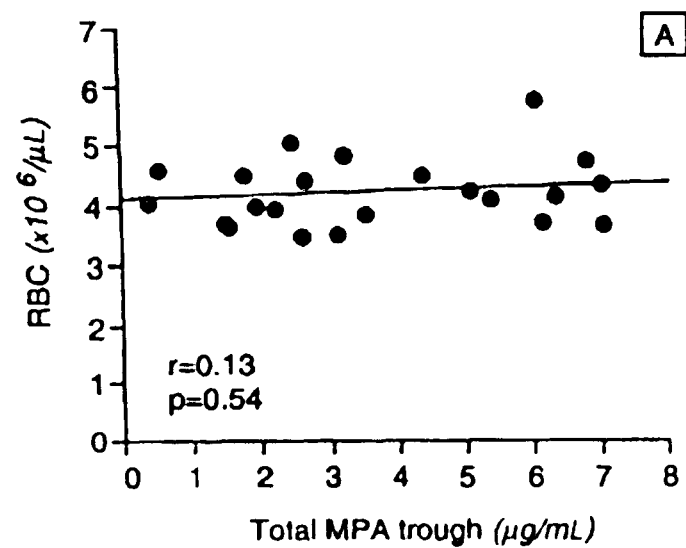
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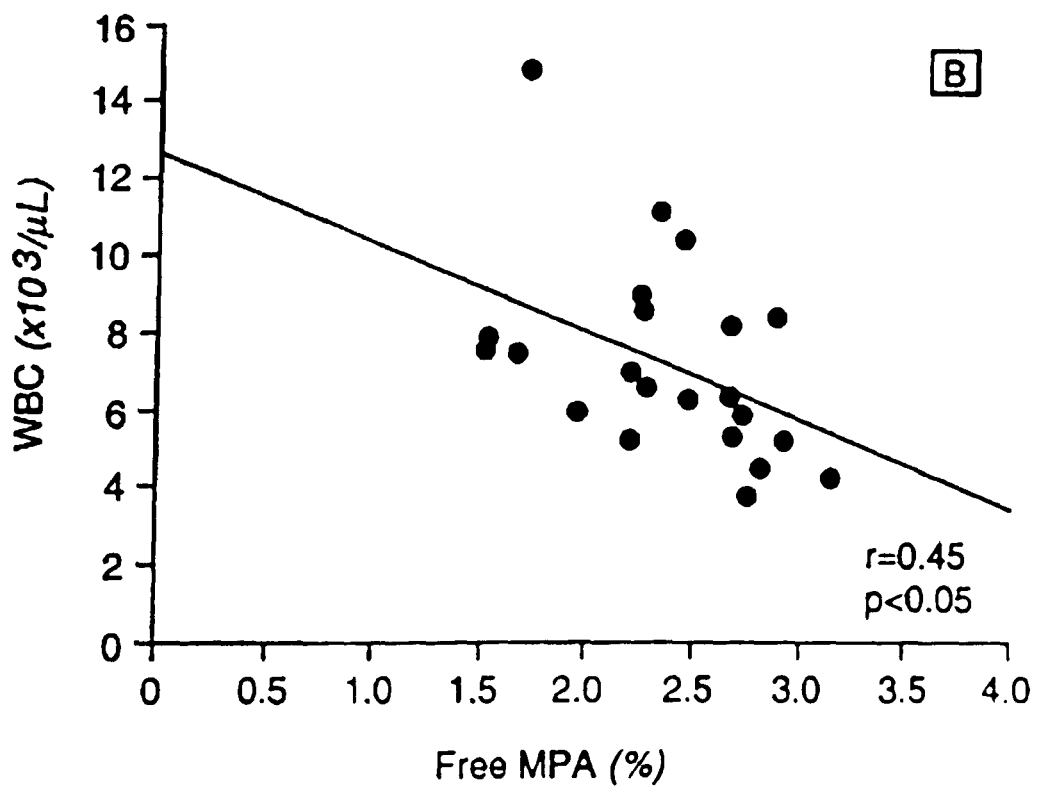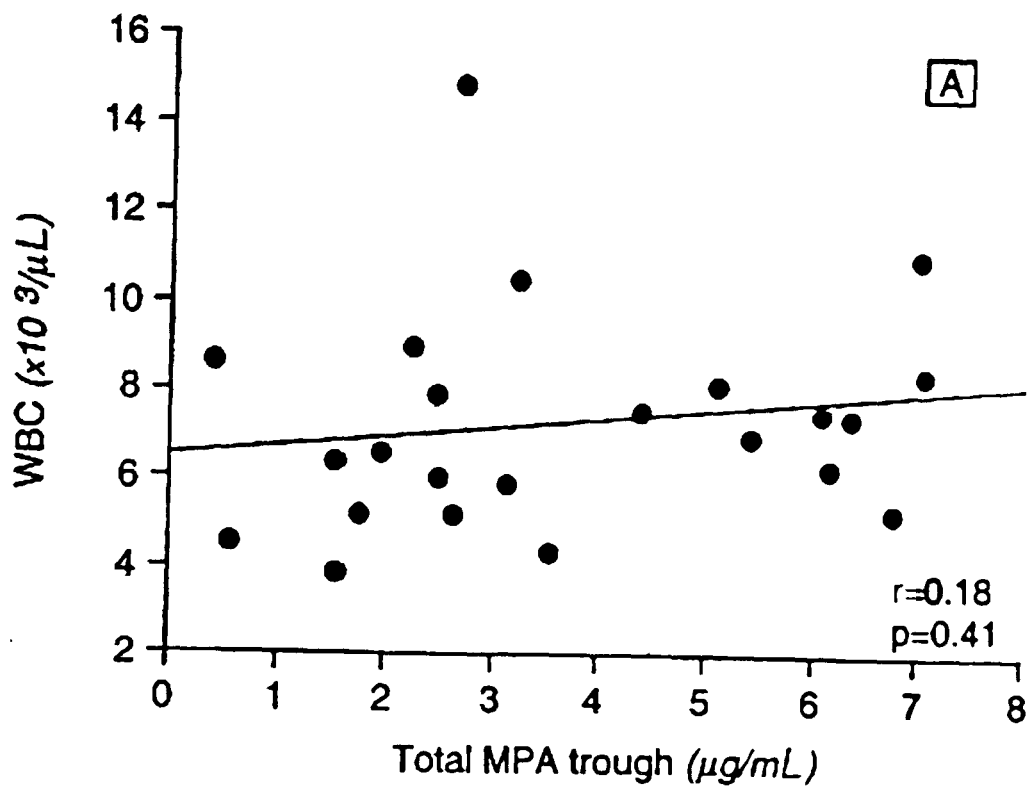
- Figure 12 -

Table 8. Patient demographics and donor characteristics

|  | Range |
| --- | --- |
| No. of patients | 46 (29 M/17 F) |
| Age (yr) | 19–61 |
| Weight (kg) | 44–97 |
| Time after Tx (months) | 6–9 |
| Cadaver/living donor | 46/0 |
| Donor age (yr) | 15–58 |

M, male; F, female; Tx, transplantation.

Table 9. Baseline graft function and MPA pharmacokinetic parameters

|  | Mean | Range |
|---|---|---|
| S.creatinine(mg/dL) | 1.4 | 0.8–2.5 |
| Creatinine clearance (mL/min) | 74.2 | 22.6–117.6 |
| S. urea (mg/dL) | 58 | 37–115 |
| C trough MPA (µg/mL) | 1.82 | 0.24–7.04 |
| MPA $AUC_{0-12h}$ (µg/mL · h) | 42.0 | 10.1–99.8 |
| MPAG (µg/mL) | 35.7 | 12.3–93.6 |

Table 10: demographics, graft function, MPA and CsA pharmacokinetic parameters in kidney transplant recipients according to MPA AYC > or < 40 mg*h/L

| | MPA $AUC_{0-12h}$ | | p-values |
|---|---|---|---|
| | >40 µg/mL · h | <40 µg/mL · h | |
| Patients (n) | 21 | 25 | |
| Age (yr) | 31.9 ± 9.0 | 39.0 ± 12.4 | p < 0.05 |
| Sex | 11 M/10 F | 18 M/6 F | – |
| Weight (kg) | 61.7 ± 11.3 | 67.0 ± 12.9 | ns |
| Rejection episodes | 2 | 3 | ns |
| Donor age (yr) | 33.0 ± 15.5 | 38.0 ± 17.0 | ns |
| C trough MPA (µg/mL) | 2.82 ± 1.59 | 0.97 ± 0.46 | p < 0.01 |
| MPA $AUC_{0-12h}$ (µg/mL · h) | 61.6 ± 18.8 | 25.6 ± 7.4 | p < 0.01 |
| S. creatinine (mg/dL) | 1.3 ± 0.3 | 1.5 ± 0.4 | p < 0.05 |
| Creatinine clearance (mL/min) | 85.7 ± 23.2 | 64.5 ± 17.5 | p < 0.01 |
| CsA dose (mg/d) | 141 ± 24 | 140 ± 34 | ns |
| C trough CsA (ng/mL) | 190 ± 51 | 197 ± 59 | ns |
| CsA $AUC_{0-12h}$ (ng/mL · h) | 5201 ± 1011 | 5340 ± 1277 | ns |

*Chapter 7*

# GLUCORTICOIDS INTERFERE WITH MYCOPHENOLATE

# MOFETIL BIOAVAILABILITY IN KIDNEY TRANSPLANTATION

# Introduction

Three large double-blind randomized trials [49,50,51] in kidney transplant recipients have shown that the addition of the novel antirejection drug MMF to an immunosuppressive regimen consisting of CsA and prednisone significantly reduced the rate of acute rejection during the first 12 months after transplantation as compared to azathioprine- or placebo-treated patients. Since then, MMF has been widely used in a fixed daily dose of 2 g, in two divided administrations, as adjunctive therapy in combination with a calcineurin inhibitor, CsA or tacrolimus, and steroids. Recent evidence, however, suggests that a fixed dose regimen of MMF might no longer be the best approach for the management of transplant patients, and drug pharmacokinetic monitoring is advised [19,37,60,66].

Following oral administration MMF is rapidly absorbed and hydrolyzed to the active compound MPA, by non-specific intestinal esterases [56]. MPA is then converted to inactive metabolites by glucuronidation mediated by the human uridine diphosphate glucuronosyltransferase (UDP-GT) enzyme family [56]. The main metabolite, 7-hydroxy-glucuronide (MPAG), is excreted in urine but may contribute to the enterohepatic circulation of MPA after excretion into the bile and hydrolysis in the gastrointestinal tract [75]. Recent reports also demonstrate that at least two acyl glucuronides of MPA can be detected in the blood and formed in vitro [76].

Modulation of MPA metabolism by concomitant administration of drugs other than MMF may, therefore, result in the modification of MPA clearance and eventually bioavailability. Previous studies in transplant recipients have indeed shown that CsA influences MPA pharmacokinetics to the extent that a significant difference was found in the MPA trough level between CsA versus non-CsA treated patients, despite they ⋅ ⋅ ⋅ ted with an identical MMF dose [71]. Changes in MPA exposure may be

ally and clinically relevant, as indicated by increasing evidence of a causal
_____ship between MPA pharmacokinetics and acute rejection [19,37,60,66] or
incidence of MPA-related side effects [73,77].

Glucocorticoids have been reported to induce glucuronosyltransferase expression,
enhancing the activity of UDP-GT in rat hepatocyte cells in culture and in vivo in
rodent animal models [78]. Both UDP-GT 1A and 2B isoforms are up-regulated by
dexamethasone in a dose- and time-dependent manner [79]. Moreover, a precocious
development of UDP-GT activity occurred in fetal rats after glucocorticoid
administration to the mothers [80]. All together these observations led us to hypothesize
that glucocorticoids, by modulating UDP-GT activity could interfere with MPA
metabolism in transplant patients given steroids and MMF as a part of their
immunosuppressive therapy. The theoretical impact of steroids on MPA
pharmacokinetics, however, has not been investigated so far.

In the current study - which is a part of a multicenter clinical trial aimed at investigating
the steroid-sparing potential of a MMF-based regimen in kidney transplants – we
sought: 1) to compare the MPA pharmacokinetic profiles in the early post-transplant
period (within the first month), when patients were given relatively high dose steroids,
with those in the later phase (6 months post-transplant) under a lower maintenance
corticosteroid regimen, while receiving a fixed daily dose of MMF; 2) to examine the
effect of steroid withdrawal on MPA bioavailability in the same kidney transplant
patients by comparing MPA pharmacokinetics at 6 month post-surgery (while on MMF,
steroids and CsA), at the end of the steroid tapering phase (9 months post-transplant)
and of follow-up (21 months post-transplant).

# Methods

## *Patients*

Twenty-six consecutive adult renal transplant patients (15 males, 11 females) – enrolled as a part of a clinical trial aimed at investigating the steroid sparing potential of a MMF-based immunosuppressive regimen [38] – were included in the present study. All were recipients of first kidney transplant from cadaver donors, and aged from 19 to 61 years. They were on triple-drug immunosuppressive regimen consisting of CsA Neoral, methylprednisolone (MP) and MMF during the first 6 months. In this period Neoral dosing was established on the basis of blood CsA trough levels targeted to 250-440 ng/mL, 200-300 ng/mL and 150-250 ng/mL from day 0 to 7, day 8 to 30, and month 2 to 6 post-transplant, respectively. After intraoperative infusion of 500 mg methylprednisolone (MP), steroid dosing was then progressively tapered to 16 mg/day up to day 12 post transplant, with maintenance 8 mg/day dose achieved by 4 months post surgery and maintained for 2 months (month 6). MP was then progressively tapered over 90 days, and then withdrawn. Patients in the control group were on triple immunosuppressive therapy with CsA, MP (8 mg/day) and MMF. All patients started with the conventional 2 g/day MMF dose, in two divided administrations. Ten of them, however, required MMF dose reduction within the first 6 months post-transplant due to adverse events. Changes in MMF dosing were performed by the attending physicians based on clinical parameters suggesting drug-induced toxicity, but not on MPA pharmacokinetics. Thereafter, MMF daily dose remained fixed, ranging from 500 mg to 2 g/day according to patient needs.

At 6 months post-transplant, patients were allowed to enter the steroid-sparing phase if the following inclusion criteria were met: serum creatinine $\leq$ 2.0 mg/dL; stable renal

function in the last 3 months; proteinuria < 1 g/day; no more than 2 acute rejection episodes in the first 6 months; no previous steroid-resistant acute rejection episodes. The study was described in detail to all patients before admission and informed consent obtained in each instance.

*Study design*

In this prospective study we first examined the effect of steroids on MPA pharmacokinetic profiles by comparing kinetic parameters early post-transplant (within the first month), when patients were given relatively high dose of steroids, with those at 6 months post-surgery under a lower maintenance steroid dosage, but still on triple immunosuppressive drug regimen (Figure 13). In the same patients we then assessed the impact of steroid withdrawal on MPA bioavailability evaluating MPA pharmacokinetic parameters and MPAG trough concentration at different time points, namely at month 9 (end of steroid tapering phase) and month 21 (end of follow-up) post-transplant (Figure 13). As control for this second phase of the study, an additional group of 12 kidney transplant recipients were also monitored at month 21 post-transplant, when they were still on triple-drug therapy with CsA Neoral, steroids, and MMF.

Moreover, in a subgroup of 12 study patients the percentage of free MPA at time 0, in addition to total MPA trough level, was determined within the first month and at 6, 9 and 21 months post-transplant.

On the morning of the pharmacokinetic studies blood samples were collected for routine biochemical analysis and for determination of trough level of plasma MPA, MPAG and blood CsA. Each patient was then given the morning dose of MMF and CsA and underwent evaluation of simplified pharmacokinetic profiles. For MPA pharmacokinetics, blood samples in EDTA-tubes were drawn at 20, 40, 75 and 120 minutes [37]. Thereafter, samples were centrifuged at 3000 g, plasma separated, and

stored at –20°C until analysis by HPLC. For CsA pharmacokinetics, blood samples were collected in heparinized tubes at 60 and 180 minutes after dosing [61] and stored at –20°C until analysis.

*MPA, MPAG and CsA measurement*

MPA (total and free) and MPAG plasma concentrations as well as CsA whole blood levels were measured by reverse phase HPLC as described in *Chapter 3*.

*Pharmacokinetic parameters*

Trough levels of MPA and MPAG were determined. The MPA $AUC_{0-12}$ was predicted using the equation of Hale et al [37]. AUC from 0 to 2 h ($AUC_{0-2}$) was calculated using the trapezoidal rule. For the 2- to 12 h interval, the concentrations ($C_t$) at 6, 8 and 12 h were estimated by the empiric equation:

$$C_t = 0.14 + 1.25 \times C_0$$

In which $C_0$ is the predose MPA level. Main MPA pharmacokinetic parameters were adjusted for the daily MMF dose and expressed as per g MMF. Free MPA was expressed as percentage rate of unbound / total MPA.

Trough level of blood CsA was determined and the CsA $AUC_{0-12}$ was calculated using a three-point strategy (sampling at 0, 1 and 3 h post CsA dosing) with the following equation:

$$AUC_{0-12} = 5.189 \times C_0 + 1.267 \times C_1 + 4.150 \times C_3 + 135.079 \ [62]$$

*Statistical Analysis*

Results are reported as means $\pm$ SD. Data were analyzed with paired and unpaired t test. Correlation between CsA $AUC_{0-12}$ and plasma MPA clearance was performed by regression analysis. The statistical significance level was defined as $p<0.05$.

# Results

*MPA and MPAG pharmacokinetics in the early post-transplant period*

Mean MMF dose was numerically but not significantly higher within the first month than at 6 months post-transplant ($1846\pm375$ vs. $1596\pm530$ mg/day). Dose-normalized MPA trough levels in the first month post-transplant tended to be lower than 6 month post-Tx values, but the difference did not reach statistical significance ($1.37\pm0.62$ versus $2.00\pm1.26$ mg/L). Similarly, a significantly lower dose-normalized maximum plasma MPA concentration in the first month as compared to 6 months post-transplant was found ($7.34\pm5.47$ vs. $17.16\pm10.01$ mg/L; $p<0.05$). As shown in Figure 14 (panel A), dose-normalized MPA $AUC_{0-12}$ was lower at 1 than 6 months post-surgery, with values achieving statistically significant difference ($32.94\pm10.98$ versus $50.87\pm22.37$ mg*h/L/g MMF; $p<0.01$). Consistent with the change in AUC, mean apparent plasma clearance of MPA was significantly higher in the first month than at 6 months post-transplant ($p<0.01$, Figure 14, panel B). When absolute, non-normalized values of MPA trough, maximum plasma concentration and AUC were considered, similar results were found.

By contrast, MPAG trough levels were significantly higher in the first month as compared to month 6 post-transplant values ($50.72\pm34.06$ versus $33.28\pm14.22$ mg/L; $p<0.05$).

In the subgroup of patients, in whom the percentage of free MPA was also measured, higher values in the first month as compared to 6 months post-surgery were found ($5.98\pm1.63$ versus $2.23\pm0.59\%$, $p<0.01$).

*MPA and MPAG pharmacokinetics after steroid withdrawal*

Table 11 summarizes the variation in MPA pharmacokinetic parameters at the end of tapering and after complete steroid discontinuation.

Dose-normalized MPA trough level progressively increased from month 6 (triple therapy) to month 9 (end tapering) and month 21 (dual therapy) post-transplant. The latter MPA trough level value was significantly higher (p<0.05) than that measured at the same time point post-surgery in control patients who were still on triple therapy with CsA, steroids and MMF. Dose-normalized peak MPA concentration also tended to increase with time as compared to month 6 values. Therefore, at the end of follow-up (month 21), peak MPA levels were significantly higher (p<0.05) than at month 6 and 9 post-transplant. In patients who discontinued steroids, higher (p<0.05) peak MPA levels than in controls on triple drug therapy at month 21 post-surgery were also found. Similarly, normalized MPA $AUC_{0-12}$ progressively increased, reaching statistical significance (p<0.05) at month 21 as compared to month 6 post-transplant. The difference in MPA $AUC_{0-12}$ values were even more significant (p<0.01) when patients who discontinued steroids and controls were compared. Apparent plasma clearance of MPA showed progressive and significant decline (p<0.01) up to month 21 post-transplant in patients with steroid withdrawal (Figure 15). A statistically significant difference in plasma MPA clearance between steroid withdrawal and control groups was also documented (p<0.01). Consistently, MPAG trough levels progressively declined in patients who discontinued steroids reaching statistical significance at month 21 as compared to month 6 post-transplant (24.30±11.43 versus 33.28±14.22 mg/L; p<0.05; Figure 16). At this time point MPAG trough levels were also significantly lower than in control group (36.43±17.65 mg/L; p<0.05).

Despite changes in these pharmacokinetic parameters associated with tapering and discontinuation of steroids, percentage of free MPA measured in the subgroup of

patients was comparable at the three observation time points (month 6: $2.23 \pm 0.59\%$; month 9: $2.40 \pm 0.53$; month 21: $2.51 \pm 0.34\%$).

*Impact of CsA on plasma MPA pharmacokinetics*

Since lowering CsA exposure per se may be a factor that affects MPA pharmacokinetics [39,71], we also measured CsA pharmacokinetic parameters in the same patients undergoing steroids tapering and withdrawal, and in controls still on triple-drug regimen up to month 21 post-transplant, and related them to changes in plasma MPA clearance. As reported in Table 12, there was a numerical reduction in CsA dose in patients who discontinued steroids from month 6 to 21 post-surgery. This was paralleled by a tendency to decline of CsA trough levels and $AUC_{0-12}$ values. Changes in CsA pharmacokinetic parameters were associated with decrease in plasma MPA clearance (Table 12). In control patients at month 21 post-transplant both blood CsA trough and CsA $AUC_{0-12}$ values were even numerically lower than those in patients with steroid withdrawal at the same time-points (Table 12). Nevertheless, control patients on triple-drug therapy had significantly higher plasma MPA clearance than patients on dual-drug regimen at month 21 post-transplant (Table 12). By regression analysis, no significant correlation was found between $AUC_{0-12}$ and plasma MPA clearance values when data of the two groups of patients were considered ($r=0.012$, $P=0.914$, Figure 17).

*Biochemical and haematological parameters*

Table 13 shows renal function, as serum creatinine and serum urea concentration, as well as serum albumin level – factors that could potentially affect MPA binding and pharmacokinetics [44,69] – in the study patients and controls. Serum concentrations of creatinine and urea did not change to a significant extent during the follow-up in patients who discontinued steroids, and mean values were comparable to those of

controls at month 21 post-transplant. Similarly, serum albumin concentration remains stable and comparable in the two groups of patients.

There was a progressive reduction of red and white blood cell count in parallel with tapering and discontinuation of steroids, which was associated with the increased MPA exposure (Table 13). A significant correlation between MPA $AUC_{0-12}$ and red blood cell count during the study period was also found (r=0.33; p<0.01).

# Discussion

Potential pharmacokinetic interactions between MPA and other immunosuppressive agents may induce significant changes in MPA exposure with relevant clinical consequences in term of efficacy and side effects.

Here we found that in cadaver kidney transplant recipients on immunosuppressive therapy with CsA Neoral, steroids and fixed dose of MMF, plasma clearance of MPA was higher early post-surgery than at 6 months when patients were still on triple-drug regimen. This translated into a lower MPA daily exposure within the first month post-transplant.

The reasons for lower MPA exposure early post-transplant could be multifactorial. Poor gastrointestinal absorption of MMF in the perioperative phase may account, at least partially, for these MPA pharmacokinetic differences. This possibility is supported by the fact that the maximum MPA concentration achieved in the plasma, which reflects the degree of drug absorption, was also lower in the first month post-transplant than at 6 month.

In addition, a potential effect of CsA on MPAG enterohepatic circulation pathway can not be excluded [39,71]. Different MPA levels could also reflect differences in the rate of drug metabolism through modulation of the glucuronosyltransferase enzyme in the liver. In this respect, in vitro evidence is available that glucocorticoids, which are usually given at high daily doses in the early postoperative period as a part of the immunosuppressive therapy, enhance the activity of UDP-glucuronosyltransferase in adult rat hepatocytes in culture and in human liver specimens [78,79,80]. These findings raise the possibility that in our patients high dose steroids early post-transplant activate pathways of MPA metabolism, eventually leading to lower than expected exposure to MPA. Induction of liver UDP-glucuronosyltransferase by glucocorticoids might also

enhance the production of the main MPA metabolite, MPAG [75], which in turn displaces MPA from the albumin binding sites increasing the free plasma fraction of the compound [44]. This was indeed what we have found in the present study in which the MPAG concentration and the percentage of free MPA were almost 2 fold higher early post-transplant than at 6 months when patients were on low maintenance steroid dose. On the other hand, the high percentage of free MPA might compensate the overall lower MPA bioavailability early post-transplant, which was expected to translate in less MMF-induced immunosuppression.

While together these results are quite supportive of the involvement of steroids in the metabolism of MPA, at this stage they are still indirect and not conclusive. We therefore sough to examine in the same kidney graft recipients the impact of steroid withdrawal on MPA pharmacokinetic parameters starting at 6 months post-surgery. Although MMF dose was unchanged, the apparent MPA plasma clearance and the MPAG trough levels progressively declined during the three month steroid tapering, and further reduced after discontinuation of the drug, resulting in higher MPA exposure. By contrast, control patients, still on triple-drug therapy including steroids at the end of follow-up, had significantly higher MPA plasma clearance and MPAG trough values. All together, these findings confirm our initial observation that steroids affect MPA pharmacokinetics.

However, an additional confounding factor in the interpretation of our results derives from published observations that also CsA may interfere with MPA metabolism [39,71]. Lowering CsA exposure, as it occurred in patients discontinuing steroids, is indeed a factor that could have decreased the MPA plasma clearance and then enhanced MPA bioavailability. To explore this possibility we compared CsA and MPA bioavailability in patients who underwent steroid withdrawal and in controls still on standard immunosuppression with CsA, steroid and MMF at 21 months post-transplant.

Although in patients who discontinued steroids CsA relative bioavailability was higher than in the control group, MPA plasma clearance was markedly lower in the former group. This indicates that the contribution of CsA to the observed MPA pharmacokinetics changes in patients discontinuing steroids was marginal, if any. Failure of CsA to affect MPA metabolism was also supported by the lack of any correlation between patient exposure to CsA and MPA plasma apparent clearance values considering the whole patient and control population, although possible deficiencies of MPA clearance calculation can not be excluded. These findings are seemingly at variance with results of MPA monitoring in kidney transplant recipients showing highly significant difference in MPA concentration between patients treated with or without a CsA-based regimen [39,71,81]. It should be considered, however, that in the latter studies the influence of CsA on MPA pharmacokinetics was documented through comparison with a completely CsA-free regimen, whereas our patients were still on CsA-treatment. This is in line with evidence by other investigators that in a comparative study on the effect of conventional CsA or tacrolimus dose, no impact of CsA on MPA trough level was reported [82,83]. Thus we can speculate that CsA may interfere with MPA pharmacokinetics only when significant variations in patient's exposure to CsA may occur, or when we compare CsA-based with tacrolimus-based regimens, under comparable treatment with steroids [84].

It is known that MPA concentrations and metabolism are influenced not only by co-medication but also by protein binding – MPA is avidly bound to human serum albumin [44] – renal dysfunction, and liver disease [69] which could have also contributed to the changes in MPA pharmacokinetics we found during follow-up. However, the patients were in good health, with normal serum albumin level and no clinical and laboratory evidence of renal or liver disease during the entire study period.

Changes in MPA exposure may be biologically and clinically relevant. Indeed, according to previous observation [74], RBC count significantly correlated with the MPA $AUC_{0-12}$ as documented by a progressive reduction in RBC values associated with increase in MPA exposure. More important, however, is the fact that normal renal function and no acute rejection episodes were reported in patients who discontinued steroids but without any changes in the remaining immunosuppressive regimen. This would imply that the enhanced MPA exposure compensates, at least in part, the apparently lower immunosuppressive level achieved with the dual therapy of CsA and MMF.

Our present findings may be of clinical value also in the light of recent data that MMF reduced the risk for development of chronic allograft failure and late renal allograft loss independently of acute rejection [85].

In conclusion, this is the first demonstration that corticosteroids interfere with MPA bioavailability in solid organ transplant patients. Discontinuation of steroid dosing reduces the apparent plasma MPA clearance and thus enhances the total bioavailability of the compound. Higher MPA exposure may help to overwhelm the lower overall immunosuppression provided by the remaining unchanged dual drug regimen, while allowing patients to safely avoid the side effects of long-term steroid administration. Whether MPA pharmacokinetic changes induced by steroid withdrawal translate into lower risk of acute graft rejection than that reported in patients on conventional azathioprine-based therapy undergoing per protocol corticosteroid discontinuation to avoid the long-term sequelae of the treatment [86], is under investigation in a prospectively designed multicenter clinical trial.

**Legend to Figures**

*Figure 13.* Study design is reported of MPA pharmacokinetic evaluation during the early period post-transplant (high dose steroids), at month 6 (low maintenance steroid dosage), at month 9 (end of tapering steroids), and at month 21 post-Tx (1 year after steroid withdrawal) in 26 kidney allograft recipients. A control group of 12 kidney transplant patients on triple immunosuppressive therapy at month 21 post-Tx was also evaluated.

*Figure 14.* AUC (panel A) and plasma clearance (panel B) of MPA within the first month after transplantation (while patients were on high dose steroids) and at month 6 post-transplant (under a lower maintenance steroid regimen) in 26 kidney graft recipients on triple immunosuppression with MMF, CsA and steroids. *$p<0.01$ versus first month post-surgery.

*Figure 15.* Changes of plasma MPA clearance during tapering and after steroid withdrawal starting at 6 month post-transplant. Month 6 post-Tx: patients on triple-drug regimen with CsA, steroids, and MMF; month 9 post-Tx: end of steroid tapering; and month 21 post-Tx: one year after steroid withdrawal. #Control patients on triple immunosuppressive therapy still at month 21 post-surgery were also included. *$p<0.01$ versus month 6 and controls; °$p<0.05$ versus month 9.

*Figure 16.* Change of plasma MPAG trough level during tapering and after steroid withdrawal starting at 6 months post-transplant. #Control patients still on triple immunosuppressive therapy at month 21 post-surgery. *$p<0.05$ versus triple therapy at month 6th and versus controls at month 21st post-transplant.

