Individualized dosing of calcineurin inhibitors in renal transplantation

Press, R.R.

8

Individualized Dosing of Calcineurin Inhibitors in Renal Transplantation – General Discussion and Perspectives

Immunosuppressive therapy to prevent kidney rejection is build around calcineurin inhibitors (CNIs). As a drawback to this therapy, patients receiving CNIs have a high risk of encountering clinical toxicity. Especially acute liver-, kidney- and neuro-toxicity are complicating factors early after transplantation. On the long term chronic damage to heart and vasculature is the primary cause of patient death. Complicating factors are glucose intolerance or diabetes and dyslipidemia [1]. Overall, the primary outcome for renal transplantation remains kidney survival, which is highly influenced by chronic damage to the kidney. This has been shown to be the result of several factors of which CNI toxicity is a principal factor [2]. At this point, it is likely the leading factor for the lack of improvement in kidney survival [3-5].

Only one aspect of immunosuppressive therapy offers a promising perspective to improve graft survival, the withdrawal of calcineurin inhibitor therapy. But, CNI therapy is a prerequisite for the transplanted kidney to survive the first weeks after transplantation, which only allows withdrawal in the context of slightly lower immunological risk, thus several weeks or months post transplantation. That must be the starting point from where non-nephrotoxic alternatives should be employed [6,7].

Calcineurin inhibitor therapy has become crucial for a successful transplantation. Considering the low frequency of acute rejection episodes, apparently the risk for rejection has become less important. This is the direct result of optimal immune-modulation with triple or quadruple therapy. The quadruple approach typically consists of induction with an IL2-blocker (i.e. basiliximab) and maintenance therapy with prednisolone, mycophenolic acid and a CNI. The decrease of the risk for toxicity and over-immunosuppression has become the most crucial aim in the first period after transplantation. Specifically, opportunistic infections exploit conditions with too much immunosuppression, with a

major role for cytomegalovirus (CMV), Epstein-Bar virus (EBV) and polyoma viruses [8]. The emphasis should be placed on the latter, which typically constitutes of BK-virus and culminates into a kidney deteriorating BK-nephropathy [9,10].
To battle the consequences of toxicity or over-immunosuppression, currently low dose CNI regimens have found to be of use. Specifically, the use of low dose tacrolimus has proven to be advantageous [6]. However, the average dose itself is not the only variable that drives patient or graft outcome. Patients display large between patient variability in response to CNI administration, partly resulting from variability in pharmacokinetics [11,12]. To circumvent pharmacokinetic variability, exposure of these drugs is routinely measured in whole blood [13]. In case the exposure deviates from the target exposure the dose of the drug is adjusted accordingly. Indeed, CNI whole blood exposure has found to be related to the risk of acute rejection and the risk for nephrotoxicity [14-16]. Although improvements in clinical transplantation have been achieved with individualization of the CNI dose using exposure measurements, it remains a fairly reactive approach which can result in a long interval between start of therapy and achieving target exposure [17]. This raises the important question how CNI dosing can be optimally individualized. To answer this question this thesis is build up with a tight structure, starting with the analysis of the pharmacokinetics of ciclosporin A and tacrolimus. In a next step an effect biomarker is developed and finally a study with a clinical endpoint has been performed.

Variability in pharmacokinetics of calcineurin inhibitors

The pharmacokinetics of the CNIs are very complex and characterized by enormous variability. Upon oral administration these highly lipophilic drugs are absorbed to different extents down to the ileum. As soon as they reach intestinal mucosa and the absorption process starts, they are metabolized by the cytochrome P450 enzyme system in the intestine as well as the liver. Specifically the CYP3A4 and CYP3A5 enzymes are involved, which act together with the efflux pump P-glycoprotein that actively transports CNIs out of the cell. The highly variable fraction of the drug that reaches the blood circulation, the bio-available fraction, distributes within the blood primarily to the red blood cell, while the remainder binds to albumin, α-acid protein and lipoproteins. Only a very small fraction is unbound in blood. When CNIs distribute to cells within (organ) tissue, they bind to immunophillins to be able to a-specifically inhibit the target enzyme calcineurin by sterical hindrance of the active site. Finally, CNIs are metabolized to an array of metabolites which are principally eliminated with bile and only a small fraction with urine [11,12]. Since large part of CNIs distribute to the red blood cell, within the blood fraction, either the plasma/serum concentration or the unbound concentration would be of interest for pharmacokinetic or exposure analysis. Technical difficulties prevented the measurement of these fractions. Therefore the whole blood concentration was chosen as a concentration biomarker to reflect pharmacokinetic variability for CNIs. Despite the fact, that it may be a poor reflection of the drug at the site of action [18,19].
To achieve target whole blood exposure early after transplantation, factors or covariates should be identified that explain variability between or within transplant recipients. With the identification of factors that can be obtained before transplantation, these could be used to predict an individual's dose prior to transplantation. This was the focus of the

research described in the Chapters 3 and 4 of this thesis. To be able to discriminate between effects of multiple covariates on the pharmacokinetics of CNIs, a population analyses is essential. Such an analysis is typically performed with *non-linear mixed effects modeling* (NONMEM) [20]. This approach distinguishes between structural and random effects. With the structural model describing the time course of the drug's concentration using parameters such as absorption rate constant, volume of distribution and clearance. The random effects parameters are used to describe variability in these parameters between and within individuals. When variability in the pharmacokinetic parameters is adequately described, a covariate model can be applied to explain the identified variability and to distinguish the effects of several covariates. An advantage of this powerful technique is the possibility to analyze rich and sparse data together, which provides a possibility to use all available data.