*Figure 17.* Regression analysis of the relationship between CsA daily exposure (AUC0-12h) and plasma MPA clearance in study and control kidney transplant patients. R=0.012; P=0.914. Closed and open circles indicate patients who discontinued steroids and controls, respectively.

Kidney-Tx
*(n=26)*

**Triple therapy**      **Tapering steroids**      **Steroid withdrawal**

*(MMF, CsA, steroids)*      *(MMF, CsA, steroids)*      *(MMF, CsA)*

1           6           9           21
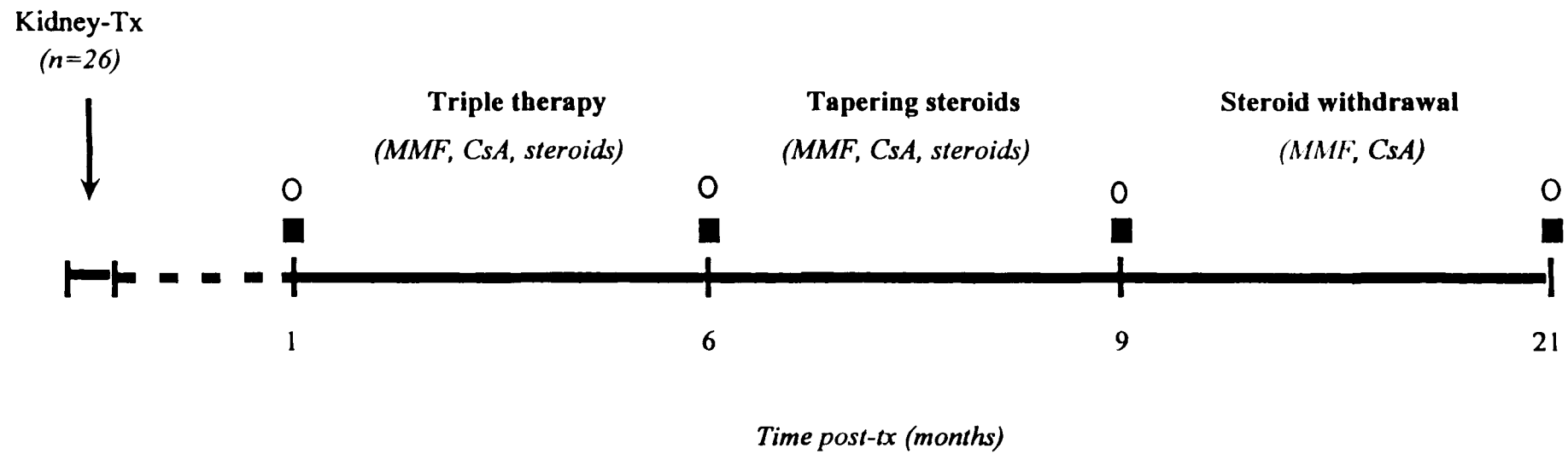
*Time post-tx (months)*

■   MPA, CsA pharmacokinetics

O   Evaluation of the percentage of free MPA in a subgroup of 12 patients

- Figure 13 -
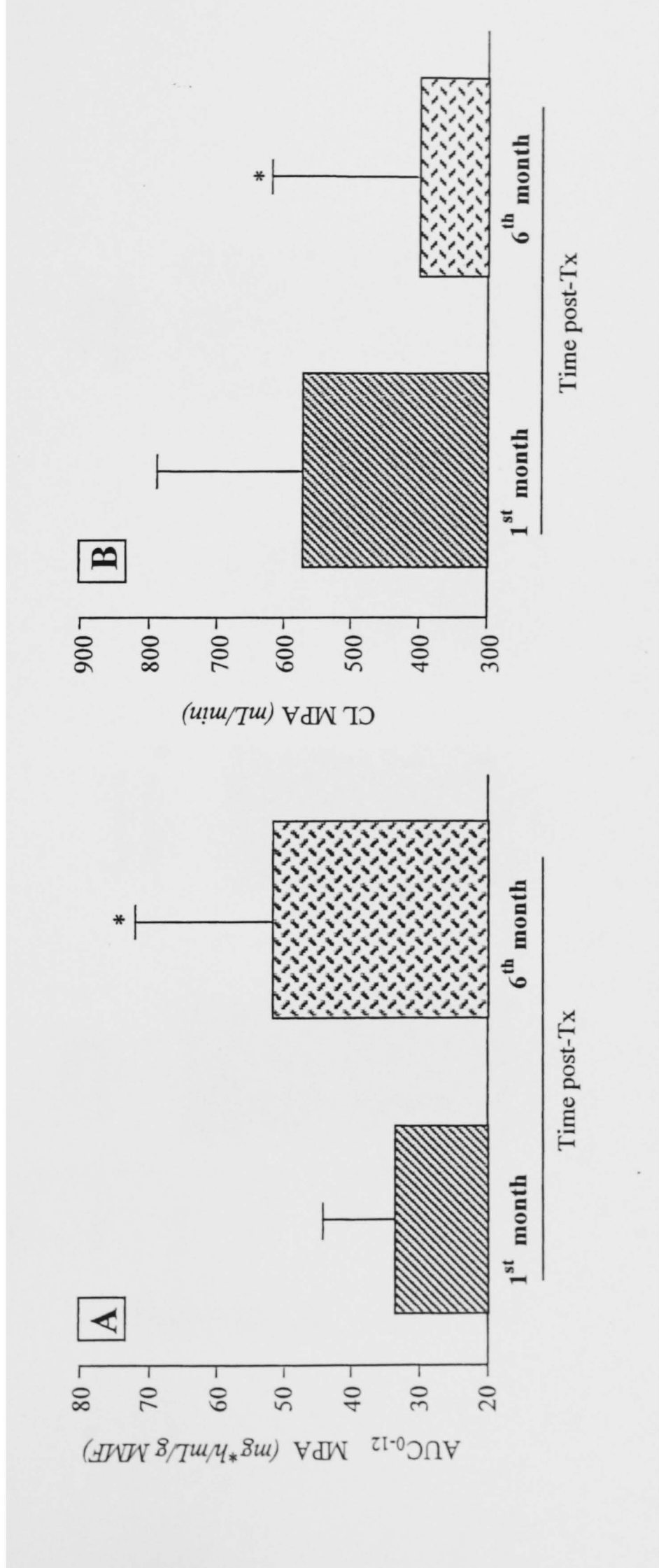
- Figure 14 -

- Figure 15 -

- Figure 16 -

CL MPA (mL/min)

CsA AUC 0-12h (ng*h/mL)

r = 0.012
p = 0.914
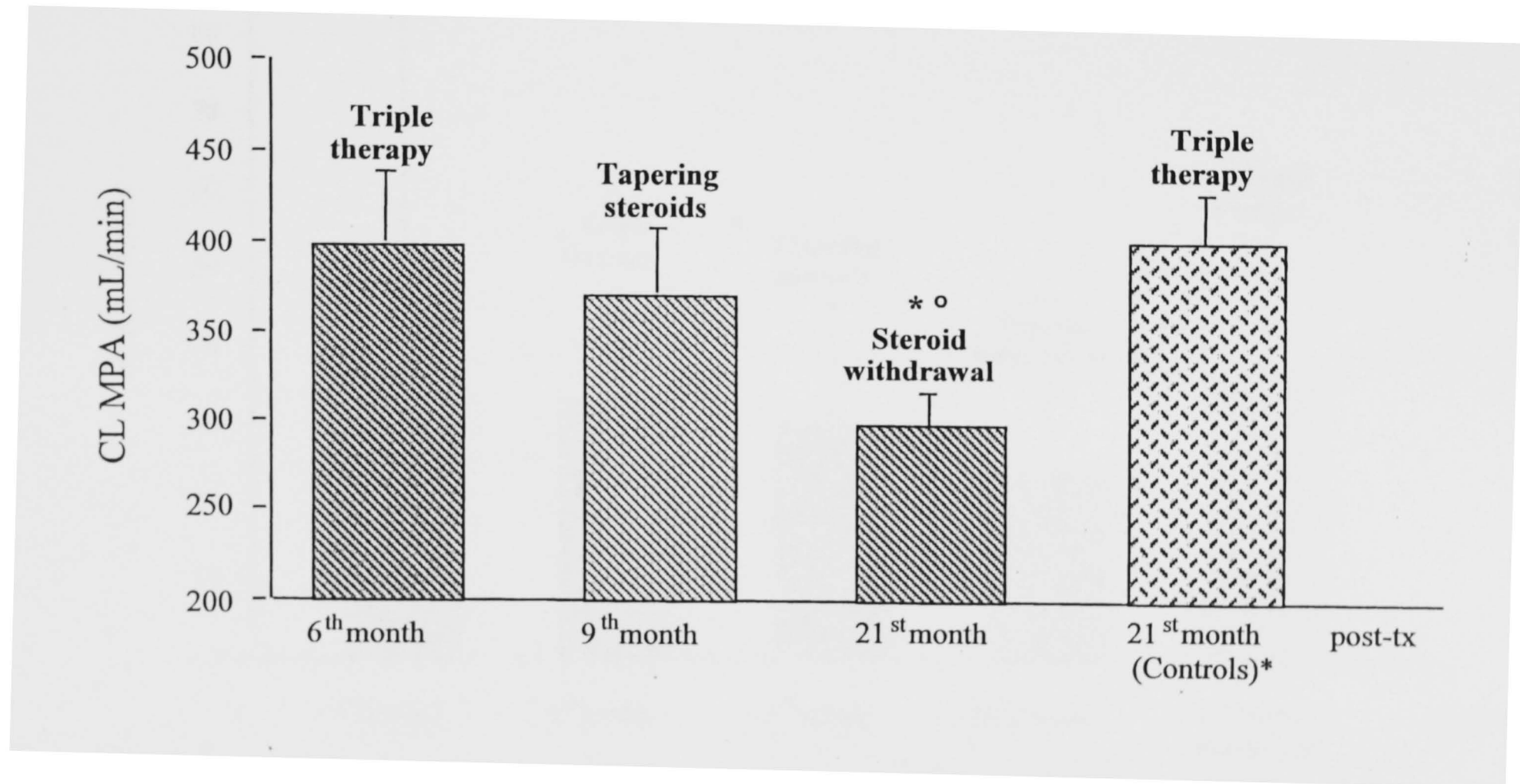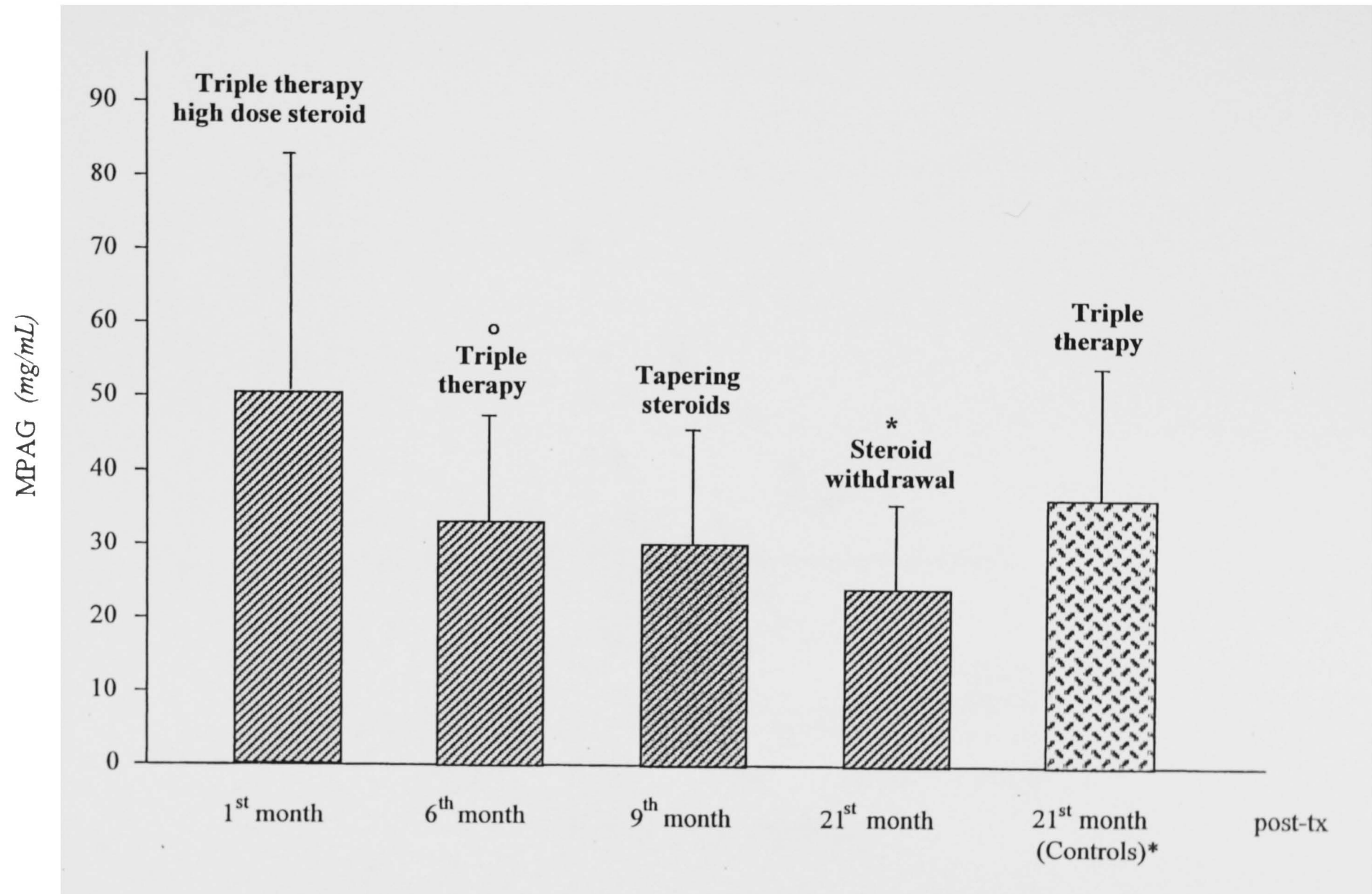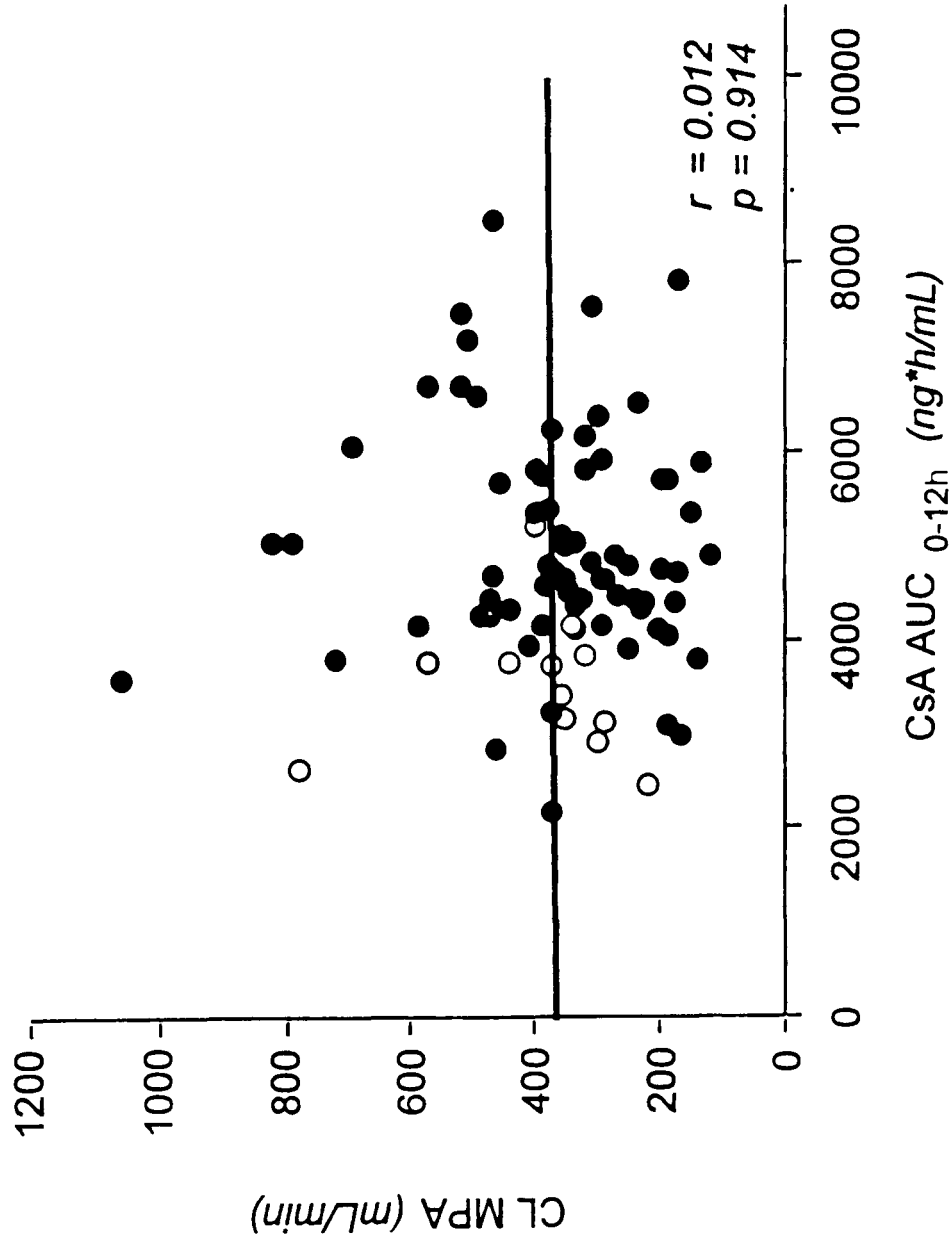
- Figure 17 -

**Table 11:** MMF dose and dose-normalized MPA pharmacokinetic parameters

| Time post-tx | MMF dose (mg/day) | Ct MPA (mg/L/g MMF) | $C_{max}$ MPA (mg/L/g MMF) | $AUC_{0-12}$ MPA (mg*h/L/g MMF) |
|---|---|---|---|---|
| 6th month | 1596 ±530 | 2.00 ±1.26 | 17.16 ±10.91 | 50.87 ±22.37 |
| 9th month | 1500 ±598 | 2.15 ±1.52 | 16.78 ±9.47 | 54.91 ±24.17 |
| 21st month | 1500 ±559 | 3.13 ±2.35 [#] | 20.49 ±8.77 [°] | 66.66 ±30.92 [* §] |
| 21st month (Controls) | 1458 ±582 | 1.64 ±0.77 | 15.23 ±5.06 | 45.51 ±13.66 |

Values are mean± SD. *p<0.05 vs month 6; § p<0.01 vs controls; #p<0.05 vs controls; °p<0.05 vs month 6, 9, and controls and controls.

Table 12: Impact of CsA pharmacokinetics on plasma MPA clearance at month 6, 9 and 21 post-transplant

| | CsA dose (mg/12h) | Ct CsA (ng/mL) | AUC$_{0-12}$ CsA (ng*h/mL) | CL MPA (mL/min) |
|---|---|---|---|---|
| 6$^{th}$month | 145±26 | 191±52 | 5313±1150 | 398.4±205.4 |
| 9$^{th}$ month | 142±31 | 197±63 | 5093±1252 | 368.0±157.5 |
| 21$^{st}$ month | 128±26 | 178±71 | 4202±869* | 298.4±120.9*#§ |
| 21$^{st}$ month (Controls) | 125±18 | 106±35* | 3536±769* | 402.9±147.2 |

Values are mean ± SD. * p<0.01 vs month 6; #p<0.01 vs month 21 (Controls) ; §p<0.05 vs month 9.

Table 13: Biochemical and hematological parameters

| Time post-tx | S.Creatinine (mg/dL) | S. Urea (mg/dL) | S. Albumin (g/dL) | RBC $(x10^6/ \mu L)$ | WBC $(x10^3/ \mu L)$ |
|---|---|---|---|---|---|
| 6 $^{th}$ month | 1.30 ± 0.33 | 53.5 ± 18.9 | 3.9 ± 0.4 | 4.7 ± 0.9 | 9.2 ± 2.5 |
| 9 $^{th}$ month | 1.42 ± 0.41 | 56.6 ± 19.1 | 3.8 ± 0.3 | 4.5 ± 0.8 | 6.8 ± 2.0 |
| 21 $^{st}$ month | 1.34 ± 0.16 | 58.3 ± 14.4 | 4.0 ± 0.3 | 4.3 ± 0.8 * | 6.9 ± 1.1 |
| 21 $^{st}$ month (Controls) | 1.33 ± 0.37 | 65.1 ± 26.3 | 3.7 ± 0.6 | 4.8 ± 0.6 | 8.0 ± 2.2 |

Values are mean + SD. *p<0.05 vs controls

*Chapter 8*

# INFLUENCE OF CO-MEDICATION ON MYCOPHENOLIC ACID

# PHARMACOKINETICS IN ORGAN TRANSPLANTATION

# Introduction

MPA, the active immunosuppressant form of the pro-drugs MMF and the new mycophenolate sodium, is a widely used component of immunosuppressive regimens in organ transplantation [87]. This immunosuppressant is commonly administered in a fixed daily dose. Recent evidence, however, suggests that a fixed dose regimen no longer might be the best approach for the management of transplant patients, and drug pharmacokinetic monitoring is advised [19,37,60,66]. Indeed, a significant predictive value for assessment of the risk for acute rejection [19,37,60], renal function [88] and drug-related side effects [74,77] has been shown for the 12-h dose interval MPA $AUC_{0-12}$ and, although the data are less precise, for the predose trough MPA concentration.

Following oral administration, MPA is converted to inactive metabolites by glucuronidation mediated by the human UDP-GT enzyme family [56,87]. The main metabolite, 7-hydroxy-glucuronide, is excreted in urine but may contribute to the enterohepatic circulation of MPA after excretion into the bile and hydrolysis in the gastrointestinal tract [75]. Recently, three carboxyl-linked additional glucuronides have also been detected in vitro and in vivo [89].

Previous studies have shown that concomitant immunosuppressive therapy significantly influences MPA bioavailability. In particular we have documented that glucocorticoids, by inducing UDP-GT expression, interfere with MMF bioavailability [90]. Other investigators reported significantly increased MPA concentrations in patients treated with MMF plus tacrolimus [81,83]. These findings led to the hypothesis that the major cause for increased MPA levels during co-administration of tacrolimus is inhibition of the glucuronidation of MPA by tacrolimus [83,91]. At variance, Others have shown that combining MMF with the other calcineurin inhibitor CsA reduced MPA exposure [39,71], suggesting that CsA may inhibit the transport of MPA metabolites into bile

from hepatocytes and reduce enterohepatic cycling of MPA, ultimately leading to a decrease in MPA concentrations in the 4 to 12-h window of the AUC profile [39].

SRL is a new immunosuppressant characterized by a unique mechanism of action and a potential ability to synergize with other antirejection drugs [35]. In the clinical practice SRL is usually given in association with low-dose calcineurin inhibitors and steroids or antimetabolites such as MMF and AZA [92]. However, the use of novel induction therapies (i.e. with the humanized anti-CD52 monoclonal antibody Campath-1 [93]), that provide enough immunosuppression, is allowing the development of different immunosuppressive-sparing regimens [93,94,95]. SRL can, therefore, may be safely used in combination with Campath-1H induction and MMF avoiding the administration of calcineurin inhibitors.

Emerging data suggest that MPA pharmacokinetics in patients receiving concomitant SRL therapy might be comparable to the situation observed for tacrolimus. Indeed some Authors have previously found that MPA trough levels were significantly higher in kidney transplant recipients treated with MMF in combination with SRL as compared with those given MMF with CsA [96,97]. However, prospective studies, with strict drug monitoring and full pharmacokinetic evaluations aimed at formally assess the effects of SRL on MPA exposure are still lacking.

The current study - which is a part of a protocol aimed at investigating the efficacy of Campath-1H induction therapy in a steroid-free regimen in kidney transplantation – was designed to test the different, if any, effects of SRL- or CsA-coadministration on MPA exposure. To address this issue we : 1) measured serially MPA trough levels in patients treated with low-dose SRL or low-dose CsA both in addition to low-dose MMF over 12 months post-operatively; and 2) compared 12-h MPA pharmacokinetic profiles in both groups performed at month 6 and 12 after surgery.

## Methods

*Patients*

Twenty-one patients (13 men; 8 women) with end-stage renal disease who underwent primary kidney transplant were enrolled under an Ethics Committee-approved protocol at the Ospedali Riuniti Bergamo, Italy, following written informed consent. They were allocated to one of the following two study groups according to a randomization design: Group 1 (n=11) was assigned to Campath-1H, low-dose SRL and low-dose MMF; group 2 (n=10) entered a regimen with Campath-1H, low-dose CsA and low-dose MMF.

Campath-1 (Alemtuzumab, Schering Plough, Milano, Italy) was given as a single intravenous infusion (30 mg, over 2 hours) intraoperatively on the day of transplant (day 0). Corticosteroids were administered for the first 2 days after transplantation. Thereafter, patients were free of steroids. Patients randomized to SRL received the drug (Wyeth, Rome, Italy) at the oral dose of 4 mg/day in a single morning administration starting on the day 1 after transplant. SRL dosing was adjusted to maintain whole blood levels within the 5-10 ng/mL range. In the CsA-based group, the drug was started just after surgery (1-2 mg/kg/day) and CsA doses were adjusted to achieve trough blood concentration of 120 to 200 ng/mL in the first month post-surgery, and of 70 to 120 ng/mL thereafter. Patients of both groups were given MMF at the oral low dose of 250 to 750 twice a day starting on day 1 postoperatively according to total blood leukocyte count. MMF dosing was adjusted according to plasma trough level targeted to MPA concentration of 0.5-1.5 mg/L.

The administration of prokinetic drugs, resins, or any agent known to interfere with MPA absorption, distribution, metabolism and/or elimination was not allowed during all the study period.

## Study Design

This prospective study first examined the effects of SRL and CsA on dose-adjusted MPA trough levels measured every 5 days starting from day 5 post-Tx, when patients reached steady-state of drug distribution, to day 90, and then at month 4, 6 and 12 post-surgery.

Moreover, at month 6 and 12 post-transplant, all patients underwent a 12-hour MPA pharmacokinetic profile. On the morning of the pharmacokinetic studies blood samples were collected for routine biochemical analysis and for the determination of trough levels of plasma MPA and blood SRL or CsA. Each patient was then given the morning dose of MMF and SRL or CsA. All the drugs were given orally (MMF and CsA b.i.d., SRL once a day). The pharmacokinetics of MPA was based on an analysis of EDTA-tubes collected from the antecubital vein at 20, 40, 75, 120 minutes and 3, 4, 5, 6, 7, 8, 10 and 12 h after drug administration. Thereafter, samples were centrifuged at 3000 g, plasma separated, and stored at -20°C until analysis. For SRL and CsA pharmacokinetics, blood samples were collected in heparinized tubes at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 hours after dosing and stored at -20°C until analysis. All drug measurements were performed by high-performance liquid-chromatography (HPLC) as previously described in *Chapters 3-5*. MPA, SRL and CsA concentration-time profile was recorded for all patients, together with the time to reach the maximum concentration ($T_{max}$) and the maximum drug concentration ($C_{max}$). The AUC from time equal to 0 to the last sampling point (12 h) was calculated by the trapezoidal rule. Experimental studies have shown that CsA, but not tacrolimus, decreased the enterohepatic cycling of MPA [17]. To test this hypothesis, we also estimated the MPA AUC from 4 h to 12 h after drug administration ($AUC_{4-12}$), an interval time corresponding to the appearance of a secondary MPA peak due to enterohepatic cycle with conversion of MPAG to MPA.

The $AUC_{0-12}$ is recognized as the best predictor of drug exposure. This approach is, however, time consuming and increases the discomfort of the patient as it requires multiple sampling analysis. As an alternative, abbreviated AUC profiles with limited sampling protocols have been proposed [98]. Therefore, as additional analysis, we tested the feasibility of a previously published equation to predict daily MPA exposure using a limited sampling strategy [37]. This approach requires only 5 samples collected in the first 2 hour after MMF administration (at 0, 20, 40, 75 and 120 min).

As stated above, MMF dose for each patient was modified according to white cell blood count. To take into account this confounding factor MPA trough levels measured from day 5 to month 6 post-surgery, as well as MPA pharmacokinetic parameters assessed at month 6 and 12 post-Tx, were adjusted for the daily MMF dose.

## Statistical analysis

Results are reported as means $\pm$ SD. Unpaired t-test was used to compare MPA levels (dose-adjusted trough concentrations throughout the study period and MPA pharmacokinetic parameters measured at month 6 and 12 post-surgery) between group 1 (given MMF with SRL) and group 2 (given MMF and CsA). The statistical significance was defined as $p < 0.05$. The ratio between each MPA value measured in the SRL group and the corresponding value measured in the CsA group was used to estimate the degree of the differences between the two groups as follows:

$$\text{Ratio} = \frac{\text{MPA Pharmacokinetic parameter } (C_0, \text{ AUC}) \text{ measured in the SRL group}}{\text{MPA Pharmacokinetic parameter } (C_0, \text{ AUC}) \text{ measured in the CsA group}}$$

Linear regression analysis between MPA $AUC_{0-12}$ predicted with the equation proposed by Hale et al [37] and measured CsA $AUC_{0-12}$ was performed. Agreement between the predicted and measured MPA AUC was estimated using the Bland and Altman approach [99,100], where the percentage difference is plotted against the mean MPA AUC between the two series. We considered acceptable values within 1 SD of the differences.

# Results

*Recipients' demographics*

All patients enrolled in the study were Caucasians. Mean age of recipients was 49 years with a range of 24 to 71 years. The majority of renal transplants were performed from cadaver donors (90%). Patients randomized to SRL- or CsA-based maintenance immunosuppression were comparable as for the distribution of baseline demographics, including age, sex, and HLA matching among the donors and the recipients (data not shown).

*Immunosuppressive drugs monitoring*

Mean immunosuppressive drug dose, whole blood trough SRL and CsA, and plasma trough MPA concentrations are shown in Table 14. As anticipated, the mean SRL trough levels fell within the planned range of 5 to 10 ng/mL. The mean dose used to achieve these levels was between 3.6 and 4.3 mg/day. Similarly, mean CsA trough levels were maintained within the expected range low target range during the 12 month follow-up (Table 14).

Mean MPA trough levels during this period were within the chosen range of 0.5-1.5 mg/L. However, we observed that, despite quite comparable MMF doses during all the study period, MPA levels were close to the high threshold for patients belonging to the SRL group and to the low threshold for those of CsA group. To better investigate this unexpected finding, we adjusted MPA trough levels for the daily drug given to each patient and then studied the temporal distribution of MPA concentrations in the two groups of patients. As shown in Figure 18, at each time point starting from day 10 post-surgery, dose-adjusted MPA trough levels were significantly higher in patients given

SRL as compared to those treated with CsA. The average ratio of dose adjusted MPA trough levels between SRL and CsA groups was 4.4.

*Pharmacokinetic studies*

As shown in Table 14, there were no differences in SRL or CsA trough levels within all the study period. Similarly, SRL $AUC_{0-24}$ (283 at month 6 vs 334 ng*h/mL at month 12, p=0.40) and CsA $AUC_{0-12}$ (3246 at month 6 vs 3080 ng*h/mL at month 12, p=0.63) were comparable during the two pharmacokinetic evaluations.

Results of the MPA 12 h pharmacokinetic studies, performed at month 6 and 12 post-transplant, are given in Tables 15 and 16. To take into account different MMF doses as potential confounding factor, we adjusted all pharmacokinetic parameters for the daily drug dose administered to each patient.

At month 6, we found that MPA trough levels were significantly higher in the SRL group compared to CsA. The ratio between the two series of MPA trough levels was 4.5. Although less pronounced, the same trend was confirmed at month 12 (2.3 $\pm$ 1.3 vs 0.9 $\pm$ 0.4 mg/L/g MMF, p=0.0151). Despite the observed significant differences of MPA trough levels, looking at the pharmacokinetic profiles (Figures 19-20), we found only slight differences for MPA $T_{max}$ and $C_{max}$ between the two groups of treatment. At variance, dose-adjusted MPA $AUC_{0-12}$ at month 6 was significantly higher in the SRL group as compared with patients given CsA. It should be pointed out, however, that the ratio between the two series of $AUC_{0-12}$ was 1.8, thus greatly inferior to that observed with dose-adjusted MPA trough levels (4.5). Again, at months 12 post-Tx, the differences of MPA $AUC_{0-12}$ were less pronounced, with a ratio between the two groups of 1.4.

*Different effects of SRL and CsA on MPA enterohepatic cycle*

As shown in Figures 19-20, the main differences in the pharmacokinetic profiles between SRL and CsA groups were found in the late phase starting 4 hours after MMF administration, whereas no significant differences were observed in the first part of the kinetic profile.

It is now well established that metabolites of MPA undergo enterohepatic recirculation (EHC) and, after hydrolysis in the gastrointestinal tract, release a secondary peak of MPA, usually 4 to 12 hours after MMF administration [75]. To assess whether SRL and CsA might affect the MPA EHC we measured MPA AUC from 4 h to 12 h after drug dosing ($AUC_{4-12}$). Using this approach we found that dose-adjusted MPA $AUC_{4-12}$ were significantly higher in patients given SRL as compared with those treated with CsA both at month 6 ($19.9 \pm 9.2$ vs $6.5 \pm 3.3$ mg*h/L) and 12 post-surgery ($17.2 \pm 6.5$ vs $6.8 \pm 2.5$, mg*h/L; Figure 21). Of note, the ratio of $AUC_{4-12}$ between SRL and CsA groups were higher (month 6: ratio=3.1; month 12: ratio=2.5) as compared with that estimated for corresponding $AUC_{0-12}$. The lack of secondary MPA peak in patients treated with CsA may therefore explain the significantly low MPA trough levels in these patients. At variance, all patients given SRL had a secondary MPA peak 4 to 12 h after MMF administration, ultimately leading to higher MPA trough levels than those given CsA.

*MPA $AUC_{0-12}$ prediction by limited sampling strategy*

The above mentioned data suggested that trough levels are not the best way to monitor daily MPA exposure. Therefore, as additional analysis, we also extrapolated $AUC_{0-12}$ using a previously published equation [37] routinely used in our Clinical Centre to monitor patient's exposure to MPA [88,90]. Despite a tendency to overestimate MPA exposure (Tables 15-16), the correlation between the measured and predicted AUC was good (r=0.90). Bland & Altman analysis [99,100] showed that 85% of values were in

the accepted range of $\pm$ 1 SD. Moreover, this approach reliably predicted the effect of concomitant immunosuppressive administration on MPA exposure, as confirmed by similar ratios of MPA $AUC_{0-12}$ between the two groups observed with the measured and predicted $AUC_{0-12}$ (month 6, measured $AUC_{0-12}$: ratio =1.8, predicted $AUC_{0-12}$: ratio = 2.1; month 12, measured $AUC_{0-12}$: ratio =1.4, predicted $AUC_{0-12}$: ratio = 1.4). Of note, there ratios were lower than that observed with the trough levels (month 6, ratio=4.5; month 12, ratio=2.6).

# Discussion

Potential pharmacokinetic interactions between MPA and other immunosuppressive agents may induce significant changes in MPA exposure with relevant clinical consequences in terms of efficacy and side effects, especially when a drug is given in a fixed dose regimen.

In this study we found that in kidney transplant recipients on immunosuppressive therapy with MMF, MPA levels were influenced by the concomitant immunosuppressive regimen. Indeed, co-administration of SRL and MMF was associated with higher dose-adjusted MPA trough levels and AUC than those measured under CsA-based regimen.

So far, only two studies have previously investigated the impact of SRL co administration on MPA levels [96,97]. However, both these observations were retrospective, considered only MPA trough levels as surrogate marker of daily drug exposure, and patients were monitored for a short time after transplantation (3 months). As additional weakness, in these studies MPA concentrations were assessed using an immunoassay, a method that cross reacts with MPA metabolites, ultimately overestimating MPA values [32]. At variance with these studies, we have formally compared two cohorts of kidney transplant recipients randomized to receive SRL or CsA in addition with MMF. Patients were monitored, starting immediately after transplantation, over a long follow-up (1 year), using a strict sampling of MPA levels measured by HPLC, universally recognized as the gold standard method to assess MPA concentrations [63]. With this approach we extended previous finding showing that dose-adjusted MPA trough levels were consistently 4- to 5-fold higher in the SRL arm than in CsA group during all the study period. Of note this difference was present also at 1 year post-transplantation.

SRL and CsA are metabolized by the cytochromes P450 3A [35] with phase I reactions, whereas MPA is mainly detoxificated by phase II metabolic reaction, without involvement of the cytochromes P450 3A [75,87]. Indeed, we excluded a potential influence of SRL or CsA on MPA metabolism.

As alternative hypothesis, we speculated that SRL and CsA may exert a different action on MPA absorption, distribution and/or elimination. Therefore, to better investigate the potential mechanisms involved in this unexpected pharmacokinetic interaction, we studied the 12 h MPA kinetic profiles in the two groups of patients, both at month 6 and 12 after transplantation, when patients were in stable clinical conditions and fixed immunosuppressive therapy. Using this approach we confirmed that dose-adjusted MPA trough levels were 3-5 fold higher in the SRL group. Subsequently, we analyzed the daily MPA exposure and found that dose-adjusted MPA $AUC_{0-12}$ was nearly 1.5-2 fold higher in patients given SRL than those treated with CsA. However, moving from time 0 to time 12 h after MMF dosing we observed no significant differences for MPA $C_{max}$ and $T_{max}$ between the two groups. This finding led us to conclude that different effects of SRL or CsA on MPA absorption, if any, were negligible.

The plasma concentration-time profile of MPA after oral MMF administration is usually characterized by a sharp initial peak around 1 hour and the occurrence of a secondary peak usually 6 to 12 hours post dose [75]. This late peak has been attributed to glucuronidated MPA metabolites that undergo enterohepatic recirculation (EHC). EHC is the process by which drug, or a drug metabolite, is excreted by the liver into the bile and is then reabsorbed back into the portal circulation [60]. In the case of conjugated drug metabolites, as MPA metabolites, deconjugation mediated by the colonic bacteria in the gut flora precedes the uptake process. After deglucuronidation of the metabolites, MPA was reabsorbed in systemic circulation as shown by the appearance of the secondary peak [75]. Any drugs, such as cholestyramine [101], able to influence EHC

of MPA metabolites may, therefore, affect daily MPA exposure. Indeed, a previous study in animals have shown that CsA, by inhibiting the transport of MPA metabolites into bile from hepatocytes, decreased the enterohepatic cycling of MPA and thereby significantly reduced MPA concentrations in the 4- to 12-h window of the AUC profile as compared with rats treated with MMF alone or in combination with tacrolimus [39]. For the first time, we have documented a similar trend observed in animals by Van Gelder et al [39] also in humans. Indeed, looking at the pharmacokinetic profile we found that, despite no significant differences in MPA AUC in the first few hours after MMF administration, patients in the CsA group experienced the absence of a second MPA peak as documented by significant low MPA $AUC_{4-12}$ levels compared to patients given SRL. According to this analysis of the data, therefore, the post-absorption, post-distribution phase of the MPA AUC profile during which enterohepatic cycling of MPA is most prominent was suppressed in the CsA-treated patients. We can therefore speculated that interruption of the EHC secondary to inhibition of MPA conjugated metabolites' transport across biliary duct epithelium into bile by CsA explained the significantly higher values for dose-adjusted MPA $C_0$ and AUC in patients receiving concomitant SRL and MMF compared to those given CsA and MMF.

At variance with animal studies [39], we did not measure MPAG levels. We did it in the past [88,90]. However, interest in measuring MPAG levels has declined in the last few years after the understanding of its pharmacological inactivity and the discovery at least other 2-3 acyl glucuronidated MPA metabolites [89]. Although data of MPAG are lacking, we are confident that our results may be strong enough to support the hypothesis that the observed differences in MPA pharmacokinetic profile in patients given SRL or CsA are mainly driven by the different effect on MPA enterohepatic recirculation.

Since EHC usually manifests 6 to 12 hours after MMF dosing, MPA levels 12 h (trough) were mainly influenced by the inhibitory action of CsA, with values 3-5 fold higher in the SRL group. Given the fact that the secondary MPA peak is usually 50-70% lower than the $C_{max}$, its contribution to daily MPA exposure is less prominent, as documented by only a 1.5-2 fold difference in MPA $AUC_{0-12}$ between the two groups. These findings suggested that trough levels may not be the best way to monitor daily MPA exposure, especially when patients are treated with different MMF-based therapies. In our routine clinical practice, we estimate MPA $AUC_{0-12}$ using an equation that requires 5 samples collected within the first 2 hours after MMF dosing [37]. In the present study we have shown a good correlation between MPA $AUC_{0-12}$ measured and predicted using the equation of Hale et al [37]. Of note, the degree of the pharmacokinetic interaction between MPA and SRL or CsA was better predicted with the estimated AUC rather then by MPA $C_0$ levels. This approach may, therefore, represent a useful tool to monitor daily MPA exposure when the full 0-12h pharmacokinetic sampling can not be performed, as usually happened in the clinical conditions.

In conclusion, to our knowledge this is the first clinical demonstration that CsA inhibit MPA enterohepatic recirculation, ultimately resulting in higher MPA daily exposure as compared with other CsA free regimens. As additional finding, the MPA $C_0$-based monitoring may overestimate the degree of this pharmacokinetic interaction. These results should be carefully taken into account when MMF-based regimen are implemented, especially when the drug is administered at a fixed daily dose and patients are switched to different poly-pharmacological therapies.

**Legend to Figures**

*Figure 18.* Mean dose-adjusted MPA trough levels from day 5 to year 1 after transplantation in kidney transplant recipients treated with SRL or CsA. both in addition to MMF. °p<0.01 vs CsA group; *p<0.05 vs CsA group.

*Figure 19.* Dose-adjusted MPA concentration-time curves from 0 to 12 hours after MMF administration in patients at month 6 post-surgery and treated with SRL or CsA. °p<0.01 vs CsA group; *p<0.05 vs CsA group.

*Figure 20.* Dose-adjusted MPA concentration-time curves from 0 to 12 hours after MMF administration in patients at month 12 post-surgery and treated with SRL or CsA. °p<0.01 vs CsA group; *p<0.05 vs CsA group.

*Figure 21.* Area under the curve from 4h to 12 h ($AUC_{4-12}$) after MMF administration in kidney transplant recipients treated with SRL or CsA.
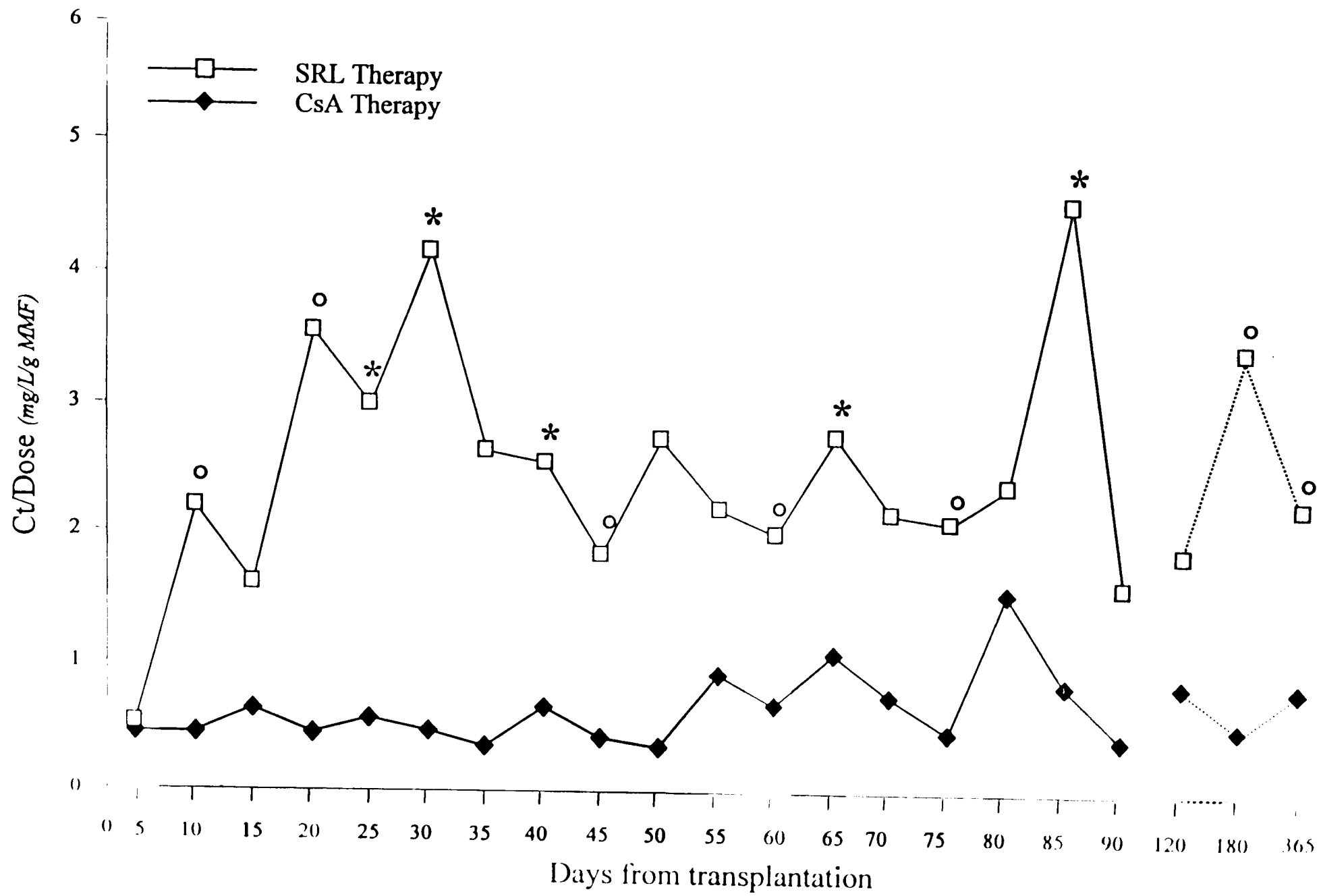
- Figure 18 -
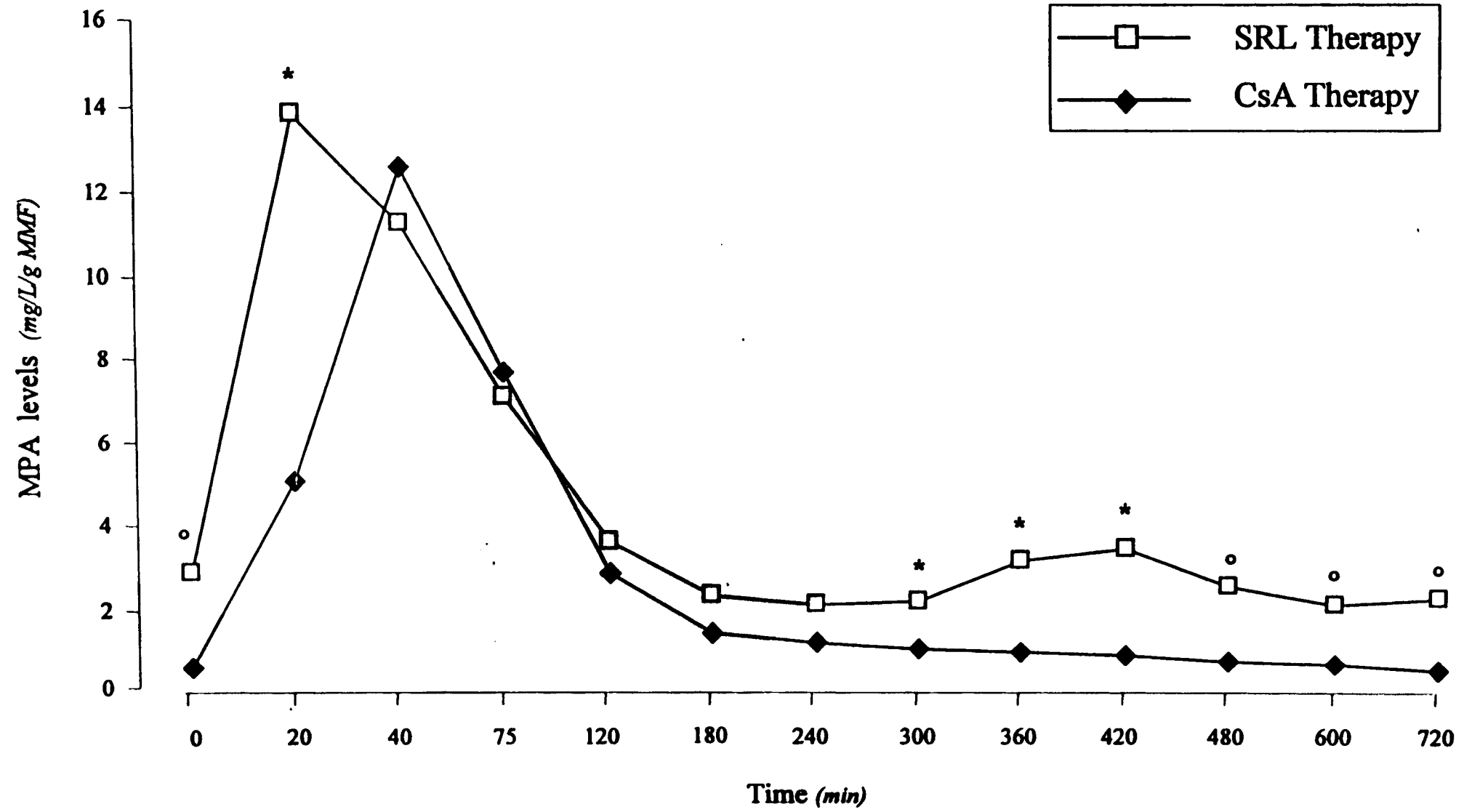
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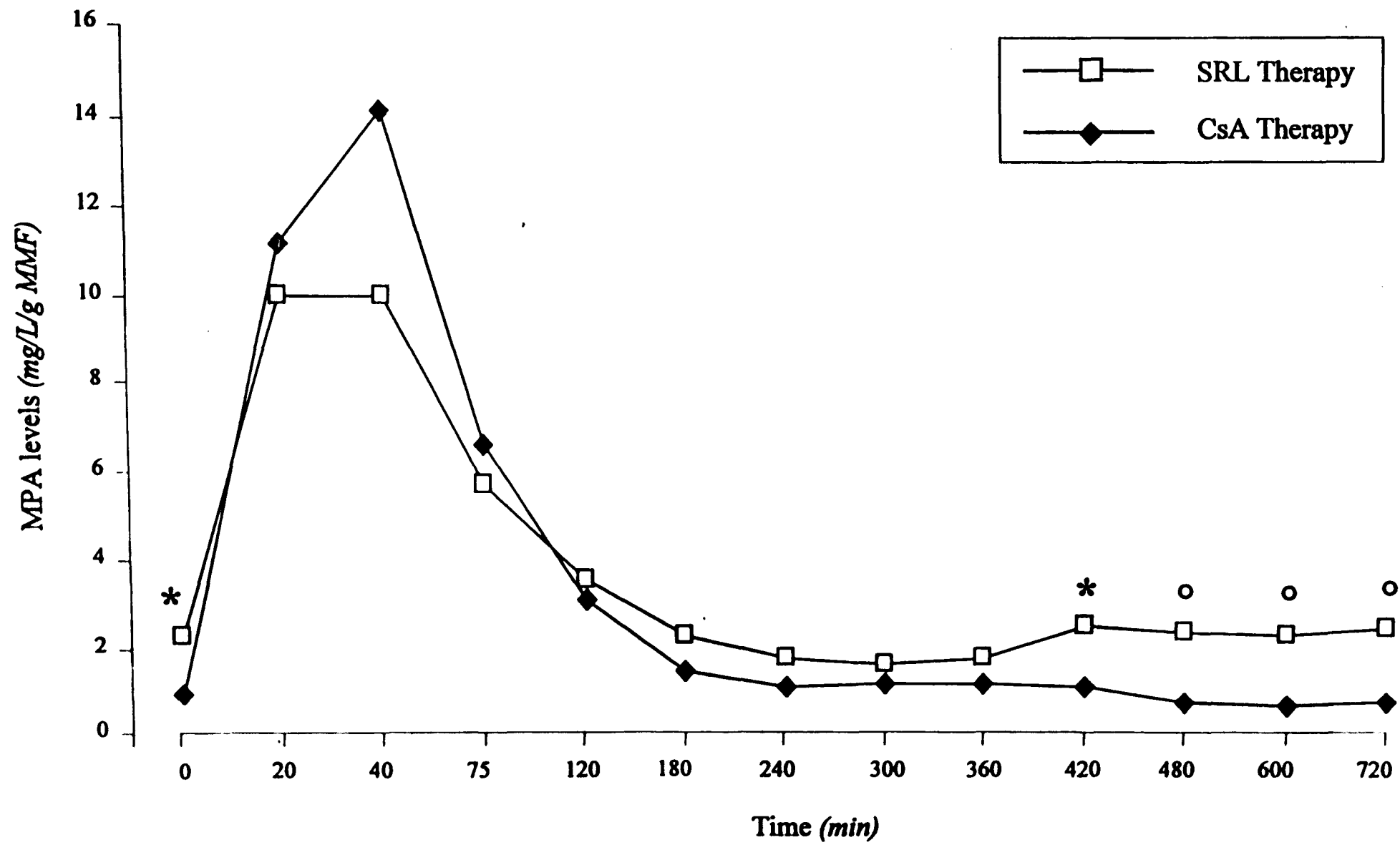
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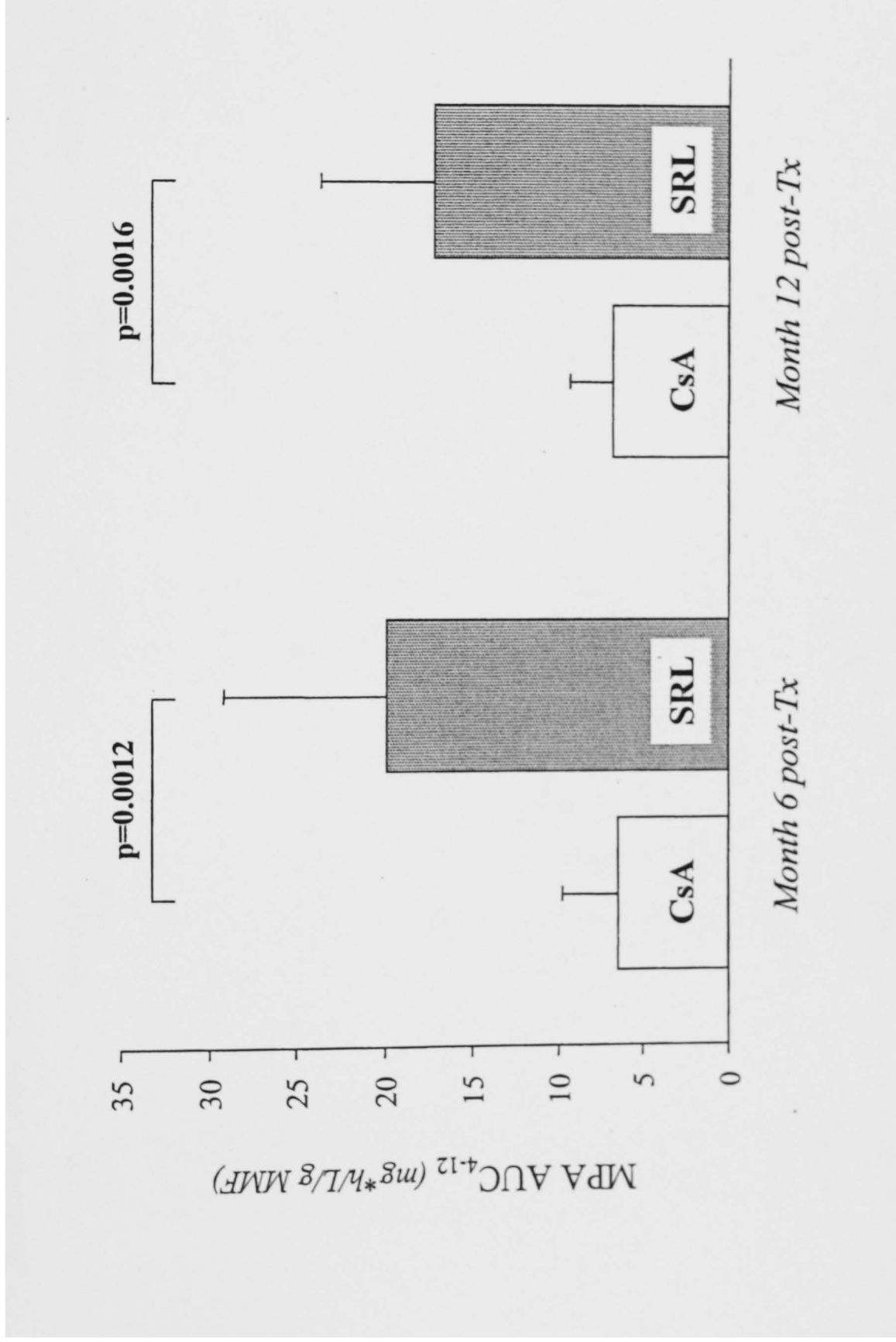
- Figure 21 -

**Table 14.** Immunosuppressive drug dosing and trough levels

*Sirolimus group (n=11)*

| Time Post-Tx | Mean SRL Dose (mg/day) | Mean SRL trough (ng/mL) | Mean MMF Dose (mg/day) | Mean MPA trough (μg/mL) |
|---|---|---|---|---|
| *1 week* | 3.6 ± 1.7 | 4.5 ± 1.9 | 542 ± 144 | 0.61 ± 0.56 |
| *2 weeks* | 4.3 ± 0.8 | 9.1 ± 2.2 | 484 ± 63 | 0.88 ± 0.63* |
| *1 month* | 4.3 ± 1.7 | 9.4 ± 3.2 | 571 ± 182 | 1.73 ± 0.94° |
| *3 months* | 4.3 ± 1.8 | 8.3 ± 2.7 | 621 ± 430 | 1.54 ± 1.05° |
| *6 months* | 4.0 ± 1.5 | 7.9 ± 2.3 | 591 ± 202 | 1.67 ± 1.12* |
| *12 months* | 4.0 ± 1.0 | 10.1 ± 4.5 | 1200 ± 447 | 2.75 ± 1.63° |

*Cyclosporine group (n=10)*

| Time Post-Tx | Mean CsA Dose (mg/day) | Mean CsA trough (ng/mL) | Mean MMF Dose (mg/day) | Mean MPA trough (μg/mL) |
|---|---|---|---|---|
| *1 week* | 343 ± 191 | 145 ± 110 | 688 ± 372 | 0.22 ± 0.14 |
| *2 weeks* | 243 ± 106 | 134 ± 59 | 550 ± 158 | 0.33 ± 0.32 |
| *1 month* | 279 ± 109 | 133 ± 61 | 559 ± 166 | 0.26 ± 0.14 |
| *3 months* | 263 ± 74 | 124 ± 52 | 608 ± 204 | 0.41 ± 0.34 |
| *6 months* | 269 ± 78 | 119 ± 34 | 750 ± 267 | 0.49 ± 0.38 |
| *12 months* | 207 ± 57 | 101 ± 31 | 813 ± 259 | 0.66 ± 0.24 |

*p<0 05 vs CsA group at same time point; °p<0 01 vs CsA group at same time point

**Table 15.** MPA pharmacokinetic parameters at month 6 post-transplantation in patients treated with SRL or CsA both in combination with MMF

| MPA Pharmacokinetic parameters | SRL Group (n=11) | CsA Group (n=10) | P-value |
|---|---|---|---|
| MMF Dose (mg/day) | 591 ± 202 | 750 ± 267 | 0.1570 |
| MPA $C_0$ (mg/L) | 1.67 ± 1.12 | 0.49 ± 0.38 | 0.0117 |
| $C_0$/Dose (mg/L/g MMF) | 2.87 ± 2.08 | 0.63 ± 0.40 | 0.0082 |
| $C_{max}$ (mg/L) | 10.66 ± 9.28 | 11.74 ± 6.56 | 0.7829 |
| $C_{max}$/Dose (mg/L/g MMF) | 17.09 ± 8.97 | 15.28 ± 5.60 | 0.6221 |
| $T_{max}$ (min) | 63 ± 100 | 46 ± 19 | 0.6444 |
| $AUC_{0-12}$ (mg*h/L) | 24.63 ± 13.88 | 18.78 ± 11.47 | 0.3441 |
| $AUC_{0-12}$/Dose (mg*h/L/g MMF) | 41.52 ± 14.76 | 23.66 ± 7.77 | 0.0006 |
| $AUC_{0-12}$° (mg*h/L) | 32.25 ± 18.41 | 19.97 ± 11.08 | 0.1129 |
| $AUC_{0-12}$/Dose° (mg*h/L/g MMF) | 54.59 ± 28.54 | 25.53 ± 7.10 | 0.0124 |
| $AUC_{4-12}$ (mg*h/L) | 11.45 ± 6.14 | 5.07 ± 3.73 | 0.0186 |
| $AUC_{4-12}$/Dose (mg*h/L/g MMF) | 19.93 ± 9.31 | 6.46 ± 3.31 | 0.0012 |

° AUC predicted using the equation by Hale et al [37]

**Table 16.** MPA pharmacokinetic parameters at month 12 post-transplantation in patients treated with SRL or CsA, both in combination with MMF.

| MPA Pharmacokinetic parameters | SRL Group (n=11) | CsA Group (n=10) | P-value |
|---|---|---|---|
| MMF Dose (mg/day) | 813 ± 259 | 1200 ± 447 | 0.0706 |
| MPA $C_0$ (mg/L) | 0.66 ± 0.24 | 2.75 ± 1.63 | 0.0037 |
| $C_0$/Dose (mg/L/g MMF) | 0.89 ± 0.44 | 2.30 ± 1.30 | 0.0151 |
| $C_{max}$ (mg/L) | 12.53 ± 3.91 | 13.46 ± 6.32 | 0.7471 |
| $C_{max}$/Dose (mg/L/g MMF) | 15.89 ± 4.30 | 11.11 ± 2.96 | 0.0528 |
| $T_{max}$ (min) | 30 ± 11 | 24 ± 9 | 0.3193 |
| $AUC_{0-12}$ (mg*h/L) | 20.37 ± 6.57 | 43.92 ± 24.60 | 0.0236 |
| $AUC_{0-12}$/Dose (mg*h/L/g MMF) | 26.00 ± 8.61 | 35.45 ± 10.02 | 0.0974 |
| $AUC_{0-12}°$ (mg*h/L) | 23.03 ± 6.08 | 52.88 ± 26.34 | 0.0092 |
| $AUC_{0-12}$/Dose° (mg*h/L/g MMF) | 30.23 ± 10.77 | 43.89 ± 16.31 | 0.0936 |
| $AUC_{4-12}$ (mg*h/L) | 5.14 ± 2.18 | 21.38 ± 13.04 | 0.0051 |
| $AUC_{4-12}$/Dose (mg*h/L/g MMF) | 6.81 ± 2.52 | 17.18 ± 6.49 | 0.0016 |

* AUC predicted using the equation by Hale et al [37]

# PHARMACOKINETICS OF THE NEW ENTERIC-COATED MYCOPHENOLATE SODIUM AND COMPARISON WITH THE TRADITIONAL MOFETIL FORMULATION IN KIDNEY TRANSPLANT RECIPIENTS

# Introduction

Mycophenolate mofetil (MMF), the ester prodrug of mycophenolic acid (MPA), is a potent immunosuppressive agent actually used as a part of standard immunosuppressive regimens in combination with a calcineurin inhibitor or sirolimus, and steroids [107]. MMF is usually administered at a fixed oral dose of 1000 mg twice daily, and therapeutic drug monitoring is not routinely performed. Recent evidence, however, suggests that a fixed dose regimen of MMF no longer might be the best approach for the management of transplant patients, and drug pharmacokinetic monitoring is advisable [19,88,90].