The absorption, distribution and elimination of tacrolimus were mathematically described with a 2-compartment model with linear first-order absorption and first-order elimination. Variability in tacrolimus clearance between patients was explained by a polymorphism in the gene encoding the cytochrome enzyme CYP3A5. Roughly 20% of the Dutch renal transplant population carries one *1 allele coding for this metabolizing enzyme which leads to a 50% higher tacrolimus clearance (Chapter 3) [21-23]. In the current clinical practice they have a 50% lower exposure in terms of area-under-the-blood-concentration *versus* time curve (AUC). Depending on the number of *1 alleles present, recipients should be dosed 50% (one allele) to 100% (2 alleles) higher compared to carriers of the *3 allele, to minimize the time to target exposure or to prevent rejection [21,24]. African-Americans have been shown to carry the CYP3A5*1 allele in over 75% of transplant recipients, while this is the case for only 10-20% of Caucasians [25,26]. Indeed, the rejection rate is higher for African-Americans [27], which may be attributed to the *CYP3A5* genotype. However, other factors (waiting time on dialysis, socio-economic status, non-compliance and co-morbidity (diabetes, hypertension)) seem to play a (more important) role as well [27,28].

Although initially tacrolimus is dosed based on a persons body weight, as advised in the package insert of tacrolimus (Prograft®), body weight was not found to be related to drug clearance in chapter 3. When a strict body weight based dosing regimen would be applied this could lead to severe tacrolimus under- and overexposure for patients with low and high body weight, respectively. Furthermore, co-administration of prednisolone in a dose of 10 mg or higher was found to increase apparent tacrolimus clearance with 15%, explaining variability in tacrolimus exposure within patients (Chapter 3).

Similar to the model for tacrolimus, ciclosporin A disposition was described with a 2 compartment distribution and elimination model. But, this time a delayed absorption was identified which was described with a transit compartment between the dose compartment and the central compartment. In contrast to tacrolimus, ciclosporin A clearance did depend on a patient's body weight. Also ciclosporin A apparent clearance was 22% higher with a concomitant prednisolone dose greater than 20 mg, which explained variability within the transplant recipient (Chapter 4).

Drug interactions are responsible in large part for patient variability in drug exposure and/or response to CNIs [12,29-31]. Prednisolone is often administered concomitantly in

varying doses in transplant medicine. In most cases high intravenous induction doses are used around transplantation, which are rapidly converted or tapered to low dose oral regimens. Prednisolone is known to induce the metabolism of tacrolimus, while it is less obvious for ciclosporin A [32-34]. Rapid tapering of prednisolone early after transplantation could decrease the inductive effect of prednisolone on tacrolimus metabolism with increasing exposure as a result. Often researchers have dedicated this to an independent factor 'time after transplantation' [12,35-37]. This factor comprises all physiological changes in a transplant recipient early after transplantation and not solely the decrease in steroids. Typically, it is assumed that an interaction works the same way for everyone within a patient group. Generally, the concept of drug-interaction was thought to be solely a drug-drug effect, disregarding the role of the host. Naturally, patients do differ in their susceptibility for an interaction. For prednisolone it was hypothesized that the interaction was the result of activating the nuclear factor pregnane X receptor (PXR). Normally, glucocorticoids regulate gene expression by activating the high affinity, low capacity glucocorticoid receptor. In the situation of high endogenous cortisol (i.e. stress) this receptor is saturated and glucocorticoids bind to the low affinity, high capacity PXR to induce its own metabolism and transport which at that time is not sufficient. Subsequently, CYP3A enzymes are induced to increase the metabolism of glucocorticoids [38]. The same is likely to occur for administration of exogenous prednisolone, which at the same time would increase the metabolism of CNIs as well. Genetic variability in *NR1I2*, the gene coding for the pregnane X receptor could then be responsible for differences in susceptibility for this inductive effect between transplant recipients. These differences could result in variability in exposure to CNIs during concomitant administration of prednisolone. A polymorphism in *NR1I2* was associated with increased tacrolimus clearance, but did not explain differences in susceptibility for the interaction between tacrolimus and steroids. Neither was a relationship found between ciclosporin pharmacokinetics and genetic variability in *NR1I2* (Chapter 3 and 4).