Despite excellent efficacy, MMF is associated with tolerability problems, particularly because of gastrointestinal (GI) adverse events such as nausea/vomiting, diarrhoea, abdominal pain and gastritis [87,103]. Additionally, also haematological toxicity has been reported after MMF use [74]. These adverse events often lead to a reduction in drug dose or discontinuation of MMF from the immunosuppressive regimen, leading to sub therapeutic dosing and impaired clinical outcomes [104,105].

To overcome the problems associated with MMF use, an enteric-coated form of mycophenolate sodium (EC-MPS, Myfortic[R]) has been recently developed, which, via its advanced formulation, has the potential to extend the therapeutic window of MPA through enhanced tolerability relative to MPA exposure [87,106]. Unlike MMF, which releases MPA in the stomach, EC-MPS releases MPA in the small intestine.

Previous studies [107,108,109] have shown that 720 mg of EC-MPS and MMF 1000 mg deliver near, equimolar doses of MPA and provide bioequivalent MPA exposure, defined as the AUC. Therefore, it is now generally accepted that these two doses are therapeutically equivalent [106,109]. Results of pivotal phase III studies have shown that EC-MPS has a comparable efficacy and safety profile to MMF in de novo and

maintenance renal transplant patients [109,110]. The incidence of GI adverse events was similar for MMF and EC-MPS, though the severity of GI side effects with EC-MPS tended to be lower in the study of maintenance patients [110]. Furthermore, a non-significant trend for fewer de novo patients receiving the new formulation requiring dose reduction, drug withdrawal, or interruption due to GI adverse events compared to the traditional MMF formulations has been observed [109,110].

Up to now, only few studies have formally compared the pharmacokinetics of MPA released from EC-MPS with that obtained after MMF administration [111,112]. Nevertheless, most of these studies were incomplete, with no baseline patient demographic or statistical analysis included, and an omission of full kinetic profiles. Moreover, they focused on the comparison between the two MPA-releasing formulations only after single drug dose administration [111] or providing only pharmacokinetic data from the early period after transplantation [112]. Therefore, complete data on the pharmacokinetics of MPA after chronic EC-MPS administration in stable kidney transplant recipients are still lacking.

Indeed, the current study was designed to: 1) investigate the full pharmacokinetics of MPA after EC-MPS administration in stable renal transplant patients at month 6 post-surgery; 2) to assess the intra- and inter-patient variability of MPA pharmacokinetics by comparing values at month 6 with those obtained at month 12 post-surgery; 3) to compare the above mentioned pharmacokinetic parameters with those observed in a control group of patients, well matched for concomitant immunosuppressive therapy and time after transplantation, given MMF instead of EC-MPS; 4) to estimate the correlation between each sampling time points and daily MPA $AUC_{0-12}$ for both formulations.

## Materials and methods

*Study population*

Ten adult renal transplant patients (4 males/6 females) referred to the Kidney Transplant Centre of the Ospedali Riuniti Bergamo, and enrolled in an intensified multi-factorial therapy open trial, were included in the present study. They were on triple immunosuppressive therapy, including CsA Neoral, corticosteroids and EC-MPS.

All patients received EC-MPS twice daily, every 12 hours in the morning and in the evening, at starting dose of 720 mg (2 enteric coated tablets of 360 mg b.i.d). EC-MPS was commenced as soon as possible after transplant at a daily dose of 1440 mg and drug dose remained fixed through all the study period. Some of the patients, however, required drug dose reduction, due to adverse events. In case of leukopenia (leukocyte count $<4000/mm^3$), neutropenia (absolute neutrophil count $<1500/mm^3$), or other moderate/severe adverse events, EC-MPS dose was reduced by 50%. In any case, changes in EC-MPS dosing were performed by the attending physicians based on clinical parameters suggesting MPA-induced toxicity, but not according to MPA pharmacokinetics. For comparison, ten adult renal transplant recipients (6 males/ 4 females) transplanted in the same period in our Transplant Unit, well matched for time post-transplantation and concomitant immunosuppressive therapy, given MMF instead of EC-MPS were also included in the pharmacokinetics evaluations.

Only patients with stable renal function in the previous 4 months (defined by less than 15% differences in serum creatinine values during monthly evaluations) were included in the present pharmacokinetic study. Patients with existence of any surgical or medical condition, other than the current transplant, which might significantly alter the absorption, distribution, metabolism or excretion of the study medications, and/or presence of severe diarrhoea or active peptic ulcer disease, were excluded from the

study. The study protocol was described in detail to the patients before admission and written informed consent to enter the study was obtained.

*Study design*

This prospective study first evaluated the MPA pharmacokinetic parameters at month 6 post-surgery in patients given EC-MPS or MMF. The MPA complete 12-hour plasma concentration-time profile was recorded for all patients, together with the time ($T_{max}$) to reach the maximum concentration ($C_{max}$) and MPA $AUC_{0-12}$, calculated with the trapezoidal rule. On the morning of the pharmacokinetic studies, blood samples were collected for routine haematological analysis and for the determination of trough levels of plasma MPA. Then, each patient was given the morning dose of EC-MPS or MMF under fasted conditions. Thereafter, no particular restrictions on food intake were applied during the day. For MPA pharmacokinetics, blood samples in EDTA-tubes were drawn at 20, 40, 75, 120 minutes, and 3, 4, 5, 6, 8, 10 and 12 hours. Thereafter, samples were centrifuged at 3800 x g, plasma separated, and stored at –20°C until analysis by HPLC as described in *Chapter 3*. At month 12 post-transplantation, all patients given EC-MPS or MMF underwent a second pharmacokinetic evaluation, together with routine haematological analyses. Using this approach, we were able to estimate both intra- and interpatient variability from both MPA-releasing formulations.

As additional analysis, each sampling time was correlated with the daily MPA exposure, expressed as MPA $AUC_{0-12}$, with the goal to identify time points useful for the development of limited sampling strategies aimed at predicting daily MPA exposure after EC-MPS administration.

Patients experiencing drug-related toxicity were allowed to modify the daily EC-MPS or MMF dose. To take into account this potential confounding factors, all the MPA pharmacokinetic parameters were adjusted for the daily drug dose, and expressed as

equivalent of MPA, assuming a 1:1 equivalence between EC-MPS 720 mg and MMF 1000 mg, as previously documented [106-112].


*Statistical analysis*

Results are reported as means $\pm$ SD. Differences in MPA pharmacokinetics within and between the two MPA-releasing formulations at month 6 and 12 post-surgery were analyzed using the ANOVA test. Within- and between-patient variability of the main MPA pharmacokinetic parameters were expressed as CV%. Correlation between plasma MPA concentrations ranging from 0 to 12 hour post dosing and MPA $AUC_{0-12}$ were performed by linear regression analysis. The statistical significance level was defined as $p < 0.05$.

# Results

## Recipients' demographics

All patients enrolled in the present study were Caucasians, and recipients of first kidney transplant from cadaver donors. As shown in Table 17, patients given EC-MPS were comparable to those given MMF as for demographics, renal and liver function, as well as for haematological profile both at month 6 and 12 post-surgery.

## Pharmacokinetics of the two MPA-releasing formulations

The mean MPA pharmacokinetic parameters for the EC-MPS and MMF groups are shown in Table 18. Absorption was slower for EC-MPS than for MMF, consistent with a functional enteric coating for EC-MPS. Indeed, at month 6 post-surgery, mean $T_{max}$ was 130 min for EC-MPS and 46 min for MMF (p<0.01). Mean MPA exposure, defined either by dose-adjusted $AUC_{0-12}$ and MPA $C_{max}$, were not different between the two groups. The same findings were confirmed also at month 12 post surgery (Table 18). However, at variance with previous observations, we found that dose-adjusted MPA trough levels were 4.7-fold higher in patients given EC-MPS than those given MMF (Table 18, Figure 22). To better investigate potential differences in the MPA pharmacokinetic profiles between the two formulations, we looked at the single kinetic curves for each patient. As shown in Figures 23 and 24, unexpected, atypical kinetic profiles of MPA in patients given EC-MPS were observed both at month 6 and 12 post-transplantation. Of note, all patients given EC-MPS presented multiple peaks of MPA (on an average of 3 peaks per patient). Interestingly, some patients had MPA $C_{max}$ at 480 minutes after drug dosing (Figure 22), whereas others had MPA $C_{max}$ peaking at time 0 (basal). Conversely, the control group of patients treated with MMF presented regular MPA pharmacokinetic profiles, consistent with previous observations [75], with

maximum peak of MPA always within 2 hours (Figure 22), and the presence of a second, flat peak at 6-12 hours post MMF dosing, corresponding to the well-known enterohepatic recirculation of MPA metabolites [75].

*Variability of MPA pharmacokinetics parameters*

All patients underwent pharmacokinetic evaluations both at month 6 and 12 after transplantation. As a measure of the intra-patient variability of plasma MPA levels, the CV% was calculated for main pharmacokinetic parameters between the two study visits in patients given EC-MPS and, for comparison, in those given MMF. We found a higher variability of MPA pharmacokinetics in patients given the new enteric coated formulation than those treated with the traditional MMF formulations (Table 19). Despite the low number of patients (n=10 for each group), these differences reached statistical significance ($C_{max}$: CV: 45.7% vs 15.1%, $p < 0.05$).

As additional analysis, we also evaluated the inter-patient variability of MPA pharmacokinetics in the formulations both at month 6 and 12 post-surgery. As documented in Table 19, we confirmed that the use of the new EC-MPS formulation was associated with significant variability in MPA pharmacokinetics as compared with MMF.

*Correlation between MPA $AUC_{0-12}$ and single sampling points*

As demonstrated in Table 20, the regression analysis between individual plasma MPA concentrations and MPA $AUC_{0-12}$ documented an overall better correlation with the MMF formulation. Of note, this findings applied for all single time points (with the only exception of the sampling at 20 minutes), with differences in the correlation coefficient (r) that, in some cases, were more than double (at 20, 75, 120, 240, and 600 minutes post drug administration) between the two MPA-releasing formulations.

Given the observed low correlation between single MPA sampling points and daily

MPA exposure, we were not able to develop feasible limited sampling strategies useful

to predict MPA $AUC_{0-12}$ in patients given EC-MPS (data not shown).

## Discussion

The main finding of the present study was that the pharmacokinetics of MPA released from the new enteric-coated formulation of mycophenolate sodium is extremely variable and irregular as compared with that found in stable kidney transplant recipients given MMF. Despite no apparent differences in mean MPA exposure expressed as MPA $AUC_{0-12}$, we observed aberrant kinetic curves in single patients, with an extremely high variability both in the $T_{max}$ and MPA $C_0$. Additionally, patients given EC-MPS presented multiple peaks of MPA, an effect never observed after MMF administration. These findings were at variance with those recently published by Arns et al [111], showing that, after single EC-MPS or MMF administration, the pharmacokinetic curves for all treatments were similar. It should be pointed out, however, that in that study only mean pharmacokinetic profiles were presented, with no data showing the variability associated with single MPA sampling points. Moreover, as stated above, only single drug administration were considered. Therefore, these results can not be applied to patients, as ours, chronically exposed to EC-MPS.

Only a very few studies have compared the pharmacokinetics of MPA released from EC-MPS with that from MMF in patients chronically treated with these drugs [111,112]. As a result of differences in the molecular weight, EC-MPS 720 mg contains a near equimolar quantity of MPA as MMF 1000 mg. Pharmacokinetic analysis have shown that the administration of EC-MPS at 720 mg and MMF 1000 mg resulted in similar MPA $C_{max}$ and AUC, documenting bioequivalence between the two formulations. Actually, the bioequivalence guidelines for approval of generic formulations require a similar average bioavailability compared with the reference formulation, with the 90% confidence interval of the relative mean AUC and $C_{max}$ of the test to reference formulation within 0.8 to 1.25 [113]. However, this approach presents

some important limitations, one of the most important being the assumption to consider average bioavailability instead of single evaluations. This concept was underlined and emphasized by our findings. Indeed, looking at mean MPA $C_{max}$ and $AUC_{0-12}$, we found no differences between EC-MPS and MMF, confirming bioequivalence between the two formulations. However, looking at the single MPA pharmacokinetic profiles, we documented enormous differences between the two formulations. In particular, patients given EC-MPS presented MPA trough levels 4- to 5-fold higher that those found in patients given MMF, despite no differences in the MPA $AUC_{0-12}$. It can be reasonably speculated that our results may have important clinical consequences when $C_0$-based monitoring is used as a guide to optimize MPA therapy, as recently suggested by international consensus conferences [114].

Enteric-coating was designed to improve the GI adverse event profile of MMF releasing MPA in the small intestine, an effect evidenced by a delay in the MPA $C_{max}$ [87,106]. Overall, this trend was confirmed also by our data, showing that mean MPA $T_{max}$ was longer in patients given EC-MPS than those given MMF. However, when we looked at the single data, we observed a very huge distribution in the $T_{max}$, covering a period from 0 to 480 min after EC-MPS administration. These unexpected findings allow us to speculate that some patients, actually via unknown mechanisms, may experience some problems in the absorption of EC-MPS, a pattern also confirmed by the presence of multiple MPA peaks. This trend resembles early observations after cimetidine administration [115], suggesting a potential influence of food intake in the absorption of EC-MPS but not MMF. Since all patients from both groups received the morning dose of the study medication under fast conditions, but had no particularly restriction to food thereafter, we were not able to draw definite conclusion on this issue.

It is now generally accepted that the measurement of MPA $AUC_{0-12}$ is the most reliably tool for the assessment of daily MPA exposure [114]. However, this approach is seldom

feasible in the routine clinical practice and, therefore, limited sampling strategies for the prediction of AUC have been early advocated for MMF [37,75,114], but are still lacking for EC-MPS. To pursue this goal we have tried to identify a few, near-the-peak, sampling points useful to predict MPA $AUC_{0-12}$ in patients given EC-MPS. However, we found a very high variability, both intra- and inter-patient, among all the pharmacokinetic parameters of MPA obtained from patients treated with mycophenolate sodium. This was also documented by poor correlations between single sampling points and MPA $AUC_{0-12}$ after EC-MPS, but not MMF, administration. For all these results, we were not able to provide an equation useful to reliably predict MPA daily exposure in patients given the new enteric-coated formulation of MPA.

In conclusion, we have shown that, despite average bioequivalence, the pharmacokinetic of MPA released from EC-MPS is extremely variable and irregular as compared with that observed after MMF administration in stable kidney transplant recipients chronically exposed to these two formulations. Given the emerging strong support for the clinical outcome benefit of MPA monitoring in transplant setting, further studies are warranted to better investigate the mechanisms underlying differences in the absorption processes between EC-MPS and MMF.

**Legend to Figures**

*Figure 22.* Distribution of MPA $T_{max}$ and dose-adjusted $C_0$ in patients treated with EC-MPS or MMF. Data are depicted as mean, minimum and maximum values for each pharmacokinetic parameter. *$p<0.01$ vs Tmax and C0 in the MMF group.

*Figure 23.* Temporal distribution (from time 0 to time 720 minutes after drug administration) of single daily MPA concentrations in kidney transplant recipients at month 6 after surgery given EC-MPS or MMF.

*Figure 24.* Temporal distribution of single daily MPA concentrations in kidney transplant recipients at month 12 after surgery given EC-MPS or MMF.

- Figure 22 -

EC-MPS (month 6)

MMF (month 6)

Time (min)

- Figure 23 -

EC-MPS (month 12)

MMF (month 12)

Time (min)
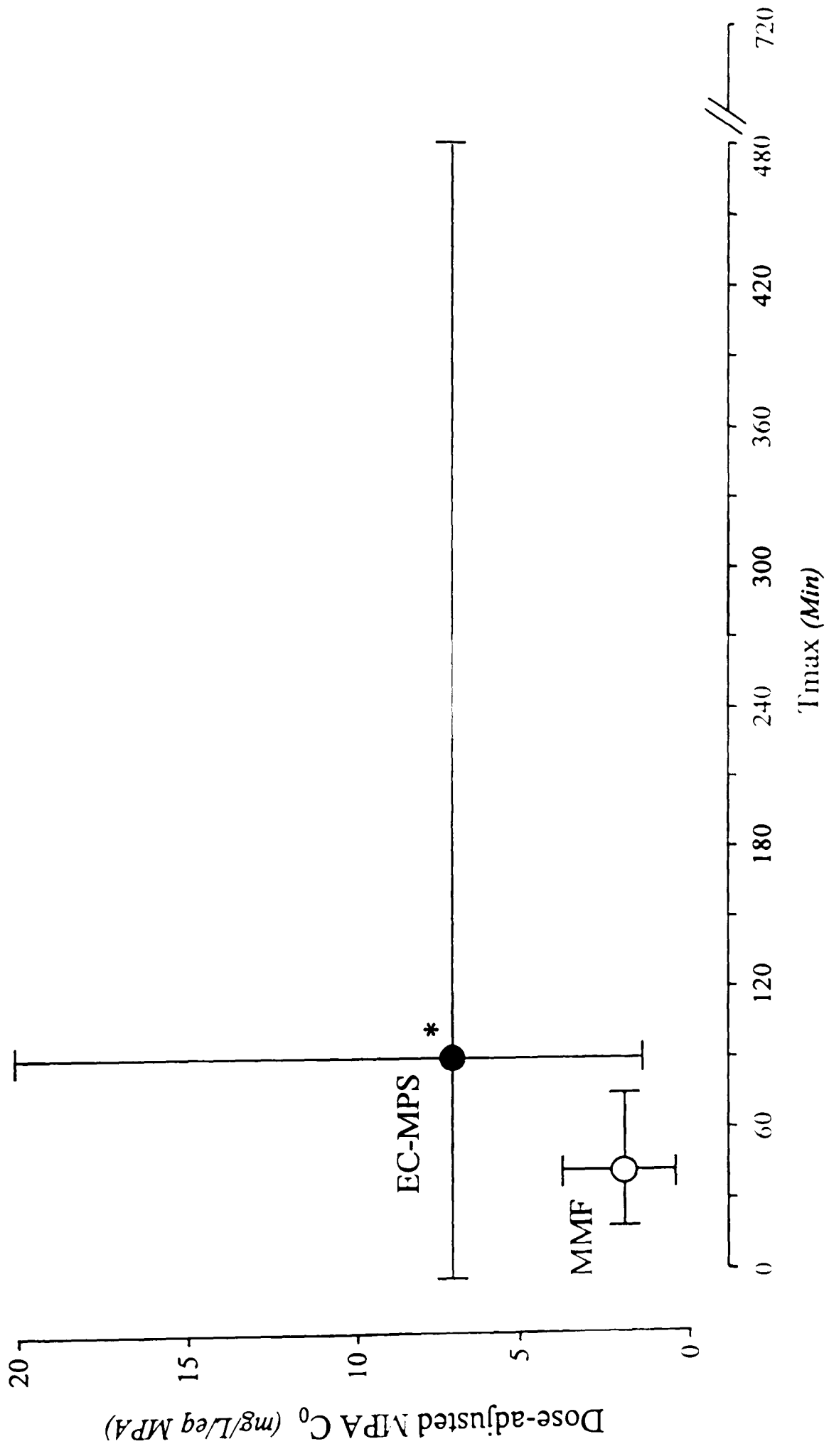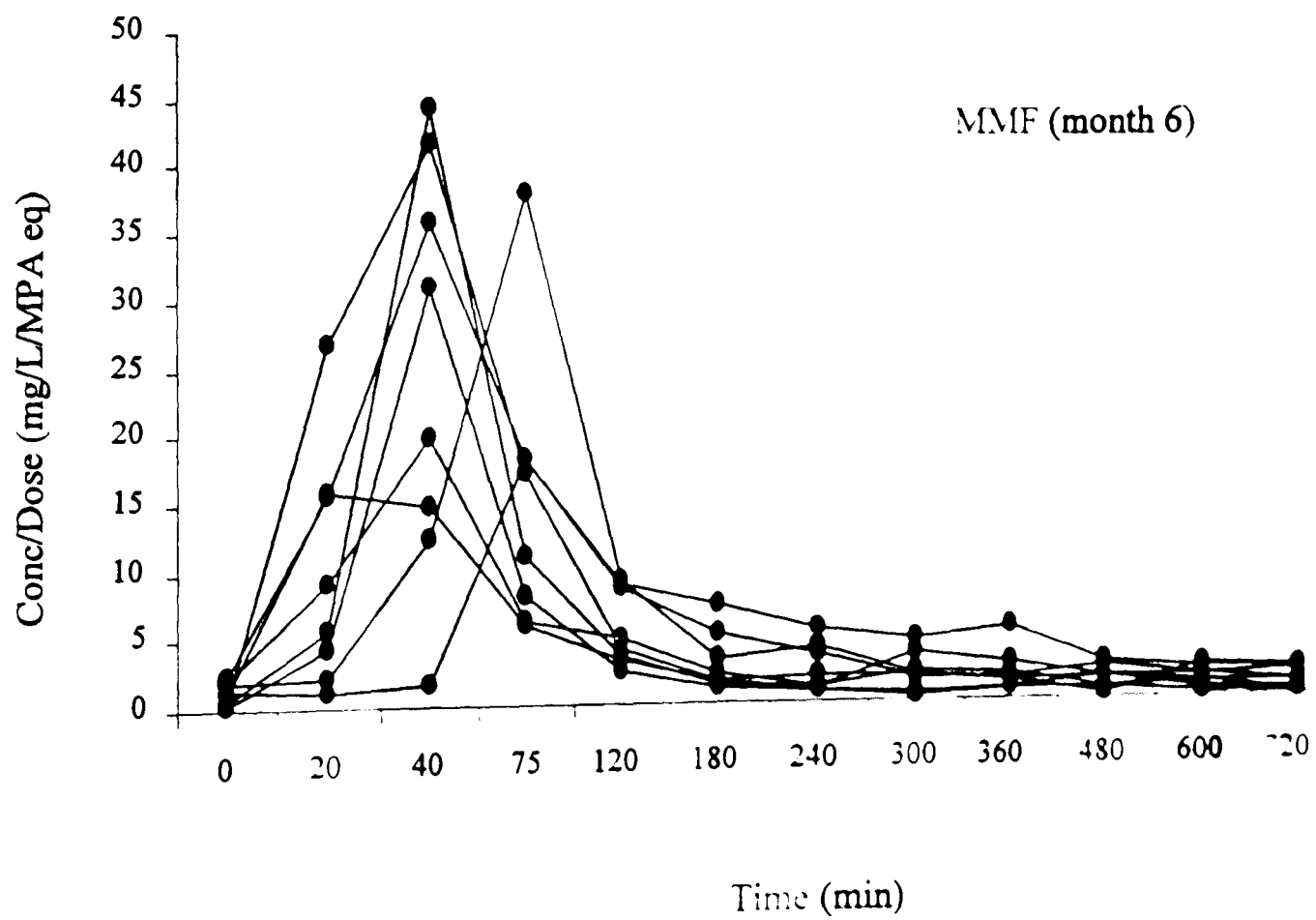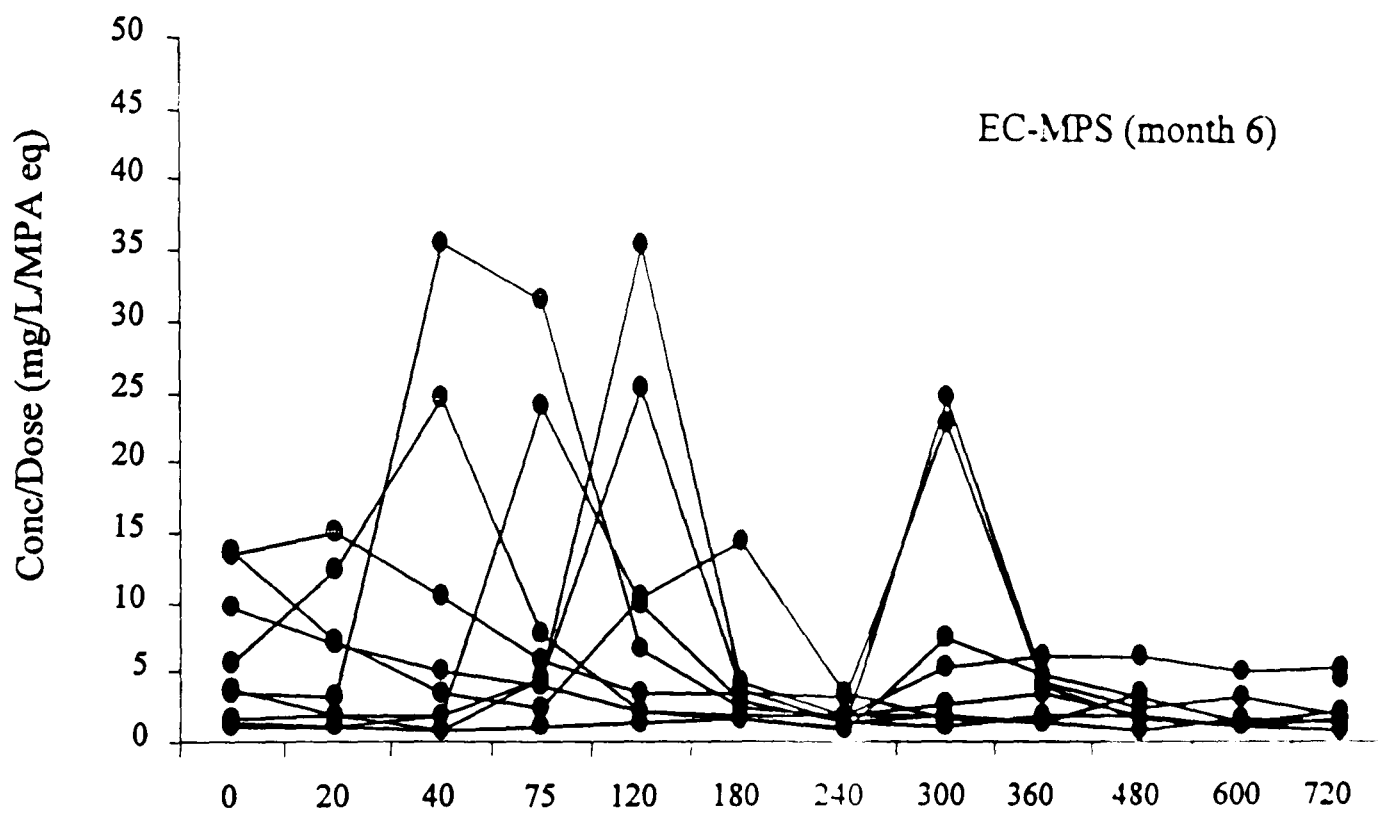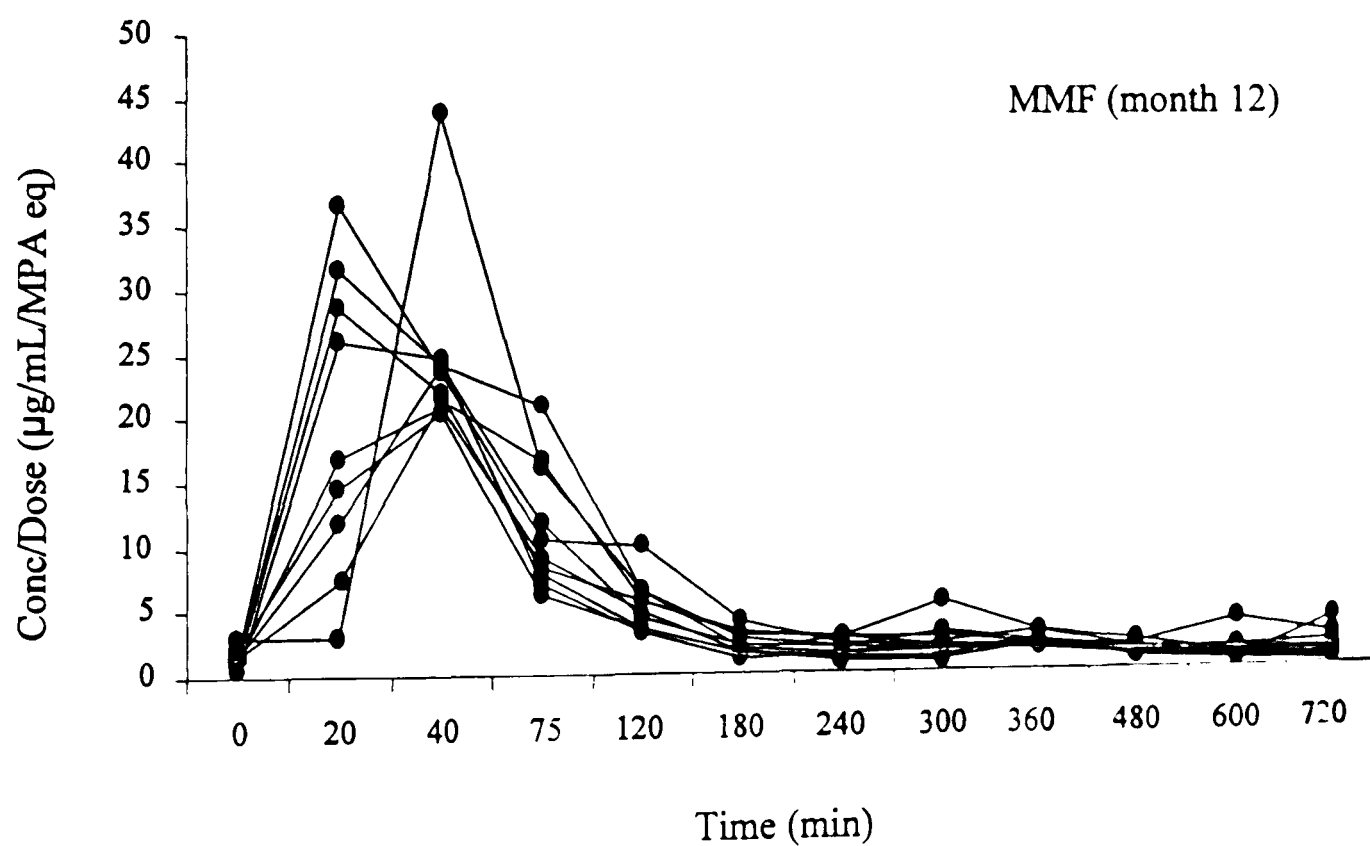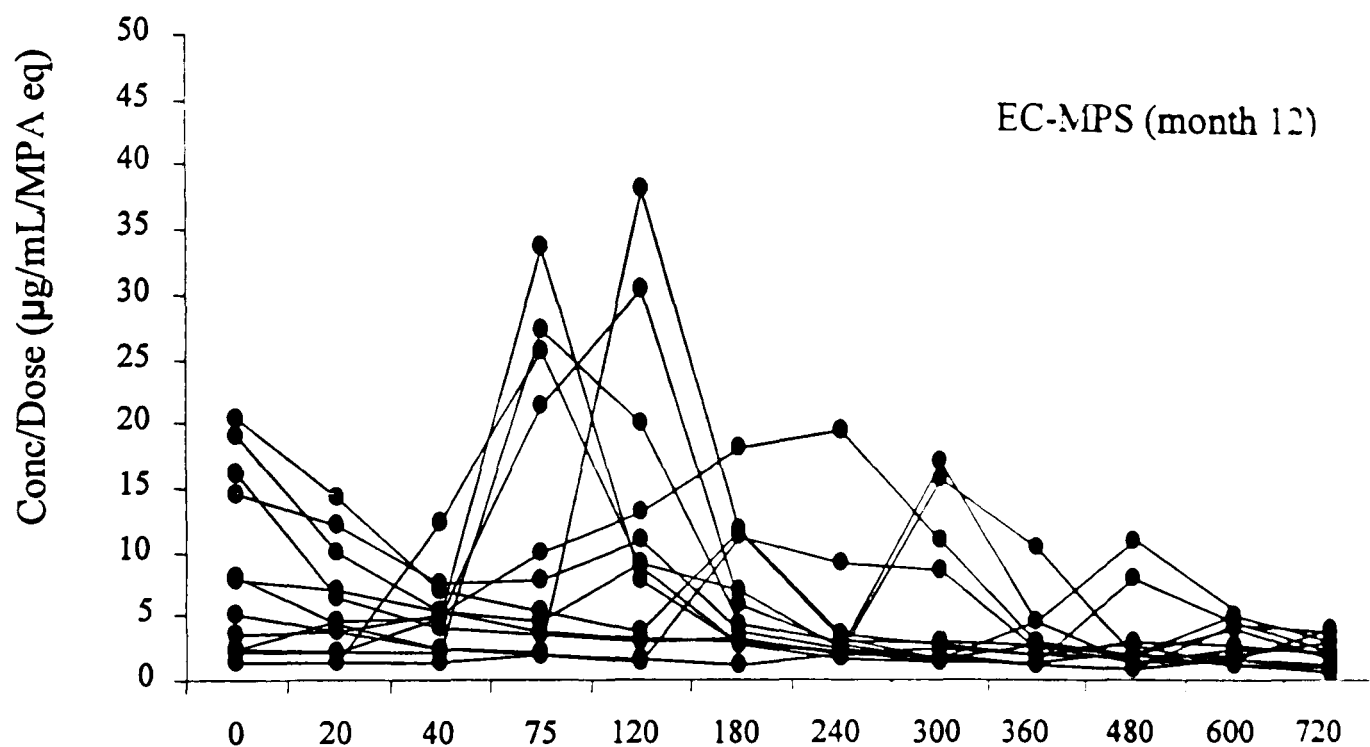
- Figure 24 -

**Table 17.** Demographic and hematological data of kidney transplant recipients given mycophenolate sodium (EC-MPS, n=10) or mycophenolate mofetil (MMF, n=10).

| | EC-MPS | | MMF | |
| --- | --- | --- | --- | --- |
| | *Month 6* | *Month 12* | *Month 6* | *Month 12* |
| Age *(yr)* | 41 ± 12 | 39 ± 13 | 44 ± 12 | 44 ± 12 |
| Body Weight *(kg)* | 72.1 ± 18.5 | 70.1 ± 16.9 | 75.3 ± 17.3 | 77.7 ± 17.3 |
| S.Creatinine *(mg/dL)* | 1.54 ± 0.42 | 1.51 ± 0.56 | 1.66 ± 0.36 | 1.56 ± 0.27 |
| Creatinine cl. *(mL/min)* | 64.3 ± 17.4 | 67.0 ± 17.8 | 55.4 ± 18.9 | 65.4 ± 17.8 |
| S.Urea *(mg/dL)* | 64 ± 13 | 59 ± 17 | 66 ± 28 | 63 ± 20 |
| AST *(IU/L)* | 17 ± 4 | 17 ± 5 | 20 ± 10 | 19 ± 4 |
| ALT *(IU/L)* | 18 ± 5 | 15 ± 4 | 27 ± 26 | 18 ± 10 |
| GGT *(IU/L)* | 18 ± 10 | 20 ± 18 | 35 ± 43 | 36 ± 33 |
| RBC *($10^6/\mu L$)* | 4.28 ± 0.71 | 4.37 ± 0.45 | 4.10 ± 0.31 | 4.37 ± 0.52 |
| WBC *($10^6/\mu L$)* | 7.70 ± 3.51 | 7.69 ± 2.81 | 5.39 ± 2.18 | 5.90 ± 1.61 |

Table 18. Pharmacokinetic parameters of kidney transplant recipients given EC-MPS (n=10) or MMF (n=10).

| Pharmacokinetic parameters | EC-MPS | | MMF | |
|---|---|---|---|---|
| | *Month 6* | *Month 12* | *Month 6* | *Month 12* |
| Dose *(MPA eq/day)* | $1040 \pm 379$ | $997 \pm 365$ | $750 \pm 267$ | $850 \pm 242$ |
| $C_0$ *(mg/L)* | $4.07 \pm 4.07^*$ | $6.50 \pm 6.56^*$ | $0.49 \pm 0.38$ | $0.72 \pm 0.26$ |
| $C_0$/Dose *(mg/L /MPA eq)* | $5.93 \pm 5.01^*$ | $7.98 \pm 7.01°$ | $1.26 \pm 0.80$ | $1.64 \pm 0.62$ |
| $C_{max}$ *(mg/L)* | $18.11 \pm 10.20$ | $15.95 \pm 7.03$ | $11.74 \pm 6.56$ | $12.23 \pm 3.54$ |
| $C_{max}$/Dose *(mg/L /MPA eq)* | $25.51 \pm 11.30$ | $25.57 \pm 14.78$ | $30.56 \pm 11.21$ | $29.84 \pm 8.67$ |
| $T_{max}$ *(min)* | $130 \pm 107^*$ | $118 \pm 129^*$ | $46 \pm 19$ | $30 \pm 11$ |
| $AUC_{0-12}$ *(mg•h/L)* | $36.65 \pm 11.78°$ . | $41.04 \pm 19.53°$ | $18.94 \pm 11.62$ | $21.14 \pm 6.08$ |
| $AUC_{0-12}$/Dose *(mg•h/L/MPA eq)* | $53.18 \pm 12.72$ | $58.86 \pm 18.29$ | $47.64 \pm 15.77$ | $51.28 \pm 15.37$ |

$^*p<0.05$ vs MMF, $°p<0.01$ vs MMF

Table 19: Intra- and inter-patient variability of main MPA pharmacokinetic parameters in kidney transplant recipients given EC-MPS or MMF at month 6 and 12 post-surgery.

| Pharmacokinetic parameters | Intrapatient variability (CV % month 6 vs 12) | | Interpatient variability (CV % at month 6) | | Interpatient variability (CV % at month 12) | |
|---|---|---|---|---|---|---|
| | EC-MPS | MMF | EC-MPS | MMF | EC-MPS | MMF |
| $C_0$/Dose (mg/L /MPA eq) | 32.8 | 38.7 | 84.4* | 63.9 | 87.8° | 37.8 |
| $C_{max}$/Dose (mg/L /MPA eq) | 45.7* | 15.1 | 44.3 | 36.7 | 57.8 | 29.1 |
| $T_{max}$ (min) | 66.7 | 32.5 | 82.4* | 41.2 | 109.4* | 35.1 |
| $AUC_{0-12}$ /Dose (mg*h/L/MPA eq) | 24.9 | 12.4 | 23.9 | 33.1 | 31.1 | 29.9 |

*p· 0 05 vs MMF; °p<0.01 vs MMF

Table 20: Correlation between single MPA sampling points and MPA $AUC_{0-12}$ in patients given EC-MPS or MMF.

| Time-point of sampling (min) | EC-MPS | | MMF | |
|---|---|---|---|---|
| | r | Regression equation | r | Regression equation |
| $C_0$ | 0.76 | $AUC = 2.21\, C_0 + 27.08$ | 0.87 | $AUC = 18.60\, C_0 + 8.87$ |
| $C_{20}$ | 0.69 | $AUC = 3.41\, C_{20} + 25.75$ | 0.16 | $AUC = 0.58\, C_{20} + 22.47$ |
| $C_{40}$ | 0.14 | $AUC = 0.57\, C_{40} + 36.92$ | 0.75 | $AUC = 1.93\, C_{40} + 0.52$ |
| $C_{75}$ | 0.18 | $AUC = -0.58\, C_{75} + 42.8$ | 0.89 | $AUC = 2.29\, C_{75} + 9.6$ |
| $C_{120}$ | 0.28 | $AUC = 0.53\, C_{120} + 35.05$ | 0.91 | $AUC = 5.16\, C_{120} + 7.65$ |
| $C_{180}$ | 053 | $AUC = 2.41\, C_{180} + 29.73$ | 0.75 | $AUC = 7.82\, C_{180} + 11.27$ |
| $C_{240}$ | 0.35 | $AUC = 2.36\, C_{240} + 33.96$ | 0.80 | $AUC = 11.52\, C_{240} + 9.18$ |
| $C_{300}$ | 0.42 | $AUC = 1.33\, C_{300} + 33.27$ | 0.59 | $AUC = 8.82\, C_{300} + 13.87$ |
| $C_{360}$ | 0.58 | $AUC = 4.29\, C_{360} + 29.75$ | 0.82 | $AUC = 19.19\, C_{360} + 4.31$ |
| $C_{480}$ | 0.59 | $AUC = 3.82\, C_{480} + 30.94$ | 0.93 | $AUC = 23.88\, C_{480} + 5.58$ |
| $C_{600}$ | 0.47 | $AUC = 6.05\, C_{600} + 27.62$ | 0.91 | $AUC = 13.04\, C_{600} + 13.61$ |
| $C_{720}$ | 0.68 | $AUC = 11.05\, C_{720} + 21.76$ | 0.88 | $AUC = 15.65\, C_{720} + 12.3$ |

*Part III*


*THERAPEUTIC DRUG MONITORING*

*OF SIROLIMUS*

*Chapter 10*

# THERAPEUTIC DRUG MONITORING OF SIROLIMUS: EFFECT OF CONCOMITANT IMMUNOSUPPRESSIVE THERAPY AND OPTIMIZATION OF DRUG DOSING

# Introduction

SRL is a macrocyclic lactone isolated from *Streptomyces hygroscopicus* characterized by a potent immunosuppressive activity [116]. Interest in the use of sirolimus in organ transplantation derives from its unique mechanism of action, its low side-effect profile, and its ability to synergize with other immunosuppressive agents [117]. SRL has no effect on calcineurin enzyme, but it reduces T-lymphocyte activation at a later stage in the cell cycle, by inhibiting the post interleukin-2 receptor mTOR signal transduction pathway [118].

The drug is metabolized by the cytochrome P450-3A4 isoenzyme (CYP3A4), a family of enzymes involved in the metabolism of several immunosuppressive agents currently used in combination as antirejection treatments in organ transplantation [24]. This may lead to potential pharmacokinetic interactions that eventually affect SRL blood concentrations and ultimately may result in over- or under-immunosuppression or toxicity [119,120]. So far, this issue remains ill defined. Monitoring blood sirolimus levels is mandatory to optimize the drug dosing regimen, since the dose is itself a poor predictor of drug exposure [121]. At variance, SRL trough levels ($C_0$) show good correlation with the daily drug exposure, defined as the area under the time-concentration curve ($AUC_{0-24}$), and with clinical outcomes, both as rejection episodes and drug-related toxicity [40]. HPLC, with either UV or MS detection, is now the only available technique for the assessment of SRL whole blood levels [35,122].

In the past two years our laboratory served as a centralized institution in Italy to measure SRL concentrations in blood samples of kidney transplant recipients on different SRL-based immunosuppressive regimens. These samples referred only to SRL trough levels, and no complete pharmacokinetic profiles were performed to study additional pharmacokinetic parameters. Although the AUC is an accurate index of

patient exposure to the drug, this measurement is quite expensive. time-consuming, and increases the discomfort for the patients, making it seldom feasible in routine clinical monitoring. Therefore, the $C_0$-based SRL monitoring is commonly used as a surrogate marker of daily drug exposure in the routine clinical practice. Thus, given the large number of SRL measurements available in our laboratory referring to different time samplings post transplant and to different settings of drug combination, we sough to investigate the possible effect of concomitant immunosuppressive agents on SRL trough levels ($C_0$) as an index of patient exposure to the drug. In particular, we examined: 1) the potential influence of CsA, tacrolimus (TRL), MMF and steroids on dose-normalized SRL trough concentration; and 2) the variability of SRL trough levels according to different time points post surgery. In addition, we tested an algorithm based on measured SRL trough levels useful to guide changes of SRL dosing.

# Materials and Methods

## Patients and blood sampling

Four hundred ninety-five transplant recipients of cadaver kidneys from 40 Italian Transplant Centres were included in the present study. Patients were at a median of 258 days post-surgery (from day 0 to 14 years), and treated with different immunosuppressive regimens that include CsA, TRL, MMF, and steroids. In most patients, SRL was given in association with a calcineurin-inhibitor (CsA 58%; TRL 19%) in dual (28%) or triple therapy with steroids (46%). Alternatively, patients were treated with SRL in combination with MMF (7%), or steroids (9%) alone, or in triple combination (8%). For a detailed description of the different immunosuppressive regimens see Table 22. The patients received SRL 4 hours after the morning CsA dose, as suggested by the drug manufacturer.

## Study design

Serial SRL blood levels centrally assayed in our Laboratory between June 2001 and October 2003 were considered (n=2,658). Samples were collected in EDTA-containing tube and sent (frozen at –20°C) to the central Laboratory. At the same time the Centres were asked to provide information on: day of transplant, actual SRL daily dose, concomitant immunosuppressive regimen and graft function as serum creatinine. Complete required data were available for 689 SRL trough levels, which belong to 189 patients. Measured SRL concentrations were initially divided in three groups according to immunosuppressive regimen: 1) SRL, CsA with or without steroids; 2) SRL, TRL with or without steroids; 3) SRL, MMF with or without steroids. The impact of CsA, TRL, MMF, or steroids on SRL trough levels was first examined. We also assessed the consistency of SRL trough levels measured at different time points post transplant with

the recently proposed guidelines of target SRL blood concentrations, according to the adopted immunosuppression regimen [123]. Moreover, similarly to what has been recently proposed for CsA Neoral dose adjustment [124], we also evaluated the feasibility of adjusting SRL dose to a given blood trough target using the following equation:

$$\text{Predicted SRL Conc.} = \text{Old SRL Conc.} \times \frac{\text{New SRL dose}}{\text{Old SRL dose}}$$

*SRL concentration measurement*

Whole blood sirolimus concentrations were measured using a validated HPLC method, already described in *Chapter 4* [122].

*Statistical analyses*

Dose-normalized SRL trough values were compared between different groups according to different immunosuppressive regimens by ANOVA. Bonferroni t-tests of difference between means were performed when ANOVA showed a significant difference ($p < 0.05$).

Agreement between blood SRL concentrations measured and predicted with the above mentioned equation was assessed using a linear regression analysis. In addition, the Bland and Altman approach [99], where the percentage difference [100] is plotted against the mean SRL value, was used. We considered acceptable values within 1 standard deviation (SD) of the difference.

# Results

*Concomitant immunosuppressive therapy and SRL levels*

In patients given CsA or TRL as a part of their immunosuppressive therapy, mean SRL dose as well as trough levels were lower than in those receiving MMF but not calcineurin inhibitors (Table 21). Normalization of SRL trough level to drug dose, however, resulted in a significantly higher trough values in patients on CsA, than in those treated with TRL ($p < 0.05$) or MMF ($p < 0.01$).

Since some patients were also given steroids, we further characterize the potential additional effects of corticosteroids on SRL trough levels by comparing dual or triple immunosuppressive regimens with or without steroids. As shown in Table 22, the addition of steroids to CsA- but not to TRL-based immunosuppressive regimen significantly lowered the dose-normalized SRL trough levels as compared to the dual regimen without steroids ($p < 0.01$). A similar trend was found for MMF-based immunosuppression, in which steroids resulted in numerically lower normalized SRL trough levels than steroid-free patients.

Considering the impact of MMF on SRL blood concentration, normalized SRL trough levels were lower with ($2.91 \pm 1.52$ ng/mL/mg SRL; $p < 0.05$) than without MMF ($4.15 \pm 2.23$ ng/mL/mg SRL) added to a dual regimen of SRL/CsA. Moreover, combining MMF with SRL resulted in a significant reduction in dose-normalized SRL trough levels ($3.26 \pm 1.86$ ng/mL/mg SRL) compared with the dual CsA-SRL regimen ($p < 0.05$). When dual SRL-based regimens were compared, dose-normalized SRL trough levels were significantly higher with CsA ($p < 0.05$) than TRL and MMF but not steroids.

*Distribution of SRL trough levels with- or without CsA*

Blood SRL trough concentrations measured by HPLC ranged from 1.0 to 46.4 ng/mL. To evaluate the agreement of SRL trough levels with current regulatory guideline ranges - according to whether patients were given or not CsA - SRL concentrations were divided based on samples from patients with or without CsA as a part of their immunosuppressive regimen. As shown in Figure 25, 71.2% of SRL trough levels from patients receiving the drug in combination with CsA fell in the proposed therapeutic range of 5-15 ng/mL. Among these samples, 72% were between 5-10 ng/mL. Conversely, only 36.7% patients without CsA fell in the proposed SRL trough range (10-20 ng/mL), while the majority of samples (75.0%) were in the range of 5-15 ng/mL.

*Modelling SRL dose according to drug levels*

In 166 patients who did not change SRL dose and concomitant immunosuppressive therapy during consecutive visits (503 determinations), the intrapatient and interpatient variability of SRL trough levels, defined by the mean coefficient of variation, were 19% and 47%, respectively (Table 23). These parameters were significantly affected by the time post-surgery, with the first week after transplantation associated with the greatest intra- and interpatient variability (Table 23).

To provide an algorithm that would help in choosing the appropriate SRL dose and ultimately limiting the drug trough level variability, a simple dose-adjustment formula recently proposed for CsA monitoring [124] was tested. To this purpose we used data from 82 patients who underwent 186 modifications of SRL dose, and correlated the measured and predicted SRL trough levels. As shown in Figure 26, a significant correlation was found between SRL concentrations measured after drug dose change and those predicted with the proposed formula. The mean percentage error of prediction was 4.1 $\pm$ 34%, with 71% of estimations within the threshold acceptable error of $\pm$ 30%.

This arbitrary value results from the sum of the mean intrapatient variability found in patients who did not undergo to SRL dose change (20%), and the performance of the method used to assess SRL levels (an imprecision and accuracy of nearly 10%). To overcome the limitation of the arbitrary estimate of this threshold in error, a more conservative approach [99], that defines as acceptable 1 SD of the mean in the percentage difference of SRL values [100], was considered. As shown in Figure 27, 73% of predicted SRL values fell in the proposed range.

# Discussion

In this study we found that in kidney transplantation the degree of patient exposure to SRL is not only influenced by drug dosing, but also by the concomitant immunosuppressive regimen. Concomitant administration of SRL and CsA was associated with higher dose-normalized SRL blood trough levels than those measured under TRL- or MMF-based regimens. These findings indicate potential pharmacokinetic interaction between different immunosuppressants. They confirmed drug interactions previously reported in animal models [125-127], and in humans [128], in which CsA was found to significantly increase SRL blood levels.

Different mechanisms may underlay this drug interaction. CsA and SRL share common transport and metabolic pathways, thus, both drugs may compete for the binding to P glycoprotein and for the metabolism by intestinal and hepatic CYP3A4 enzyme [129]. Because the molar quantity of CsA in a typical daily dose far exceeds that of SRL (>10 times), it might be expected that CsA preferentially interacts with CYP3A4 and thus inhibits SRL metabolism [35]. In addition, CsA acts not only as substrate but also as inhibitor of CYP3A4 [130]. Both these mechanisms explain the high normalized SRL trough levels we found when the drug was given as part of a CsA based immunosuppressive regimen. Conversely, TRL is only a substrate but not an inhibitor of CYP3A4 [130]. Moreover, therapeutic blood concentrations of TRL are in the molar quantity close to those of SRL. This implies that one molecule of TRL may compete with one molecule of SRL for CYP3A4. Thus, the likelihood of TRL to interact with this enzyme is similar to that of SRL, but far lower than that of CsA. This ultimately may explain why the influence of TRL on SRL metabolism, if any, is less evident. This is further supported by studies in liver and kidney-pancreas transplant recipients showing that neither pharmacokinetic profiles of SRL nor those of TRL were altered by

simultaneous administration of the two drugs [131]. Similarly, Ciancio et al have recently shown that dose-normalized SRL trough levels, expressed as bioavailability index, were significantly higher in patients receiving CsA, than in those given TRL [128].

Intestinal CYP3A4-mediated drug metabolism, P-gp expression, and cell membrane permeability are factors that most likely affect the bioavailability of SRL, CsA and TRL. It is therefore possible that the drugs interact not only at hepatic level but also at the small intestinal level where drug absorption occurs. Experimental studies in rats have indeed shown marked pharmacokinetic interactions when SRL and CsA were administered simultaneously by gavage but not via continuous intravenous infusion, suggesting that the gastrointestinal surface may be an important site of drug interaction [127]. Moreover, in healthy volunteers given a single oral dose of SRL the rate and extent of SRL absorption and exposure were significantly affected not only after simultaneous but also after staggered drug administration with Neoral [132]. This was also true in kidney transplant recipients [133]. The finding that in our patients – who were given SRL dose 4 hours after CsA morning dose – SRL trough levels were still affected by CsA administration, confirm previous observations and implies that the interaction between the two drugs occurs not only in the liver but, at least in part, at the gastrointestinal level.

At variance with CsA, co-administration of MMF resulted in a significant reduction of dose-normalized SRL trough levels. Indirect evidence of mutual pharmacokinetic interaction between MMF and SRL is available in transplant patients. A multicenter clinical trial [96] has shown that, despite low MMF dose employed, patients treated with MMF and SRL have MPA (the active compound of the prodrug MMF) trough levels significantly higher than in patients treated with a calcineurin-inhibitor and normal MMF dose. Similarly, others have found in renal transplant recipients that MMF

may be given at lower dose than conventional when used in association with SRL in renal transplant recipients [134]. Both these studies provide clear evidence that SRL significantly affect MPA pharmacokinetics. Conversely, we found that co-administration of MMF and SRL resulted in a significant reduction of dose-normalized SRL trough levels. The mechanisms of MMF-SRL interaction, however, remain ill defined. The possibility exists that MPA, by affecting cytokine production [135], may induce the expression of P glycoprotein and/or CYP3A4, resulting in a significant reduction in dose-normalized SRL trough levels. Alternatively, but not mutually exclusive, MPA and SRL may interact at metabolic level. This is supported by the fact that a phase I metabolite of MPA produced by CYP3A4/5 (the same enzyme involved in SRL metabolism) has been recently identified [136], although in the past MPA was thought to be metabolized exclusively by phase II reactions.

Here we also documented that even steroids influence SRL trough concentration. So far, available data on the effects of steroids on SRL patient exposure are conflicting, with some studies [137] but not others [138] showing pharmacokinetic interactions between SRL and prednisolone. In vitro and in vivo evidence show that glucocorticoids increase the expression and activity of CYP3A4 [139,140]. These findings raise the possibility that steroids activate pathways of SRL metabolism, eventually leading to a lower exposure than expected to SRL. This was indeed what we found in the present study. The addition of steroids to a dual therapy regimen of CsA and SRL resulted in a significant reduction in dose-normalized SRL trough levels; the same trend was observed when this class of drugs was associated with MMF and SRL. Conversely, the combination of steroids with TRL and SRL, did not result in a significant reduction in dose-normalized SRL concentrations. Several confounding factors may have, however, influenced the latter observation. Indeed, it has been recently shown that steroids influence TRL pharmacokinetics [141], suggesting a complex scenario of interactions

between TRL, steroids and possibly SRL. Moreover, our patients on triple therapy with steroids, TRL and SRL had higher degree of renal dysfunction (measured as serum creatinine levels) than those given only TRL and SRL, which could have resulted in SRL accumulation and higher than expected SRL blood levels. The accumulation of SRL due to renal dysfunction may have ultimately masked the possible trend to lower SRL levels secondary to TRL/SRL pharmacokinetic interaction.

We then investigated whether the distribution of SRL trough levels in patients given the drug with or without CsA, meets the registration guidelines for SRL target blood ranges [35,40,123,142]. This was feasible since our samples did belong from different Transplant Centres, each with a given drug combination and SRL blood target protocol for routine drug monitoring. The recommended therapeutic window for SRL trough levels, when the drug is given concomitantly with a calcineurin inhibitor, is 5-15 ng/mL [35,40]. More than 70% of the samples assayed in our study fell within this range, although most of them were in the lower 5 to 10 ng/ml window. Two double blind randomized clinical trials [123,142] have recently shown that CsA can be safely discontinued three months after kidney transplantation, provided that SRL dose is increased to target blood trough levels of 20 to 30 ng/mL (if measured by immunoassay) [123] or of 10 to 20 ng/mL (if assayed by HPLC) [142]. Such difference in the target range relates to the fact that the immunoassay gives cross-reaction with SRL metabolites resulting in a significant overestimation [143]. Here we showed by HPLC that most of patients who 'per protocol' discontinued CsA had SRL trough levels lower than the recommend blood window of 10 to 20 ng/mL or more close to the expected 5 to 15 ng/mL range for triple therapy including CsA. These findings underline the tendency of most transplant physicians to underdosing SRL in the dual immunosuppressive regimen of SRL and steroid, when CsA has been withdrawn from the initial triple therapy. While this strategy may still be safe in respect to the risk of

acute rejection and/or on long-term graft outcome, formal data derived from *ad hoc* designed study are not yet available. Therefore, they should go forward warily in adopting this SRL regimen without a clear rationale.

As previously reported [144], SRL levels exhibited a great intra-patient variation that further supports the need of therapeutic drug monitoring for this novel immunosuppressant. A correct drug monitoring approach should rely on reliable methods to assess drug level and on adequate tools to calculate the drug dose required to obtain a given drug level. Here we described and provided evidence of reliability of a prediction equation to calculate the new SRL dose required to target a given therapeutic range, when adjustment of SRL dosing is needed to meet clinical requirements. To our knowledge, this is the first attempt to provide such a tool for the improvement of SRL monitoring in transplant patients.

The present study has certainly some shortcomings. This is a retrospective analysis of the available data of SRL trough levels provided by different transplant Units. They, however, did not include doses and levels of the other immunosuppressive agents co-administered with SRL. With this limitation, it should be pointed out that so far data on the effects of currently used immunosuppressive agents on SRL trough levels are scanty. This is the first demonstration that not only CsA and TRL, but also MMF and steroids may affect SRL blood levels in kidney transplant patients.

An additional shortcoming is that the apparent concentration of the drug measured in the blood, as in our study, may not accurately reflect the drug level in the lymphoid organs and in the graft. Alternative approaches have been recently proposed to tailor the best immunosuppressive regimen. For instance, pharmacodynamic monitoring involves measurement of the biological effect of the drug at its target site [15], and pharmacogenomics, a genome-wide approach aimed at identifying the network of genes that govern an individual's response to drug therapy [41]. These novel strategies hold

many promises for the future. However, they will never replace traditional therapeutic drug monitoring completely but, rather, act as complementary fields resulting in a better patient management.

Prospectively designed multicenter clinical trials are now needed to assess whether SRL pharmacokinetic changes related to drug-drug interaction would translate to a different risk of rejection or graft outcome.

**Legend to Figures**

*Figure 25.* Frequency distribution of the dose-normalized SRL trough levels in patients treated with SRL and with- (panel A), or without CsA (panel B). Shaded areas represent SRL therapeutic windows, according to the immunosuppressive therapy (panel A: 5-15 ng/mL; panel B: 10-20 ng/mL).

*Figure 26.* Linear regression plot between SRL levels measured with a HPLC method and predicted with a proposed formula in 82 patients who underwent 186 modifications of SRL dose.

*Figure 27.* Bland and Altman plot for measured and predicted SRL concentrations. The percentage difference from the measured SRL concentration is plotted against the mean SRL value. The mean percent difference is displayed by a bold solid line. Dotted lines represent $\pm$ 1 SD of difference.

- With CsA -

SRL Trough level (ng/mL)

- Without CsA -

SRL trough level (ng/mL)

- Figure 25 -

- Figure 26 -

Percent difference

Mean SRL Conc. (ng/mL.)
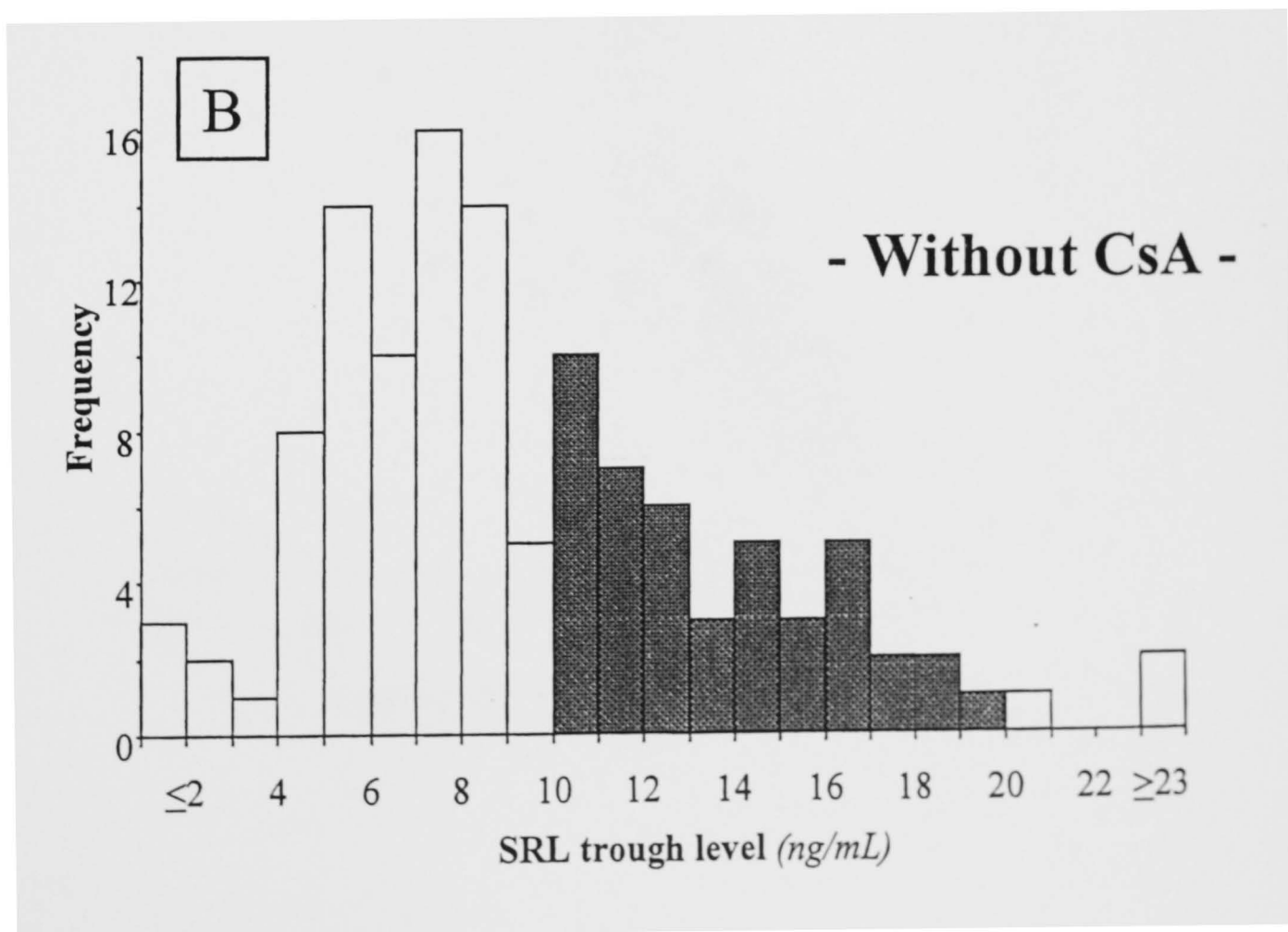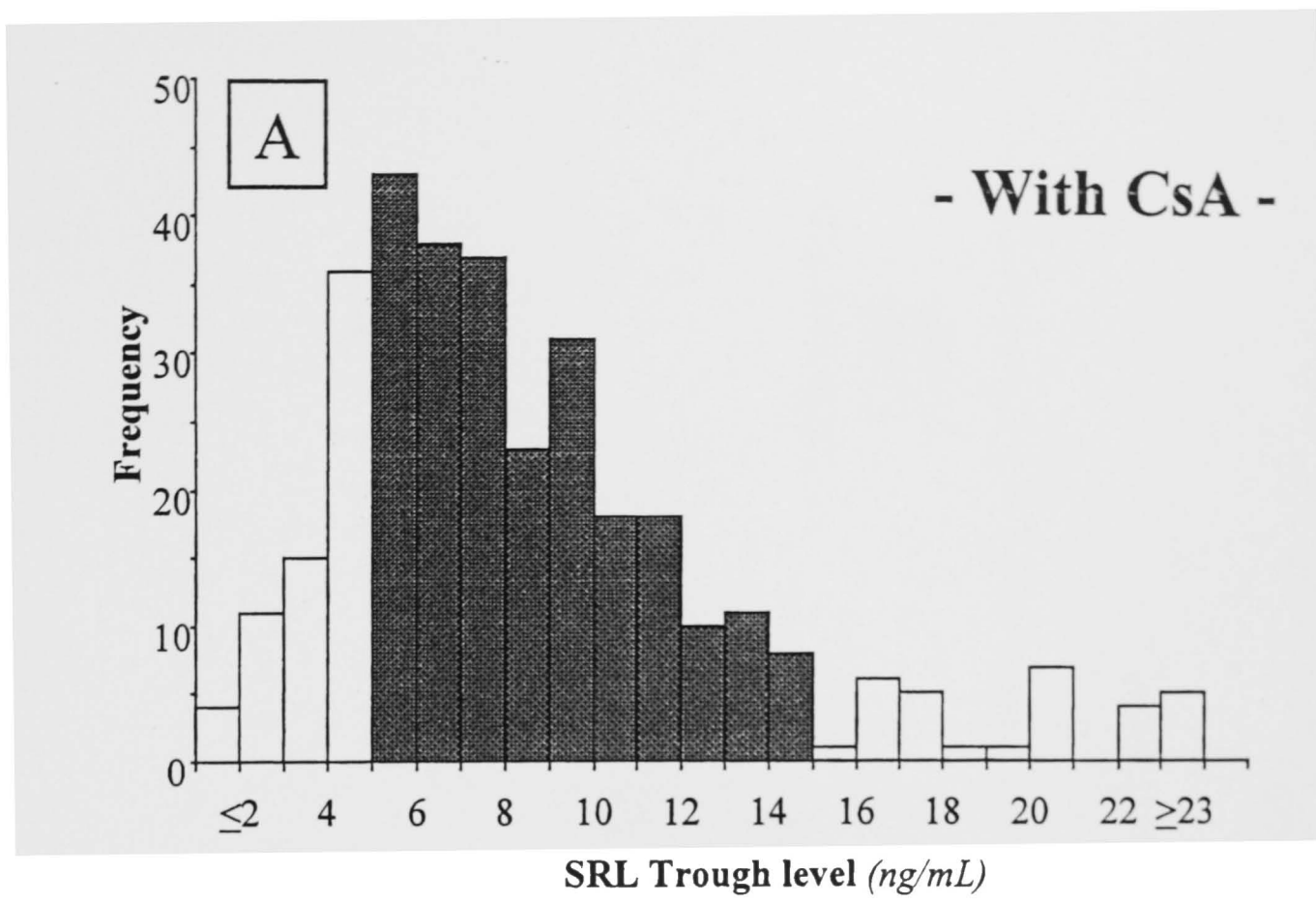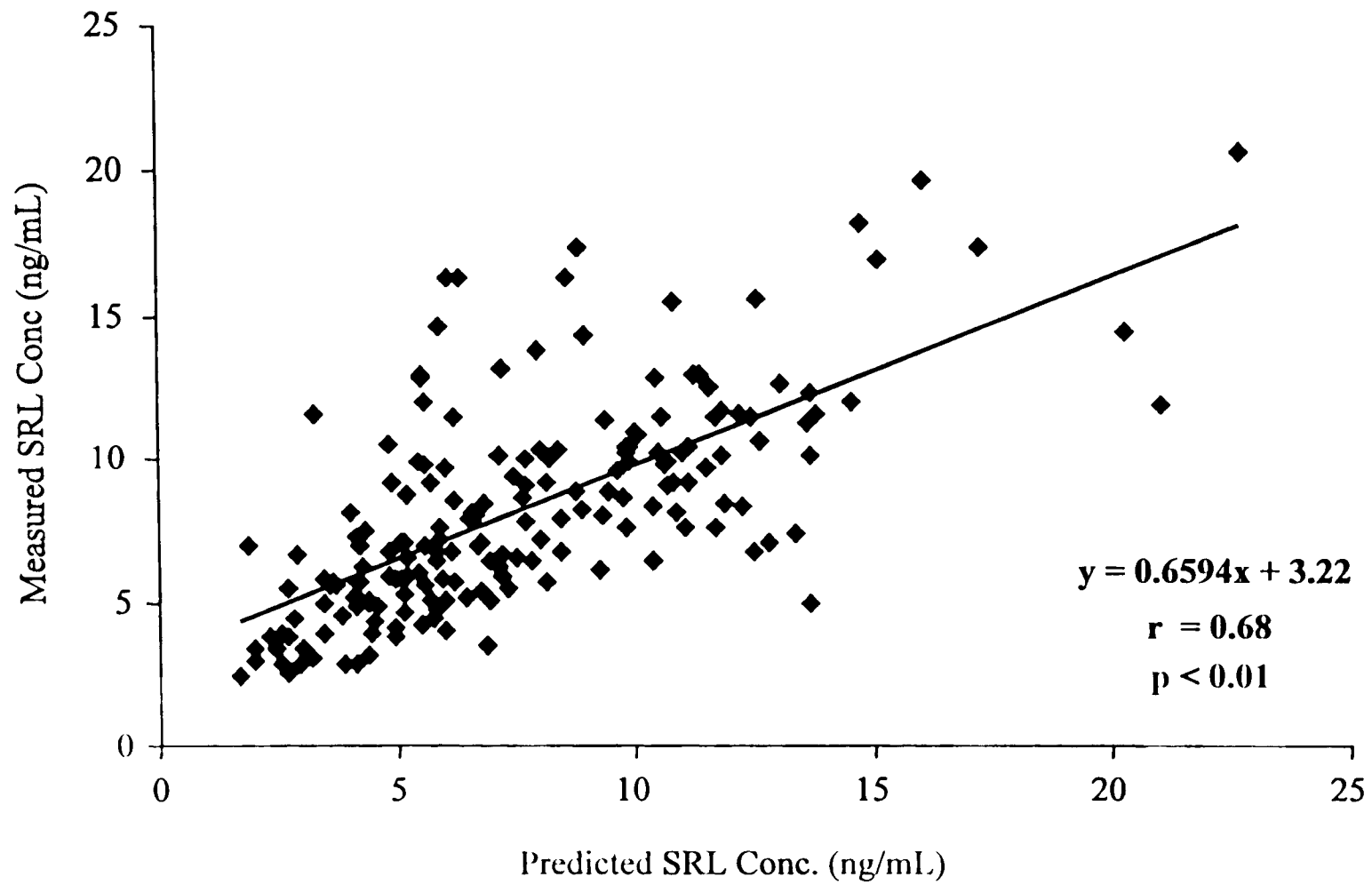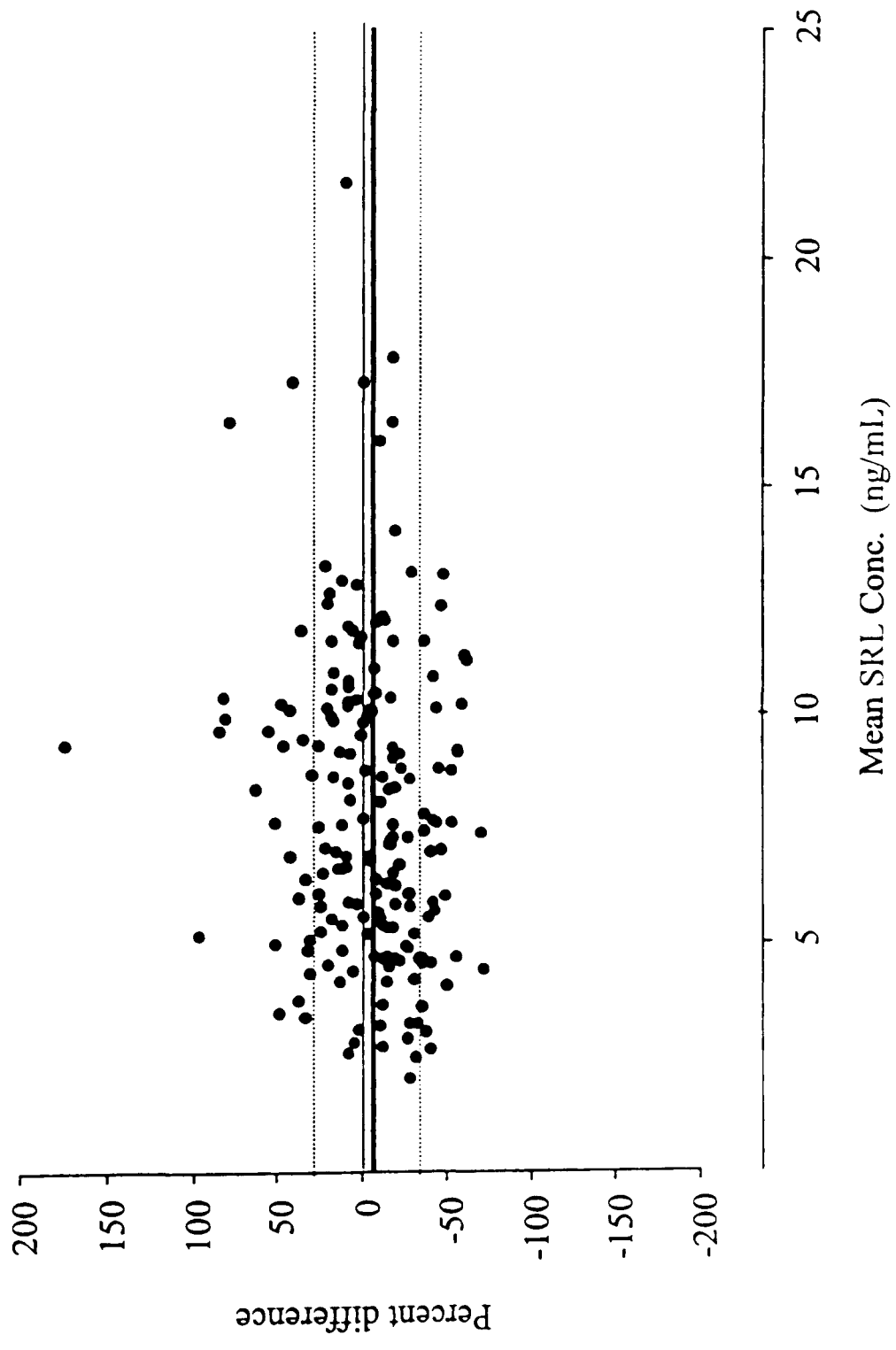
- Figure 27 -

Table 21. SRL blood trough levels according to the immunosuppressive regimens

| Therapy | SRL Dose (mg/day) | SRL $C_t$ (ng/mL) | $C_t$ / Dose (ng/mL/mg SRL) |
|---------|---------|---------|---------|
| SRL + CsA *(n=400)* | 2.7 ± 1.4 | 8.58 ± 4.38 | 3.77 ± 2.35 * ° |
| SRL + TRL *(n=128)* | 3.1 ± 1.5 | 8.09 ± 4.44 | 3.24 ± 2.16 |
| SRL + MMF *(n=102)* | 4.3 ± 1.6 | 10.57 ± 4.93 | 2.84 ± 1.81 |

* $p<0.01$ vs SRL+MMF
° $p<0.05$ vs SRL+TRL

Table 22. SRL blood trough levels according to different immunosuppressive regimens

| Therapy | SRL Dose (mg/day) | SRL $C_t$ (ng/mL) | $C_t$ / Dose (ng/mL/mg SRL) | S. Creat (mg/dL) |
|---|---|---|---|---|
| SRL + CsA + Ster (n=221) | 2.7 ± 1.7 | 7.92 ± 4.07 | 3.53 ± 2.45 | 2.21 ± 1.59 |
| SRL + CsA (n=165) | 2.6 ± 1.0 | 9.58 ± 4.66 | 4.15 ± 2.23 [a,b] | 2.03 ± 1.55 |
| SRL + CsA+ MMF (n=14) | 2.8 ± 1.3 | 7.21 ± 3.48 | 2.91 ± 1.52 | 2.16 ± 0.80 |
| SRL + TRL + Ster (n=98) | 3.1 ± 1.4 | 8.15 ± 4.34 | 3.26 ± 2.22 | 2.64 ± 1.08 [o] |
| SRL + TRL (n=30) | 3.1 ± 1.8 | 7.89 ± 4.84 | 3.18 ± 1.97 | 2.00 ± 0.69 |
| SRL + MMF + Ster (n=57) | 4.5 ± 1.1 | 10.54 ± 5.38 | 2.52 ± 1.73 [c,d] | 2.32 ± 1.09 |
| SRL + MMF (n=45) | 3.9 ± 2.0 | 10.60 ± 4.35 | 3.26 ± 1.86 | 2.16 ± 1.20 |
| SRL + Ster (n=59) | 3.1 ± 2.0 | 8.46 ± 3.77 | 3.85 ± 2.91 | 2.77 ± 1.19 [*] |

p<0.01 vs SRL+CsA+Ster, SRL+TRL+Ster   $^b$p<0.05 vs SRL+TRL, SRL+MMF, and SRL+CsA+MMF   $^*$p<0.01 vs SRL+CsA
p<0.01 vs SRL+CsA+Ster, SRL+CsA, and SRL+Ster   $^d$p<0.05 vs SRL+TRL+Ster   $^o$p<0.05 vs SRL+CsA

Table 23. Intra- and interpatient variability of SRL blood trough levels according to time post-surgery

| Time post-surgery | SRL Dose (mg/day) | SRL $C_t$ (ng/mL) | Intrapatient CV% | Interpatient CV% |
|---|---|---|---|---|
| 1 week (n=19) | 2.6 ± 1.0 | 7.2 ± 4.5 | 27% * | 63%° |
| 1 month (n=27) | 2.9 ± 1.2 | 7.5 ± 3.9 | 16% | 52% |
| 3 monts (n=21) | 2.9 ± 1.2 | 8.2 ± 3.9 | 15% | 48% |
| 6 months (n=29) | 2.6 ± 1.6 | 7.9 ± 3.9 | 17% | 49% |
| > 6 months (n=70) | 2.9 ± 1.6 | 8.2 ± 3.7 | 18% | 45% |
| Overall (n=166) | 2.9 ± 1.4 | 8.0 ± 3.8 | 19% | 47% |

* $p < 0.01$ vs month 3; $p < 0.05$ vs months 1, 6 and > 6    ° $p<0.05$ vs other groups

*Part IV*


# PHARMACOGENETIC-BASED THERAPEUTIC

# DRUG MONITORING: PRELIMINARY

# EXPERIENCE WITH CYCLOSPORINE

Immunosuppressive drugs are often associated with high risk of serious adverse events, including infections and cancer [145]. The narrow therapeutic index unique to each patient, as well as variable absorption, distribution and elimination properties give reason for individual response and risk of side effects related to the treatment with immunosuppressants. Therefore, periodic monitoring of plasma/blood concentrations of drugs is required. The goal is to maintain levels of immunosuppressive drugs within their therapeutic ranges, as variations outside these limits are often associated with adverse clinical outcomes or under treatment [64]. Moreover, as emphasized in the previous chapters, pharmacokinetic-based therapeutic drug monitoring may be helpful also to study and eventually predict potential drug-to-drug interactions. It should be pointed out, however that, although the extensive literature on immunosuppressive drug monitoring to guide therapy [17,64], there is still controversy amongst the effective impact of a concentration-controlled based immunosuppressive regimen [14], since in a significant proportion of patients acute rejection or drug-related toxicity occur despite drug levels fell within the therapeutic range [146]. Therefore, we are in need of novel strategies for a better management of transplant recipients, which could support the conventional TDM.

By examining the genetic factors that contribute to variability in drug response in individual patients, pharmacogenetics could provide a promising and complementary tool in this field. The field of pharmacogenetics could not have evolved without the development of new technological resources that allow scientists to access and process the enormous amount of information embedded in the human genome. Actually, the most diffuse technological advances are focused on single-nucleotide polymorphisms (SNPs), which form the backbone of molecular genetics technology. SNPs are common variations in the structure of a gene and consist of one-nucleotide alteration in the sequence of a gene [147]. It has been estimated that there are 30 millions of SNPs in the

human genome, accounting for 90% of all inter-individual variations. These genetic variations can result in altered catalytic activity of enzymes, with gain or loss of function by gene replication, gene deletion or gene miss-splicing. Certain SNP's will also exert a more indirect influence by altering genes or promoter regions that determine transcription and translation of the gene of interest. Thus, SNPs offer a potentially useful tool for pharmacogenomics to identify genetic markers that might be predictive of toxicity, side effects or lack of response to a drug. However, it should be pointed out that this approach is expected to be clinically relevant for those SNP's that are strongly linked to a corresponding phenotype [148], whereas those characterized by a bell-shaped distribution [149] and less evident phenotypes might have a limited clinical impact. These promising technologies could be useful tools to address the main challenge of pharmacogenetics: to predict which individual will benefit most from which drug. This task applies for all drugs but is of particular importance for those characterized by a NTI, such as the immunosuppressive agents that represent the ideal candidates for pharmacogenomic approaches.

Today, the key-point is to understand whether clinical pharmacogenetics is already applicable today to organ transplantation. Early observations focused simply on drug metabolism [150,151]. However, there is also great interest in the full spectrum of drug disposition, including absorption, distribution and pharmacological targets. One of the potential advantages of this type of approach lies on the common metabolic pathways for several immunosuppressive agents. CsA, TRL, SRL and the novel rapamycin-analogue everolimus are all mainly transported and metabolized by the cytochrome 3A-P-glycoprotein system [24]. Thus, the study of patient's genotype can provide predictive value for multiple drug therapy as in transplant patients. In particular, P-glycoprotein, a member of the ATP-binding cassette family encoded by the multidrug resistant gene (MDR1) regulates the cellular efflux of several substrates, including the

immunosuppressive drugs [152]. Several SNP have been identified for MDR1 gene, all potentially important in predicting the efficacy and/or toxicity of these drugs. The presence of variants in genes encoding for drug targets or other genetic polymorphisms with indirect effects on drug response are less known. Thus, they represent an exciting challenge for the future.

Following there are preliminary results of a study aimed at investigating whether the identification of SNPs in the MDR1 gene could be used as useful tool to predict drug exposure in organ transplant recipients. *Chapter 11* focused on CsA. However, it can be reasonably speculated that the same approach could be applied in the near future also for other immunosuppressants [41].

*Chapter 11*

# MDR1 POLYMORPHISMS IN EXON 26, BUT NOT 12, INFLUENCES INDIVIDUAL CYCLOSPORINE LEVELS IN KIDNEY TRANSPLANT RECIPIENTS

# Introduction

CsA is one of the main immunosuppressant currently used to prevent graft rejection, which has significantly contributed to improve allograft and patient survival [153]. This drug is, however, characterized by a narrow therapeutic index, an unpredictable absorption profile, and important side effects [11,154]. Given the appreciable inter-individual variation in blood CsA concentration despite the same drug dosage, major efforts have been devoted in the past to individualize CsA dose to maximize the efficacy and minimize toxicity of the treatment. To this purpose, several approaches have been proposed to monitor daily CsA exposure as useful tools to tailor drug dosage for each patient [20]. In particular, two blood sampling points, at time zero ($C_0$) or at 2 hours after CsA dosing ($C_2$) are currently used in the clinical practice [25]. However, this pharmacokinetic-based therapeutic drug monitoring is not without pitfalls. Indeed, some Authors failed to document a significant association between CsA pharmacokinetic parameters and patient's clinical outcome [14]. Therefore, complementary strategies have been advocated.

The advent of the genomic era has provided new insights into the molecular basis of human genetic disorders, and pharmacogenetics – a science that studies how the genome may affect the full spectrum of drug disposition – is undoubtedly a potential source of additional information, as complementary field to the traditional pharmacokinetics. In the past few years, large number of studies has shown that genetic polymorphisms in drug-metabolizing enzymes and transporters may predict drug efficacy as well as drug toxicity [23,41].

CsA disposition is mainly influenced by the P-glycoprotein (P-gp), the product of the MDR1 gene [155]. This protein is an efflux pump, which removes lipophilic drugs, like CsA, from the intracellular space. P-gp is mainly found in the hepatocytes, kidney

proximal tubular cells, and the brush border surface of enterocytes [155]. The function and the anatomic localization of P-gp suggest that this transporter acts as a protective barrier to keep toxins (drugs or xenobiotics) out of the body by excreting these compounds into bile, urine, and the intestinal lumen. To date, about 30 SNPs have been reported for the MDR1 gene [155]. However, only SNPs in exons 12, 21 and 26 were associated with lower P-gp expression/activity. SNP in exon 12 is silent, whereas SNPs at exon 21 result in two distinct amino acid changes, namely, Ala863Ser (G2677T) and Ala893Thr (G2677A). The silent SNP in exon 26 (C3435T) was the first variant to be associated with altered protein expression [156]. More recently, also SNPs in exons 12 and 21 were found to be associated with altered drug disposition [157,158].

Genetic polymorphisms of MDR1 gene in transplant recipients may therefore result in significant reduction in P-gp expression, eventually increasing the exposure of patients to immunosuppressants, such as CsA, with potential clinical consequences in terms of efficacy and adverse effects. So far, only few studies have investigated the impact of MDR1 SNPs on the bioavailability of immunosuppressive agents. Whereas studies focusing on TRL have consistently shown that genetic polymorphisms of the MDR1 gene in exons 12, 21 and 26 can predict drug bioavailability and toxicity [159], those dealing with CsA led to contradictory results [41,159], possibly due to the limited number of patients studied or inadequate pharmacokinetic monitoring.

Therefore, the present study was designed to assess whether single nucleotide polymorphisms in MDR1 gene (exon 12 C1236T and exon 26 C3435T), alone or in combination (as haplotypes and genotypes), were associated with variable CsA levels (at week 1 and at month 1, 3 and 6 after transplantation) in a large cohort of 120 kidney transplant recipients enrolled in the MYcophenolate Steroid-Sparing (MY.S.S.) Trial [38].

# Material and Methods

*Patients*

120 Caucasian patients (≥17 years old), enrolled in the MY.S.S. Trial - a prospective, randomized, multicenter, European study of first cadaver renal transplant recipients randomly allocated to receive azathioprine or mycophenolate mofetil in addition to the Neoral microemulsion formulation of CsA and steroids for the first six months post-surgery (for a detailed description of the study see reference [38]) – entered this pharmacogenetic study.

CsA was initially infused intravenously (on average 4 mg/kg daily) from the day of surgery (day 0) to day 3 and then, given orally twice a day (Neoral Novartis, Basel, CH). CsA dose was adjusted to maintain blood drug trough levels within 250-440 ng/mL from day 0 to 7, and within 200-300 ng/mL from day 8 to the end of the first month. Thereafter, CsA trough concentrations were targeted to 150-250 ng/mL up to 6 months postoperatively. Patients were randomly assigned to receive treatment with mycophenolate mofetil (1 g twice a day), or azathioprine (100 or 150 mg/day according to body weight ≤ or > of 75 Kg). Corticosteroid dosing early post-transplant was dictated by the participating centre's practice, whereas all patients received 16 mg/day of methylprednisolone from day 12 to 60 post-transplant tapered to 8 mg/day thereafter. None of the patients was given medications known to interfere with P-gp function (inducers or inhibitors). A control group of 100 healthy volunteers was also considered to assess the frequency distribution of MDR1 SNPs (exon 12 and 26). All study participants (kidney transplant recipients and controls) provided written informed consent for genetic testing.

*CsA pharmacokinetic parameters*

Whole blood samples were collected immediately before ($C_0$) and at 2 hours after CsA administration ($C_2$) daily within the first week after surgery, and every two weeks until the end of month 6 post-transplant. To limit intra-individual variability of CsA values, blood drug concentration was calculated as the mean of three replicate measurements by HPLC, using a validated method described in *Chapter 5*. Each CsA concentration was adjusted for the corresponding 12-h drug dose, expressed as mg/kg body weight. CsA exposure, defined by the area under the time-concentration curve ($AUC_{0-12}$), was estimated using the two-point equation proposed by Keown et al [160], as follows: $AUC = 12.34 \times (C_0) + 2.48 \times (C_2) + 441.42$. The apparent clearance of CsA was calculated as the ratio between CsA dose and CsA AUC.

*DNA collection and isolation*

Blood from kidney transplant recipients and healthy volunteers was drawn from an anticubital vein into vacutainer containing $K_3EDTA$. One vacutainer was collected for each patient (4 mL of whole blood), and the samples were placed at -20°C. For genotype determination, genomic DNA was isolated from EDTA-anticoagulated whole blood using the Nucleon BACC2 kit (Amersham, Biosciences, Buckinghamshire, UK). According to the Manufacturer instruction, in order to minimise damage to DNA in collected blood samples, blood should be extracted within 8-12 months of collection. All the samples used for the present study were processed within one month of collection. Using as aseptic procedure we added 4 times the volume of reagent A to the blood sample, then we rotary mixed 4 minutes at room temperature and centrifuged at 3000 RPM for 8 min. The supernatant was discarded and to the pellet we added 2 mL of reagent B. We vortexed briefly to resuspend the pellet and transferred the suspension to a 15 mL screw capped propropylene centrifuge tube. Following resuspension and

transfer to a clean tube we added 3 µL of a 10 µg/mL RNase solution and incubated the tube in a water bath at 37°C for 30 minutes. Subsequently we added 500 µL of sodium perchlorate solution (for the deproteinisation), mixed by hand, inverting the capped tube at least 7 times. For DNA extraction 2 mL of chloroform was added and each sample was mixed by hand, inverting the capped tube at least 7 times. Without remixing the phases we added 300 µL of Nucleon resin and centrifuged at 2800 RPM for 5 min. For DNA precipitation, we transferred the upper phase (holding the tube vertically, without disturbing the brown Nucleon resin layer) to a clean tube of minimum volume 7.5 mL and added 2 volumes of cold absolute ethanol. The tube was mixed by inversion until the DNA precipitate appeared. For DNA washing, we centrifuged at top speed for 5 min to pellet the DNA and discarded the supernatant. We added 2 mL cold 70% (v/v) ethanol, mixed several time by inversion, re-centrifuged and discarded again the supernatant (this step can be repeated if necessary). Finally, we aired dry the pellet for 10 min (ensuring that all the ethanol has been removed), re-dissolved DNA in an appropriate volume of water or TE buffer (TRIS/HCl and EDTA). The DNA should re-dissolve within 2 h when using a rotary mixer.

## Genotyping

MDR1 exon 12 (C1236T) and exon 26 (C3435T) SNPs were considered in the present study. The genotypes of MDR1 were identified by a Polymerase Chain Reaction-Single Stranded Conformational Polymorphism analysis (PCR-SSCP). This analysis is based on two steps. First the DNA sequence of interest is PCR-amplified, and second, the amplified DNA is heat-denaturated and size-fractionated by native polyacrylamide gel electrophoresis (native PAGE). After heat denaturation the mobility of single-stranded DNA fragments is size-and sequence-dependent with single-stranded DNA molecules adopting secondary structure conformations by intramolecular base pairing. For a given

double-stranded fragment there will be two bands identified following SCCP, one corresponding to each of the two original DNA strands. If the two fragments differ by as little as single base pair the denaturated strands are likely to adopt different conformations and therefore to be distinguishable following native PAGE. The difference is identified by a shift in mobility of one or both of the mutant bands relative to the wild-type control strands. The migration of single-stranded DNA and the conformational changes are influenced by the percentage of acrylamide, the electrophoresis temperature and the ionic strength of the electrophoresis buffer. Therefore, before to analyse our samples, I worked to identify the appropriate and reproducible conditions for each of the given fragments from exon 12 and 26.

*PCR-SCCP*

PCR is a process that can amplify minute amounts of nucleic acid, thus generating ample material for further analysis. Subsequently PCR products are analyzed by gel electrophoresis for size separation. Scan for sequence alterations were performed using SCCP and confirmed by direct sequencing. Conventional PCR thermocycling is based on three steps: denaturation (separation of the complementary strands), annealing (the oligonucleotide primers hybridize to the template) and extension (polymerization of the fragment of interest), as three separate reactions that occur after reaching equilibrium at defined temperatures.

For the PCR we need two oligonucleotide primers for each of the exons, which act as sites for initiation of the replication, and the DNA polymerase enzyme, for the amplification of the region of the template DNA that will be copied.

The sense primer: 5'-AGTCAGTTCCTATATCCTGTGTCTGTGA-3' and antisense: 5'-GCAGTCACATTGCACATCTTTCT-3' for exon 12 as well as sense primer: 5'-GACTGCAGCATTGCTGAGAACA-3' and antisense: 5'-

AATTTCTCTTCACTTCTGGGAGACC-3' for exon 26 (Sigma-Aldrich, UK) were designed using the Gene-Jockey Software. For DNA polymerization we used AmpliTaq Gold (PE Applied Biosystems, Foster City, CA, USA), which is a thermostable DNA polymerase. Upon receipt, the AmpliTaq Gold and reagents (GeneAmp PCR Buffer and $MgCl_2$ solutions) were stored at -20°C.

PCR reaction was performed in a 20 µL volume, containing 100 ng DNA, 15 pmol of each primer, 16 nmol deoxynucleoside triphosphates (dNTP), 2.25 mmol/L magnesium chloride, 1 U AmpliTaq Gold polymerase. The PCR thermal cycling conditions for exon 12 and 26 consisted of a 10 min denaturation at 94°C, then 35 PCR cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 45 s, followed by a 10 min extension at 72°C. Reaction products were mixed with 20 µL of loading buffer, denatured at 65°C for 10 min and electrophoresed onto non-denaturing 6% (62/1 acryl/bis) acrylamide gel in TAE buffer (pH 6.8) at 35 Watt for 3-5 h at 4°C. SCCP bands were visualized on the gels by silver staining.

Direct sequencing (ABI 377 sequencer, 8000CEQ Beckman Coulter, Fullerton, USA) was used to confirm the results in three individuals (one homozygous, one heterozygous, and one homozygous mutated on SCCP) for each of the two exons, used as controls for genotyping. The different genotypes for C1236T and C3435T SNPs were classified as follows: wild type (C/C), heterozygous (C/T) and homozygous for the allelic variant (T/T).

*Haplotype and combined genotype analysis*

Haplotype and combined genotype analysis included the SNPs 1236C>T and 3435C>T. The term haplotype was originally referred to indicate set of genes that are closely linked, however, it has been also recently used to consider different SNPs allocated in the same gene [161]. Here, haplotype analysis included the SNPs C1236T and C3435T.

Each genotype was assigned a haplotype pair. With the assumption that each haplotype is predominantly inherited, comparisons were performed between carriers and noncarriers of a given haplotype. For haplotype coding "1" is referred as "identical to the reference sequence (1236C and 3435C) and "2" as "different from the reference sequence". The first digit refers to position 1236, the second digit refers to position 3435. For genotype coding "0" refers as "homozygous identical to the reference sequence (1236C, 3535C), "1" as "heterozygous" and "2" as "homozygous different from the reference sequence". Different allelic combinations of both variants of SNPs 1236 and 3435 can result in four possible haplotypes and nine possible genotypes, as shown in Table 24.

*Statistical Analysis*

For analysis of continuous pharmacologic variables, patient genotypes were used as categorical independent variables. The values of CsA pharmacokinetic parameters are expressed as the mean ± standard deviation (SD). Between-group comparisons for MDR1 SNPs and genotypes were performed by one-way ANOVA using, as the multiple comparisons post-test, the Bonferroni test. P value less than 0.05 was considered statistically significant.

# Results

*Frequency of MDR1 variants in renal transplant patients*

The genotype and allelic frequencies for exon 12 and 26 are shown in Table 25. Of the 120 kidney transplant recipients, 35% had the MDR1 wild type genotype in exon 12 (C/C), whereas 42% were heterozygous (C/T) and 23% were homozygous for the polymorphic variant (T/T). The frequency of MDR1 SNP in exon 26 (C3435T) was 39.0% (C/C), 42% (C/T), and 19% (T/T). These frequencies were in agreement with those observed in a control population of healthy subjects (Table 25) and in the available literature [156,158,162].

*Effect of MDR1 SNPs on CsA pharmacokinetics*

We first examined the potential relationship between each MDR1 SNP and CsA pharmacokinetic parameters. As shown in Table 26, there was no significant difference in dose-adjusted CsA levels among the different exon 12 genotypes (C1236T) both in the early phase (week 1) and in the stable phase (first month) post transplantation. Similarly, no difference was found at the end of month 3 and 6 post surgery. These results were confirmed also when patients carrying the wild-type exon 12 genotypes were compared with those bearing at least one mutant allele (C/C vs C/T + T/T, data not shown).

At variance, mean CsA $C_0$ and $C_2$ concentration/dose ratio correlated with exon 26 C3435T SNP (Table 27). Indeed, dose-adjusted CsA levels were significantly lower in patients with the wild-type genotype than in those carrying one or two mutants alleles at 1, 3, and 6 months post transplantation (Table 27). This pattern was confirmed also considering the $AUC_{0-12}$ as surrogate marker of daily CsA exposure.

Since it has been previously shown that the T allele was associated with reduced P-gp activity [156], we also compared CsA pharmacokinetic parameters between wild-type subjects (C/C) and those with at least one mutant allele for exon 26 (C/T + T/T). At month 1 post surgery, dose-adjusted CsA $C_0$ and $C_2$ levels were significantly lower in wild-type patients than in those carrying the mutant allele (CsA $C_0$: 35 ± 14 ng/mL/mg/kg *vs* 46 ± 15 ng/mL/mg/kg, p<0.001; CsA $C_2$: 191 ± 80 ng/mL/mg/kg *vs* 238 ± 89 ng/mL/mg/kg, p<0.001, Figure 28). The influence of exon 26 C3435T SNP was confirmed also when CsA AUC and oral clearance (CL) were considered (CsA $AUC_{0-12}$: 6193 ± 1470 ng*h/mL *vs* 6874 ± 1294 ng*h/mL, p=0.0121; CsA CL: 9.1 ± 5.3 mL/min/kg *vs* 7.6 ± 4.7 mL/min/kg, p=0.0125, Figure 29).

*Haplotype analysis*

We then examined by haplotype analysis whether the combined SNPs in exon 12 and 26 were predictive of CsA exposure. Different allelic combinations of both variants of SNPs 1326 (exon 12) and 3435 (exon 26) resulted in nine possible genotypes and 4 haplotypes (Table 24). All possible genotypes and haplotypes were detected in the study population. CsA pharmacokinetic parameters were compared between carriers of different SNP variants and different genotypes derived from haplotype pairs, as well as between carriers and noncarriers of each haplotype. As shown in Table 28, at week 1 and month 1 post-transplant patients homozygous wild-types for both SNPs carrying the genotype 22 (TT1236, TT3435) had comparable CsA levels to those with the genotype 00 (CC1236, CC3435), homozygous for the variants in exons 12 and 26. Similarly, no significant differences were observed between genotype 01 (CC1236, CT3435) and genotype 21 (TT1236, CT3435), and between other genotypes. Only a patient carrying the 02 genotype (CC1236/TT3435) showed dose-adjusted CsA trough blood levels 2 to 3-fold higher than with all other genotypes, both at week 1 and at month 1 post-

transplant. Together these findings confirmed that SNPs in exon 12 were not predictive of CsA concentrations even when considered together with SNP in exon 26. No significant difference was observed between different haplotype carriers, suggesting that this analysis may be of poor clinical relevance for the prediction of CsA levels, at least when exons 12 and 26 are considered.

## Discussion

The present study shows that in 120 kidney transplant recipients CsA pharmacokinetic parameters significantly correlated with exon 26 (C3445T), but not with exon 12 (C1236T) SNP of the MDR1 gene. Patients carrying at least one variant allele of this gene had higher dose-adjusted CsA $C_0$, $C_2$ and AUC levels than wild-type subjects. This effect was marginal in the first week post-transplant, but became highly significant in the more stable phase postoperatively, namely at months 1, 3 and 6.

P-glycoprotein (P-gp), the product of the MDR1 gene, is an efflux transporter highly expressed in the enterocyte, and, to a lesser extent, in the liver, kidneys, brain, testis, muscle and adrenals [155]. Its absence or altered expression results in higher than normal absorption of a given drug from the intestine and its accumulation in different tissues, an effect of great relevance for narrow therapeutic index agents, such as CsA [41]. It has been previously shown that some SNPs in the MDR1 gene affect P-gp expression/activity [156]. In particular, patients carrying at least one variant allele in exon 26 of the MDR1 gene (C3435T) had marked reduction in P-gp expression [156]. Since this glycoprotein offers resistance to drug crossing from the enterocyte to the bloodstream, patients with impaired P-gp expression are at risk of being exposed to drug levels higher than expected for a given drug dose, as early documented for digoxin [156].

This could have been occurred in our patients who were given CsA as part of their immunosuppressive therapy initially as i.v. infusion and then orally from day 4 post-surgery. This possibility is supported by the finding that MDR1 SNP in exon 26 was significantly related to CsA levels only later on in a more stable phase, but not in the early post-transplant period. Early postoperatively, when the patients were receiving CsA intravenously, the contribution of MDR1 to CsA exposure by the modulation of

intestinal drug absorption would be negligible. Moreover during the first few days of oral CsA administration, CsA concentrations might be influenced by environmental non-genetic factors, more than MDR1 gene polymorphisms, such as the normalization of gastrointestinal motility, the level of graft function, and the pharmacokinetic interactions with co-administered drugs [17]. Only later, when gastrointestinal function normalizes, graft function stabilizes, and concomitant therapy is fixed, the variants in MDR1 gene become relevant in regulating patients' exposure to CsA by affecting P-gp expression/activity and eventually drug absorption.

So far, only few studies have investigated the impact of MDR1 SNPs on the bioavailability of CsA, and most of them with contradictory results. Early pharmacogenetic studies failed to document an association between MDR1 exon 26 SNP and CsA pharmacokinetic parameters in healthy volunteers [163], as well as in stable renal and heart transplant recipients [164-166]. However, in some of these studies a trend, although not significant, of higher CsA values in subjects carrying C/T and T/T in exon 26 as compared with the C/C group was reported. Therefore, it can not be excluded that the low number of patients considered, weakness in study design and in pharmacokinetic parameters considered, may have biased the results. At variance with these studies, we have prospectively followed a large cohort of kidney transplant recipients, monitored immediately after surgery and in a more stable phase, considering both the traditional $C_0$-based sampling time and the recently proposed $C_2$-based monitoring, together with a predicted AUC, as surrogate marker of daily CsA exposure. Of note, to reduce intrapatient variability, CsA levels were calculated as the mean of three consecutive measurements and adjusted to the daily CsA dose. These approaches led us to limit the influence of potential confounding factors, and underline the role of MDR1 exon 26 C3435T polymorphism as a major determinant of CsA concentrations. Our findings are in agreement with those recently observed in a small cohort of liver

transplant recipients given CsA as a part of the immunosuppressive regimen [167]. As further support to our results, different studies have documented a significant influence of polymorphisms of the MDR1 gene on tacrolimus bioavailability [159]. Indeed, it has been found that SNPs in exon 21, and 26 were positive predictors of tacrolimus exposure and drug toxicity in liver [168], heart [169] and kidney [158] transplant recipients. As far as exon 26, these studies confirmed that patients carrying at least one T allele had dose-adjusted tacrolimus levels significantly higher than wild-types.

In the past few years, it has been hypothesised that haplotypes, by considering more SNPs together, may provide more information than individual SNPs do. Studies in heart [27] and kidney [28] transplant recipients have shown that CsA levels significantly correlated with the MDR1 haplotype, suggesting that haplotypes, in addition to single SNP genotypes, could influence CsA disposition. Others [170,171], however, argued against this conclusion, showing that MDR1 haplotypes did not affect CsA pharmacokinetics. In our study population four different haplotypes and nine different genotypes were found. Analysis that consider together SNPs from exon 12 and 26, found no statistical differences between carriers of different MDR1 haplotypes as far as pharmacokinetic parameters. Nevertheless, among the 120 studied patients, one was homozygous CC for exon 12 and TT for exon 26 (genotype 02). This patient had the highest mean dose-adjusted $C_0$ values at the end of first week and first month post-surgery compared to other genotypes. Although we can not exclude that this observation could be biased by several confounding factors (i.e. gastrointestinal disorders, altered CsA metabolism, etc), this atypical pattern merits further investigations, as it might potentially affect CsA pharmacokinetics and ultimately clinical outcome.

In conclusion, the present study has shown that patients carrying mutant alleles for exon 26, but not exon 12 of MDR1 gene, are exposed to higher CsA levels compared to wild-type subjects, with potential increased risk to experience drug-related toxicity. The

identification of variant MDR1 alleles performed before transplantation, while patients are in the waiting list, could provide useful information to tailor CsA dose as early as possible after transplantation, with the ultimate goal to improve efficacy, decrease toxicity and increase long term graft survival.

**Legend to Figures**

*Figure 28.* Correlation between exon 26 SNP (C3435T) and dose-adjusted CsA levels ($C_0$ and $C_2$) recorded in 120 kidney transplant recipients 1 month after surgery. The box plot showed CsA concentrations, clustered according to the allelic variation in MDR1 exon 26 (pure wild-type versus patients with at least one mutant allele).

*Figure 29.* Correlation between exon 26 SNP (C3435T), CsA $AUC_{0-12}$ and CsA oral clearance recorded in 120 kidney transplant recipients 1 month after surgery. The box plot showed CsA pharmacokinetic parameters. clustered according to the allelic variation in MDR1 exon 26 (pure wild-type versus patients with at least one mutant allele).

# MDR1 exon 26 (C3435T)
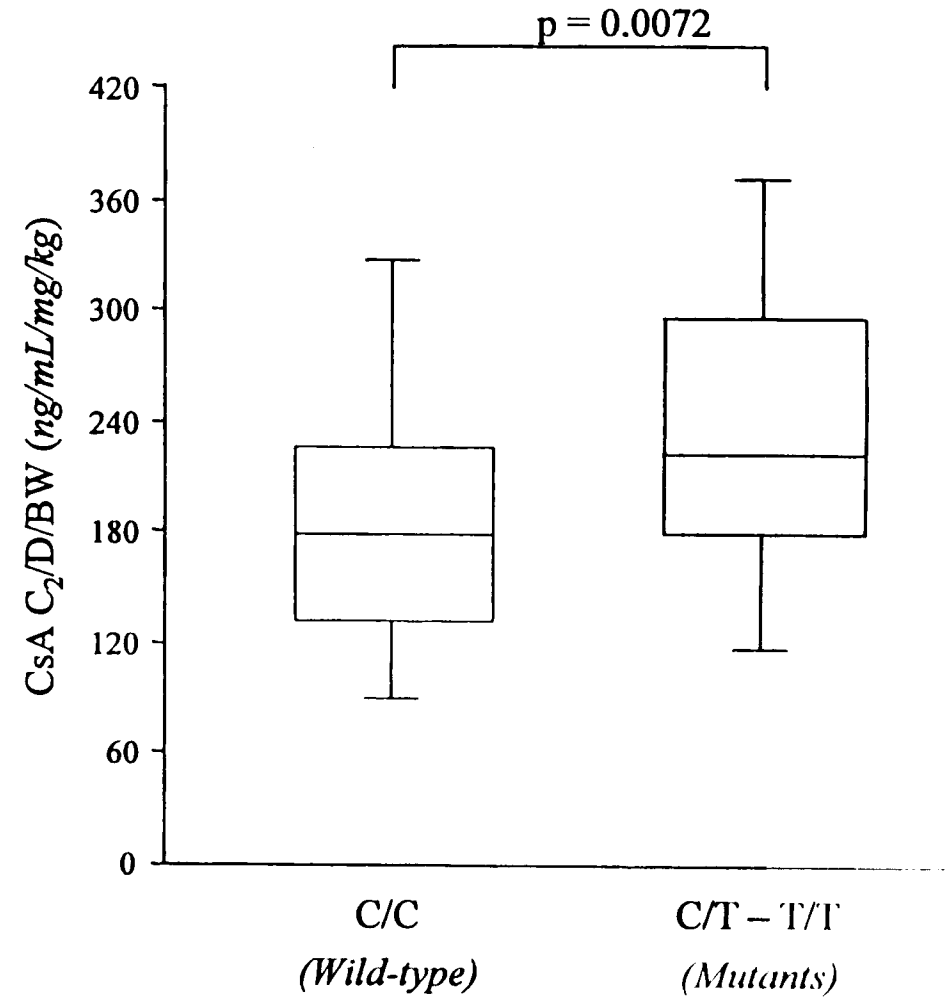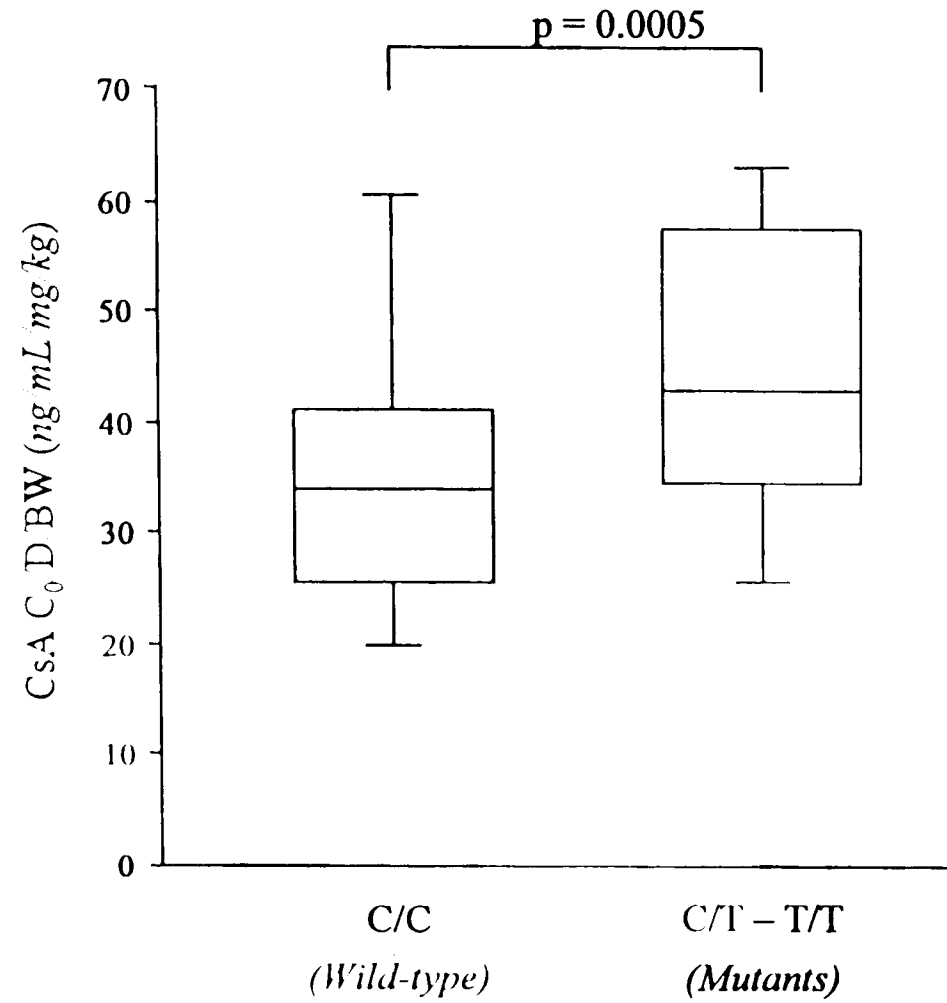


- Figure 28 -

MDR1 exon 26 (C3435T)

- Figure 29 -

Table 24 :Nine genotypes and four haplotypes of MDR1 derived from SNP C1236T (exon 12) and SNP C3435T (exon 26)

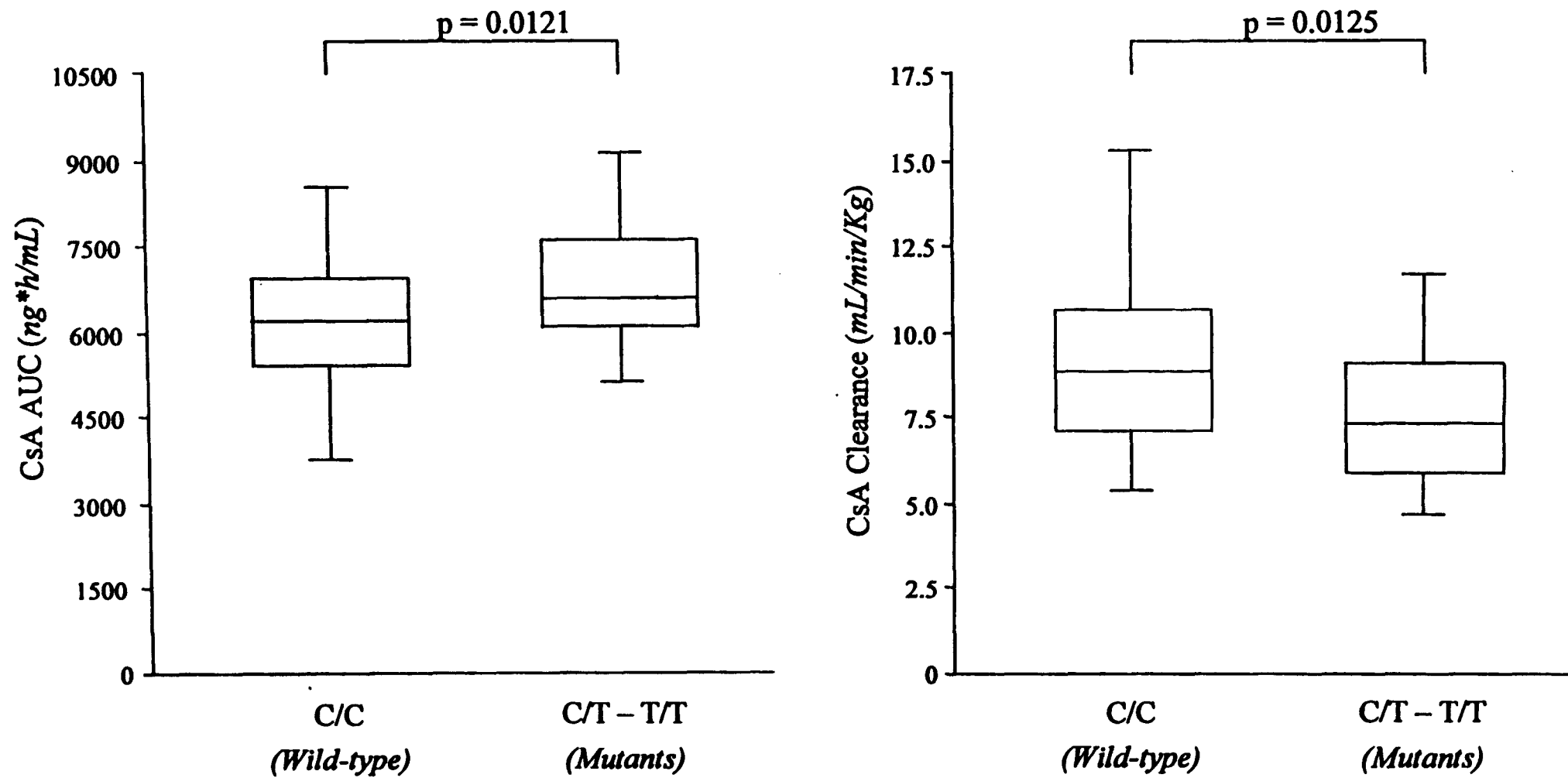| | Genotype 00 | | Genotype 01 | | Genotype 02 | | Genotype 10 | | Genotype 11 | | Genotype 12 | | Genotype 20 | | Genotype 21 | | Genotype 22 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pos 1236 | C | C | C | C | C | C | C | T | C | T | C | T | T | T | T | T | T | T |
| Pos 3435 | C | C | C | T | T | T | C | C | C | T | T | T | C | C | C | T | T | T |
| *Haplotype* | 11 | 11 | 11 | 12 | 12 | 12 | 11 | 21 | 11 | 22 | 12 | 22 | 21 | 21 | 21 | 22 | 22 | 22 |

Genotype coding: 0, homozygous identical to the reference sequence (1236C, 3435C); 1, heterozygous; 2, homozygous different from reference sequence. The first digit refers to position 1236, the second digit to position 3435. Haplotype coding: 1, identical to the reference sequence (1236C, 3435C); 2, different from reference sequence. The first digit refers to position 1236, the second digit to position 3435. For genotype 11 a second haplotype pair (12/21) is possible, but haplotype pair 11/22 is much more likely based on previous haplotype frequencies.

Table 25: Frequencies of the two SNPs on MDR1 gene evaluated in renal transplant recipients and in healthy controls

| | Exon 12 (C1236T) | | | Exon 26 (C3435T) | | |
|---|---|---|---|---|---|---|
| | C/C | C/T | T/T | C/C | C/T | T/T |
| Patient genotype (n=120) | 42 (35.0%) | 50 (41.7%) | 28 (23.3%) | 42 (35.0%) | 49 (40.8%) | 29 (24.2%) |
| Control genotype (n=100) | 30 (30.0%) | 44 (44.0%) | 26 (26.0%) | 31 (31.0%) | 51 (51.0%) | 18 (18.0%) |

Table 26 :distribution of CsA pharmacokinetic parameters in 120 kindey transplant recipients according to MDR1 exon 12 genotype (C1236T)

| CsA pharmacokinetic parameters | MDR1 exon 12 genotype | | |
|---|---|---|---|
| **Week 1 post-TX** | C/C | C/T | T/T |
| Mean $C_0$ (ng/mL) | 252 ± 87 | 253 ± 75 | 265 ± 114 |
| Mean $C_2$ (ng/mL) | 1139 ± 352* | 1404 ± 455 | 1402 ± 482 |
| Mean dose (mg/day) | 511 ± 130 | 486 ± 110 | 501 ± 150 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 32 ± 13 | 35 ± 13 | 36 ± 14 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 189 ± 70 | 196 ± 70 | 186 ± 80 |
| Mean AUC (ng*h/mL) | 6901 ± 1423 | 6956 ± 1711 | 7212 ± 2284 |
| **Month 1 post-TX** | C/C | C/T | T/T |
| Mean $C_0$ (ng/mL) | 234 ± 62 | 249 ± 67 | 245 ± 63 |
| Mean $C_2$ (ng/mL) | 1293 ± 357 | 1289 ± 344 | 1262 ± 479 |
| Mean dose (mg/day) | 395 ± 98 | 406 ± 133 | 382 ± 132 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 39 ± 13 | 42 ± 17 | 46 ± 13 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 217 ± 73 | 216 ± 87 | 238 ± 123 |
| Mean AUC (ng*h/mL) | 6541 ± 1304 | 6711 ± 1371 | 6599 ± 1915 |

*ANOVA Test: p 0.04"

Table 27 : distribution of CsA pharmacokinetic parameters in 120 kindey transplant recipients according to MDR1 exon 26 genotype (C3435T)

| CsA pharmacokinetic parameters | MDR1 exon 26 genotype | | |
|---|---|---|---|
| | C/C | C/T | T/T |
| **Week 1 post-TX** | | | |
| Mean $C_0$ (ng/mL) | 253 ± 97^ | 250 ± 69 | 296 ± 128 |
| Mean $C_2$ (ng/mL) | 1345 ± 365 | 1434 ± 537 | 1380 ± 509 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 34 ± 14 | 36 ± 15 | 41 ± 18 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 186 ± 80 | 202 ± 73 | 189 ± 70 |
| **Month 1 post-TX** | | | |
| Mean $C_0$ (ng/mL) | 222 ± 70* | 258 ± 53 | 251 ± 70 |
| Mean dose (mg/day) | 412 ± 118 | 403 ± 127 | 376 ± 103 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 35 ± 11* | 44 ± 15 | 44 ± 14 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 199 ± 80° | 234 ± 80 | 231 ± 109 |
| **Month 3 post-TX** | | | |
| Mean $C_0$ (ng/mL) | 200 ± 61 | 217 ± 64 | 197 ± 46 |
| Mean $C_2$ (ng/mL) | 1135 ± 315 | 1106 ± 281 | 1215 ± 377 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 40 ± 11* | 51 ± 19 | 51 ± 18 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 231 ± 67° | 261 ± 99 | 285 ± 94 |
| **Month 6 post-TX** | | | |
| Mean $C_0$ (ng/mL) | 169 ± 50 | 185 ± 61 | 169 ± 37 |
| Mean $C_2$ (ng/mL) | 907 ± 291 | 920 ± 261 | 902 ± 189 |
| Mean $C_0$/dose/bw (ng/mL/mg/kg) | 36 ± 21* | 46 ± 17 | 47 ± 14 |
| Mean $C_2$/dose/bw (ng/mL/mg/kg) | 193 ± 95° | 225 ± 77 | 249 ± 77 |

^$p<0.05$ vs TT; *$p<0.05$ vs CT and TT; °$p<0.01$ vs CT and TT; ●$p<0.05$ vs CT; §$p<0.05$ vs CT, $p<0.01$ vs TT

Table 28 :CsA Pharmacokinetic parameters in 120 kidney transplant recipients with different MDR1 genotypes and haplotypes

| | n | Week I | | | Month I | | |
|---|---|---|---|---|---|---|---|
| | | $C_0/D/BW$ $(ng/mL/mg/Kg)$ | $C_2/D/BW$ $(ng/mL/mg/Kg)$ | AUC $(ng*h/mL)$ | $C_0/D/BW$ $(ng/mL/mg/Kg)$ | $C_2/D/BW$ $(ng/mL/mg/Kg)$ | AUC $(ng*h/mL)$ |
| **Genotype** | | | | | | | |
| 00 | 32 | 34 ± 13 | 188 ± 82 | 6958 ± 1435 | 37 ± 13 | 201 ± 75 | 6303 ± 1446 |
| 01 | 14 | 44 ± 24 | 189 ± 21 | 6084 ± 2911 | 44 ± 13 | 278 ± 4 | 6887 ± 917 |
| 02 | 1 | 97 ± 5* | 144 ± 16 | 9977 ± 212 | 71 ± 8°^ | 178 ± 11 | 7398 ± 58 |
| 10 | 6 | 22 ± 4 | 144 ± 20 | 5817 ± 834 | 28 ± 13 | 164 ± 59 | 6091 ± 2376 |
| 11 | 33 | 35 ± 13 | 200 ± 71 | 6741 ± 1589 | 46 ± 17 | 225 ± 91 | 6981 ± 1129 |
| 12 | 12 | 41 ± 14 | 208 ± 77 | 7838 ± 1741 | 36 ± 14 | 221 ± 85 | 6160 ± 1007 |
| 20 | 4 | 39 ± 16 | 224 ± 78 | 7645 ± 1451 | 45 ± 16 | 241 ± 133 | 5558 ± 817 |
| 21 | 2 | 33 ± 15 | 196 ± 40 | 7313 ± 622 | 46 ± 15 | 216 ± 31 | 6566 ± 214 |
| 22 | 16 | 36 ± 14 | 179 ± 66 | 7002 ± 2615 | 46 ± 14 | 239 ± 125 | 6893 ± 2190 |
| **Haplotype** | | | | | | | |
| Carrier 11 | 85 | 35 ± 14 | 194 ± 76 | 6857 ± 1613 | 40 ± 15 | 216 ± 80 | 6660 ± 1363 |
| Noncarrier 11 | 35 | 40 ± 18 | 195 ± 89 | 7556 ± 2162 | 44 ± 14 | 231 ± 14 | 6517 ± 1695 |
| Carrier 12 | 27 | 43 ± 20 | 204 ± 79 | 7664 ± 2029 | 41 ± 14 | 241 ± 68 | 6741 ± 924 |
| Noncarrier 12 | 93 | 34 ± 13 | 191 ± 72 | 6873 ± 1702 | 42 ± 15 | 216 ± 92 | 6591 ± 1559 |
| Carrier 21 | 12 | 30 ± 13 | 185 ± 64 | 6872 ± 1437 | 36 ± 16 | 199 ± 88 | 6003 ± 1697 |
| Noncarrier 21 | 108 | 37 ± 15 | 195 ± 75 | 7078 ± 1845 | 42 ± 15 | 223 ± 88 | 6702 ± 1409 |
| Carrier 22 | 63 | 36 ± 13 | 196 ± 69 | 7032 ± 1852 | 44 ± 15 | 228 ± 97 | 6827 ± 1527 |
| Noncarrier 22 | 57 | 36 ± 17 | 191 ± 78 | 7087 ± 1707 | 38 ± 14 | 213 ± 78 | 6413 ± 1430 |

*ANOVA test $p<0.001$: Bonferroni test $p<0.001$ vs all other genotypes at week I
*ANOVA test = 0.0057: Bonferroni test $p<0.001$ vs genotype 10; ^$p<0.05$ vs genotype 00 at month I

*Chapter 12*

**CONCLUSION AND FUTURE PERSPECTIVES**

During the past decade, as increasing number of more powerful immunosuppressive agents became available, the short-term rate of organ survival significantly improved, yet the long-term results did not. Although graft survival rate at 1 year post-surgery is now exceeding 90%, after a decade it drops to nearly 40% [172]. The initial excitement that followed a report that long-term survival of renal allografts may be improving, especially in recipients who had never had an episode of acute rejection [173], has faded as newer data [174] showed that the long-term risk of graft loss has not improved. The reasons for chronic allograft dysfunction involve many factors that concerned variable tissue injury at the time of transplantation or during subsequent episode of rejection. However, even in the case of grafts with good function in the early years after transplantation, progressive tissue damage and slow decrements in function may develop, most often with considerable vasculopathy. Chronic allograft dysfunction affects all transplanted organs and is the most common cause of graft loss in the long-term.

Both immunologic and non-immunologic factors play a key role in promoting poor long-term graft survival. Among these, long-term use of immunosuppressive agents does contribute to chronic graft injury [175]. Paradoxically, a transplanted organ initially protected from the immunological injury by immunosuppressive drugs may be subsequently damaged and lost to chronic toxicity caused by these same agents. As a matter of fact, all anti-rejection drugs have specific side effects and additively contribute to an overall state of immunosuppression, which leads to an increased risk of various malignant conditions [176], most commonly lymphoproliferative disorders or squamous cell carcinoma of the skin. Such drugs contribute also to the increased risk of cardiovascular disease, which is the most common cause of premature death in transplant recipients [177]. Moreover, excessive total immunosuppression causes a susceptibility to infectious disease [178], such as cytomegalovirus, Epstein-Barr virus,

and the more recently recognized polyomavirus, which causes nephropathy and renal allograft loss [179]. Indeed, over the past decades major efforts have been done to optimize the use of immunosuppressive agents reducing side effects yet maintaining graft survival. Nevertheless, this individualization depends on the judgement of the physicians, as robust treatment guidelines have not yet been established.

## Major finding of the present research project

Pharmacokinetic studies in humans often involve analysis of large number of samples and therefore require simple, rapid and reliable analytical methods. Furthermore, daily analysis of an increasing number of drugs as part of the routine drug monitoring activity, urges the need for HPLC methods that can be easily run and rapidly set up. Therefore, the first part of my research activity was devoted to the development and validation of analytical methods which could be used to measure plasma levels of MPA as well as blood levels of SRL and CsA.

To date several HPLC methods have been described for determination of total MPA. As potential advantages, the method described in *Chapter 3* utilizes a simple extraction procedures using solid phase extraction (SPE) columns and does not involve any centrifugation, use of vacuum or drying of residues following extraction. Additionally, the use of PTA as IS, which is the first analytical peak eluted, allow us to stop the chromatographic run immediately after the elution of MPA (at 8 min), and to proceed fast with a new injection. Moreover, the robustness of the method makes it easy to generate reproducible results. More difficult is the assessment of free MPA, since we have to manage a signal which is less than 5% of that observed with total MPA. To overcome this problem, we have moved from a wavelength of 254 nm to 215 nm, doubled the injection volume and used a gradient, with a great improvement in the signal. As additional shortcoming, the ultrafiltration procedure is extremely variable.

Indeed, we have observed that small variation in the temperature (i.e., 1°C) and/or in the time of the centrifugation, were associated with great difference in the volume of the ultrafiltrate obtained. To measure unbound MPA it is, therefore, mandatory to strictly monitor the operational conditions. It should be pointed, however, that, despite a greater variability in inaccuracy and imprecision than that observed for total MPA, the performance of the described method for the assessment of free MPA was still in agreement with the FDA Guidelines [36].

Quantification of SRL in biological matrices is not easy, given the very low dose of the drug routinely employed that reflects its high potency. Moreover, the assay may be affected by several variables including the recovery of the analyte during sample processing and potential interfering peaks in the chromatograms. Here, we developed a new method for SRL determination in whole blood specimens, which is robust enough to be run on standard HPLC equipment, using only basic analytical reagents [122]. As shown in *Chapter 4*, the assay has adequate sensitivity, precision and accuracy for therapeutic monitoring of the drug in transplant patients. Taking into account the sample preparation step and the chromatographic time, at least 60 samples per day can be extracted and analyzed by a single technician, using two Vac Elut Manifold chambers. This method overcomes the analytical difficulties and the long time required for sample preparation described in the previously published procedures together with the need of using special (light protected) or treated (silanized) glassware [35]. As previously documented for total MPA assessment, the Bond-Elut cartridges with 200 mg of sorbent applied also for the determination of SRL allowed the use of very small volumes for either cleaning-up and elution steps, thus speeding up the solid/liquid extraction step and further reducing the time of drug measurement.

Although the present research has mainly focused on TDM of MPA and SRL, some additional aims required the assessment of CsA concentrations (see *Chapters* 6-11).

Thus, as described in *Chapter 5*, I have also described and validated a HPLC method with UV detection for the measurement of CsA in whole blood. It must be pointed out, however, that the liquid-liquid extraction procedure, used in this method, presents some problems, mainly related to the use of glass tubes and toxic solvents, as well as a very long time for sample processing. Moreover, this method is associated with a poor recovery. Therefore, we have very recently planned to develop a HPLC method with SPE extraction also for the assessment of CsA concentrations in whole blood, given alone or in combination with everolimus, a new immunosuppressive agent with a structural formula close to that of SRL [180].

The performance of the methods presented in *Chapters 3, 4,* and *5* was in agreement with the FDA Guidelines [36]. Thus, they were applied for TDM of novel immunosuppressants (namely MPA and SRL), as described in the second and third parts of the thesis (*Chapters 6-10*).

Although there is little controversy amongst clinicians prescribing CsA that measuring this drug in blood is a useful adjunct to its optimal administration, the same concept does not apply to MMF, a new immunosuppressant characterized by a narrow therapeutic index. Actually, this drug is widely used in a fixed daily dose, despite growing evidence showing that this regimen no longer might be the best approach for the management of transplant patients [181,192]. Indeed, we have explored the possibility to optimize MMF dosing by MPA pharmacokinetic monitoring by studying 46 stable adult kidney transplant patients receiving CsA, steroids and MMF at a fixed daily dose, guided by haematological parameters [88]. As first finding, we observed an over 10-fold variability of MPA dose-adjusted pharmacokinetic parameters, allowing us to speculate that the knowledge of the daily MMF dose was no guide to drug exposure. Moreover, we also found a significant correlation between MPA AUC and graft function. Indeed, patients exposed to higher concentrations of MPA presented lower

serum creatinine values and higher creatinine clearance than those with lower MPA exposure, providing a strong rationale for measuring MPA levels to guide MMF dosing (*Chapter 6*).

Pharmacokinetic-based monitoring may be useful not only to tailor dosage but also to unmask potential drug-drug interactions. This concept applies generally to all NTI drugs but is of particular relevance in the transplant setting, where patients are chronically treated with several combinations of drugs. For instance, modulation of MPA absorption, distribution, metabolism and/or excretion by concomitant administration of drugs other than MMF might affect MPA bioavailability and eventually clinical outcome [39,71,81]. Indeed, as a part of a steroid-sparing clinical trial, we have found that glucocorticoids, by inducing enzymes involved in MPA metabolism, interfere with drug bioavailability [90]. In particular, steroid reduction and discontinuation paralleled with a progressive increase of MPA exposure (*Chapter 7*). These results are of particular relevance for those centres that are adopting steroid-sparing regimens with the goal to limit drug-related chronic adverse events. If these approaches are pursued using MMF at fixed daily dose, progressive steroid reduction may, therefore, results in excessive MPA exposure with increased MMF-related toxicity.

Beside steroids, MMF is usually given in combination with CsA or TRL. However, the introduction in the clinical practice of a new class of immunosuppressive agents, namely rapamycins (SRL and everolimus), is allowing the development of calcineurin inhibitor-free regimens that combine MMF with sirolimus. As a part of a protocol aimed at investigating the efficacy of induction therapy with a new monoclonal antibody, Campath-1H, we have found that SRL and CsA exert different effects on MPA exposure. Co-administration of SRL and MMF was associated with higher dose-adjusted MPA levels and AUC than those measured under CsA-based regimens (*Chapter 8*). As working hypothesis, we suggest that CsA reduces the enterohepatic

recirculation of MPA from MPAG, its glucuronidated metabolite, whereas SRL did not. This was confirmed by the lack of a secondary peak of MPA in almost all patients treated with CsA and MMF [182]. Again, these results should be carefully taken into account when MMF is given at fixed doses and patients are switched to different poly-pharmacological therapies.

As additional confounding factor, a new formulation of MPA, the enteric coated sodium salt (EC-MPS), is now in development. A formal comparison of the pharmacokinetic profiles of these two formulations, as we did (*Chapter 9*) has evidenced dramatic differences, especially in the absorption process [183]. In particular, patients given EC-MPS had MPA trough levels 4-fold higher than those found in patients given MMF. Our results may have important clinical consequences when $C_0$-based MPA monitoring is used to optimize MPA therapy, as recently suggested by International Consensus Conferences [114]. The great variability of MPA $C_0$ levels after EC-MPS administration does not allow the implementation of trough-based TDM in patients chronically treated with the novel MPA-releasing formulation, an event that might potentially translate in a sub-optimal clinical outcome. Similarly, the very high variability, both intra- and inter-patient, among all MPA pharmacokinetic parameters from patients treated with EC-MPS and the poor correlations between single sampling points and MPA $AUC_{0-12}$ after the administration of the enteric-coating formulation did not allowed the development of a suitable equation to reliably predict MPA daily exposure in these patients. Therefore, at variance with MMF [37,114], these results argue against the possibility to implement a limited sampling strategy-based therapeutic drug monitoring of MPA in patients given EC-MPS, with potential detrimental effects for the patient and for the graft in the long term.

As mentioned above, a new class of immunosuppressive agents is now available on the market, referred as "rapamycins" (namely SRL and everolimus). For SRL, measurement

of the drug levels is actually a license requirement in Europe and is recommended in specific clinical settings in the USA [184]. In the past 3 years our laboratory served as a centralized institution in Italy to measure SRL trough samples from more than 40 Italian Transplant Units. These samples corresponded to nearly 500 kidney transplant recipients treated with different SRL-based immunosuppressive regimens. A retrospective analysis of all the data evidenced some important pharmacokinetic interaction that involved SRL. In particular, we found that dose-adjusted SRL trough levels were significantly higher in patients given CsA than in those given tacrolimus [185]. Moreover, we observed that MMF and/or steroids reduced SRL bioavailability. As additional analysis, we proposed a dose-adjustment formula which can be used to guide SRL dose changes, with the goal to reduce variability in SRL exposure, a condition known to affect clinical outcome (*Chapter 10*).

Everolimus is a macrolide bearing a 2-hydroxyethyl chain substitution at position 40 on the SRL structure, rationally developed to improve the pharmacokinetic characteristics of the innovator compound. We have recently developed and validated an HPLC-UV method for determining everolimus concentrations in human whole blood [186]. Unfortunately, this novel immunosuppressant is not yet available on the Italian market. We have, therefore no data on TDM studies focusing on everolimus. As future research, we will apply our recently developed method to measure daily fluctuations of everolimus concentrations, with the goal to assess whether the introduction of the chemical modification in position 40, may effectively translate in a better pharmacokinetic profile of everolimus as compared with SRL.

All together, these results pointed pharmacokinetics as the most useful approach to monitor and optimize drug dosing not only for traditional immunosuppressants (such as CsA and TRL), but also for novel anti-rejection agents, namely MPA and SRL.

It should be pointed, however, that drug concentration measurements are only a part of the decision tree for dose adjustment. They must be viewed in the context of other complementary fields that include biochemical and clinical analysis, as well as novel therapeutic monitoring approaches. As an example of this concept, we have conducted a randomized trial aimed at investigating whether per-protocol biopsies, in combination with routine pharmacokinetic studies, could be useful tools to implement steroid or CsA sparing regimens in kidney transplant recipients [187]. As main result, we have found that per-protocol biopsy more than one year after transplantation is a safe procedure to guide change of immunosuppressive regimen and to lower the risk of major drug-related side effects.

Alternative approaches, beside pharmacokinetics, have been recently proposed to tailor the best immunosuppressive regimen for each patient. For instance, pharmacodynamic monitoring involves measurement of the biological effect of the drug at its target site [21]. Indeed, I have also involved in the development of a method for the assessment of calcineurin, the pharmacological target of CsA, in whole blood. When we applied this assay to monitor kidney transplant recipients we found that CsA levels did not predict daily calcineurin (CN) activity, whereas a single determination of CN at baseline was a useful surrogate for the daily inhibition of the enzyme by CsA [188]. It should be pointed out, however, that pharmacodynamic tests are actually too complex for clinical use and often require radioactive materials. As additional drawbacks, studies aimed at investigating the potential predictivity of pharmacodynamic approaches in terms of rejection and/or drug toxicity are still lacking. Therefore, alternative roads, beside pharmacodynamics, should be travelled.

Those involved in TDM are now realizing that individual patient's exposure to immunosuppressive agents can be influenced by the genetic background. Indeed, it has been proposed that interinvidual differences in drug response may be due, at least in

part, to sequence variants in genes encoding drug-metabolizing enzymes, drug transporters, or drug targets [41,159]. Accordingly, the identification of an individual's genetic make-up may be useful to choose the best treatment for each patient. As compared to pharmacokinetic studies, pharmacogenetic studies can be conducted even before the beginning of treatment, they do not require the assumption of steady-state conditions, are constant for an individual's lifetime and can provide predictive value for multiple drugs. Hence, as last part of my research activity, I have attempted to assess the role of pharmacogenetics in the field of organ transplantation. To perform this study we took advantage from the large amount of pharmacokinetic data from patients enrolled in the MY.S.S. trial [28,38]. Over 2,000 CsA trough and $C_2$ measurements, collected during the first 6 months post-surgery, were available. As preliminary analysis we studied the potential association between CsA pharmacokinetics parameters and genetic variants in the MDR1, the gene encoding for the P-gp (*Chapter 11*). This protein works as an efflux pump, which removes lipophilic drugs, like CsA, from the intracellular space. As working hypothesis we speculated that genetic polymorphisms of the MDR1 gene could result in significant reduction in P-gp expression, eventually increasing the exposure of these patients to CsA. Indeed, preliminary results, performed in 120 out of the 350 patients enrolled in the MY.S.S. trial, documented that a polymorphism in the exon 26 of the MDR1 (C3435T) was associated with higher CsA levels than wild types, suggesting that also genetic assessments can be considered useful tools to guide drug dosing [189]. Assuming that P-gp exerts its activity on many lipophilic substrates, it can be reasonably speculated that these findings could applied also to other immunosuppressive agents, such as tacrolimus, SRL and everolimus.

# Future directions

Transplantation has transformed the treatment of patients with organ failure in a number of clinical settings, and immunosuppressive drug therapy is fundamental to its success. During the past decade several new potent anti-rejection molecules with different modes of action and different side-effect profiles have become available, and some will be available in the near future [190,191]. They include agents that deplete T cell (anti CD3, CD52-specific monoclonal antibodies) or B cell (CD20-specific monoclonal antibodies) signal transduction pathways, blockers of co stimulatory pathways (CD40-specific antibodies, CTLA4Ig, LEA29Y), agents that interfere with lymphocyte trafficking (LFA-1-specific antibodies, FTY720) and small-molecules drugs (SRL, everolimus, MPA-releasing formulations, mizoribine, leflunomide, FK778, JAK3 inhibitors and the new CsA analogue ISA247). Nowadays immunosuppression after renal transplantation is no longer one single regimen applicable to all patients. In the selection of the optimal immunosuppressive protocol, individual drug-related toxicity, recipient-donor related risk factors as well as all the available information have to be taken into account. It can be reasonably speculated that interest in the measurement of immunosuppressive drugs as a guide to therapy will show no signs of waning. As the number of immunosuppressive drug combinations increases, so does the complexity of interpretating the concentration data. Indeed, also for some of the new immunosuppressive agents mentioned above (such as FK778, mizoribine and ISA247) will benefit from pharmacokinetic-based TDM as a guide to tailor the best dosage for each patient. Periodical evaluations of drug exposure can be able to detect external influences (i.e. drug-drug interactions, hormonal levels, kidney and/or liver dysfunction, etc) that can vary over time. From this point of view, traditional pharmacokinetic-based TDM will undoubtedly play a key role to optimal drug prescription also for the next years.

However, it is expected that, in the future, in addition to targeting a patient's drug concentrations within a therapeutic range as in traditional TDM, physicians are more than likely to be making dosage recommendations for individual drugs based also on a large amount of additional information, such as pharmacodynamic profiles, as well as according to individual patient's genotype. On pure mechanistic grounds, measurement of pharmacodynamic parameters (for example, the biological activity of a drug) may more closely correlate with clinical outcomes than pharmacokinetic parameters. This approach has shown great analytical limitations when applied to assess calcineurin activity after CsA or tacrolimus administration or in the attempts to measure P70S6 kinase in patients chronically given SRL or everolimus. At variance, still promising is the assessment of IMPDH activity in peripheral blood mononuclear cells [192]. Indeed, preliminary studies have reported significant associations between IMPDH activity and risk of acute rejection as well as MMF-related toxicity. More data are now needed to fully establish the prognostic value of IMPDH activity, and longitudinal studies are eagerly awaited.

As exemplified in *Chapter 11*, pharmacogenetics is another promising science which could provide important information for the individualization of the immunosuppressive regimen. Almost all the available literature in this field focuses on AZA, CsA and TRL [41,159], whereas data on novel immunosuppressive agents are scanty. To date, there are only two studies which have investigated the role of SNPs in the MDR1 and CYP3A genes on SRL exposure [193,194]. Results from these observations, derived using SRL trough levels as surrogate markers of daily drug exposure, were controversial. Indeed, both these studies have shown that, unlike TRL, SRL trough concentrations and dose requirements were not affected by MDR1 polymorphisms, whereas the predicting value of CYP3As genotyping was documented by Anglicheau et al [193], but not by Mourad [194]. These findings were not conclusive and provide a rationale for future

investigations focusing on genetic determinants of SRL and everolimus exposure. On the same line, pharmacogenetic studies on MPA are largely inadequate. It is well know that MPA is extensively glucuronidated by several UDP-GTs, a family of enzymes characterized by several genetic polymorphisms [195,196]. In the only article available, it has been shown that the T-275A and C-2152T SNPs of the UGT1A9 gene promoter are associated with significantly lower MPA exposure in renal transplant recipients treated with the fixed 2 g MMF daily dose and part of this effect was caused by interruption in the EHC of MPA [197]. These observations demonstrated for the first time that, in vivo, interindividual variability in the pharmacokinetics of MPA can be partially explained by genetic variation. Given the high allelic frequency of the UGT1A9 SNPs (approximately 15% in white subjects), as well as the 2-fold reduction in MPA exposure in comparison with noncarriers, these findings are also likely to be clinically relevant and offer both a rationale and a means for a personalization of MMF treatment. As additional confounding factor, it has been recently reported that MPA exposure is influenced by the activity of the multidrug resistance-associated protein 2 (MRP2), a protein expressed at the apical surface of hepatocytes, where it functions to excrete conjugation products (such as MPA glucuronidated metabolites) into bile [198]. Similarly to what has been reported for MDR1, also SNPs in the MRP2 gene have been reported [199]. Therefore, it can be reasonably expected that future pharmacogenetic studies will focused also on the potential association between MRP2 allelic variants and MPA exposure.

To strengthen the role of pharmacogenetics in the field of organ transplantation it is important that genetic studies will not include only information on inheritance variants in gene encoding for enzymes involved in drug disposition but, even more importantly, also in genes involved in the pharmacological response. To date, no publications were available on SNPs in the pharmacogical targets of immunosuppressive agents

(calcineurin, IMPDH, P70S6 kinase, etc). However, it has been recently demonstrated that SNPs in the gene encoding for cytokines actively involved in the modulation of the immune system influence the outcome of kidney transplantation [200]. These results provide indirect evidence that, beside genes encoding for metabolizing enzymes, also allelic variants in the pharmacological target might affect drug response, ultimately affecting the outcome of the graft and patient survival.

It is expected that the availability of complete information on patient's status (pharmacokinetics, dynamics, genomics, and clinical data) will allow us move from a diagnosis-directed approach to one that is prevention-directed and patient-tailored. However, to pursue this ambitious project, we urge need the development of algorithms working within neural networks able to incorporate the large amount of data from kinetic, clinical, dynamic and genetic testing and providing predictive dosage algorithms useful for personalised therapies. If we succeed, we will be able to identify not only the best drug to be administered to a particular patient, but also the most effective and safest dosage from the outset of therapy, already before transplant surgery. Nevertheless, it should be reminded that, regardless of whether we are referring to traditional TDM or that of the future, we need to ensure that we are providing ideal immunosuppression for our patients and are helping to improve the quality of health care. Indeed, the ultimate goal of personalized therapy is the improvement of long term organ survival, eventually leading to indefinite tolerance of the graft.

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# LIST OF ABBREVIATIONS

| ANOVA: | Analysis of variance |
|---|---|
| AUC: | Area under the time concentration curve |
| AZA: | Azathioprine |
| Bk: | Blank sample |
| $C_0$: | Trough concentration |
| $C_2$: | Concentration at two-hour post-dose |
| $C_{max}$: | peak of maximum drug concentration |
| CN: | Calcineurin |
| CsA: | cyclosporine |
| CV : | Coefficient of variation |
| CYP3A4: | Cytochrome 3A4 |
| CYP3A5: | Cytochrome 3A5 |
| EC-MPS: | Mycophenolate sodium |
| EDTA: | Etylen-tetraaminoacetic acid |
| EHC: | Enterohepatic recirculation |
| FDA: | Food and drug administration |
| GI: | Gastrointestinal |
| GT: | Glucuronyltransferase |
| HLA: | Humal lymphocyte antigen |
| HPLC: | high performance liquid chromatography |
| IMPDH: | Inosine monophosphate dehydrogenase |
| IQC: | Internal quality controls |
| IPTS: | International proficiency testing scheme |
| IS: | Internal standard |
| KS: | Kaposi's sarcoma |
| LC-MS: | Liquid chromatography-mass spectrometry |

| LLOD: | Lower limit of detection |
|---|---|
| LLOQ : | Lower limit of quantification |
| LSS: | Limited sampling strategy |
| MDR1: | Multidrug resistant 1 gene |
| MMF: | Mycophenolate mofetil |
| MP: | Methylprednisolone |
| MPA: | Mycophenolic acid |
| MPAG: | Mycophenolic acid glucuronide |
| MS: | Mass spectrometry |
| mTOR: | Mammalian target of rapamycins |
| MY.S.S.: | Mycophenolate steroid sparing study |
| NTI: | Narrow therapeutic index |
| PAGE: | Poly acrylamide gel electrophoresis |
| PCR: | Polymerase chain reaction |
| P-gp: | P glycoprotein |
| QC: | Quality control samples |
| R: | Coefficient of correlation |
| $R^2$: | Coefficient of regression |
| RBC: | Red blood cell |
| SD: | Standard deviation |
| SE: | Standard error |
| SNP: | Single nucleotide polymorphism |
| SPE: | solid-phase extraction |
| SSCP: | Single strand conformational polymorphism |
| SRL: | Sirolimus |
| TDM: | Therapeutic drug monitoring |

$T_{max}$:   Time corresponding to the maximum drug concentration

TRL:   Tacrolimus

UDP-GT:   uridine diphosphate glucuronosyltransferase

UV:   Ultraviolet

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*"...grazie mamma e papà..."*

# MATHERIAL PUBLISHED OR SUBMITTED FOR PUBLICATION

# CONTAINED IN THE THESIS

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