With this in mind, the best tacrolimus dosing strategy in current clinical practice would be a genotype based fixed dose, for instance 5 mg for carriers of CYP3A5 *3/*3 genotype and 8 mg for patients carrying a single *1 allele. In case a transplant recipient is a homozygous CYP3A5*1 carrier even higher doses would be necessary. Yet, one should be cautious with these high tacrolimus doses for two reasons. First, whole blood target exposure of the parent drug is likely to be attained early with this approach, but this coincides with a relatively high exposure to tacrolimus metabolites. At least 8 metabolites have been identified of which one has demonstrated pharmacological activity *in vitro* [39-43]. Although no relationships of these metabolites with clinical toxicity have been reported, it is advised that clinicians carefully observe these CYP3A5*1 carriers during clinical follow-up. An alternative approach could be switching homozygous patients with CYP3A5*1/*1 genotype, or possibly *1 allele carriers in general, to ciclosporin A. An additional third, but non clinical, reason is related to (lower) costs, with the higher costs of a double tacrolimus dose being replaced by a standard ciclosporin dose.

Whereas CYP3A5*1 is related to high tacrolimus clearance, predictive factors for low tacrolimus clearance, hence high drug exposure, early after transplantation remain unidentified. A single nucleotide polymorphism in the promoter region of *ABCB1*, T-129C was only weakly

associated with low tacrolimus clearance (Chapter 3). With the absence of strong predictors for overexposure one should use an alternative approach to prevent this from occurring. Using tacrolimus as an example, excessive exposure such as can be seen in Figure 1 should be prevented. To prevent tacrolimus overexposure shortly after transplantation currently in clinical practice, an early trough concentration measurement should be performed at day 2, after 3 or 4 tacrolimus administrations. When considering a target tacrolimus exposure of 160 µg×h/L the first 6 weeks after transplantation, which is about to be used in our center, trough concentrations greater than 15 µg/L should result in pre-emptive dose reduction (Figure 2). As can be derived from the figure 2, this trough concentration reflects an $AUC_{0\text{-}12h}$ of 175-300 µg×h/L. Subsequently, AUC-monitoring should be performed just before discharge from the hospital, which nowadays occurs 1 week after transplantation. In the weeks thereafter any gradual decrease in CNI dose should be corrected for tapering the concomitant prednisolone dose, which causes the inductive effect to fade away. Therefore, a relatively larger dose reduction is necessary to obtain target CNI exposure. This early trough concentration approach could also be useful for ciclosporin which is reasonably dosed on body weight in most cases. To detect extreme low or high exposure an early trough concentration measurement is the only marker available at this point.

In conclusion, to obtain whole blood target exposure early after transplantation a strict TDM strategy is not sufficient. The CNI starting dose should be individualized using other factors besides body weight, such as genotyping for the presence of a CYP3A5*1 allele for tacrolimus or accounting for co-administration of prednisolone.

As has been described in chapter 5 AUC-monitoring has a clear advantage over trough concentration monitoring. This is supported by the 3-5 fold difference in trough concentration at a certain AUC target value. Preferably, the monitoring approach should be kept as practical as possible. Therefore, with chronic CNI use (arbitrarily longer than 2 months, monitoring could potentially be reduced by introducing a patient specific trough concentration. In case two or more AUCs of a renal transplant recipient are obtained during follow up and when there is an acceptable relationship between trough concentration and AUC for an individual, a patient specific trough concentration can be defined. Despite large variability between AUC and trough concentration more specific analytical techniques have come available, such as LC-MS/MS [44]. With this method the accuracy and precision of the concentration measurements have increased. The combination of these factors with already a relatively low within patient variability in pharmacokinetics for CNIs, provides a promising tool for developing the concept of a patient specific trough concentration (Chapter 5).

Although the use of the concentration biomarker in whole blood has improved therapy with CNIs in renal transplantation, more precise biomarkers are required to further optimize CNI therapy. First of all a more precise concentration measurement is desired, which is able to quantify CNIs closer to the site of action, for instance within the T-lymphocyte. When concentrations are more precisely measured in for instance plasma or the T-lymphocyte it remains unclear to what extent the concentration reflects the actual unbound CNI concentration or the CNI-immunophilin complex at the site of action, the calcineurin enzyme. Besides, variability on the pharmacodynamic level may have consequences for the response to CNIs, which will be discussed in the next section.

Variability in pharmacodynamics of calcineurin inhibitors

Unfortunately, concentration measurement in whole blood is not the Holy Grail for CNI dose optimization. Blood concentration does not entail an individual's drug response solely. Variability in the exposure *versus* response relationship for CNIs (i.e. differences in potency and maximum effect) determine susceptibility as well. This currently manifests clinically when patients, at target whole blood exposure, still encounter toxicity or rejection episodes. In contrast, patients at very high or very low exposure not necessarily develop toxicity or a rejection event, respectively.

The reason for this is twofold. First, CNI concentration measurements are performed on whole blood. This has evolved over the years because CNI measurements in the routinely used plasma samples were highly variable. CNIs are primarily bound within erythrocytes and to plasma proteins. To overcome technical issues the whole blood matrix was introduced [18,19,45-47]. The whole blood concentration may be a poor reflection of the concentration at the site of action, the donor specific T-cell. In pharmacology it is believed that the free or unbound drug concentration in blood would reflect the concentration at the site of action. Probably, in most instances this is the case, but for CNIs transport enzymes on the cell membranes of lymphocytes, such as P-glycoprotein (*ABCB1*), are likely to disturb this relationship [48,49]. Therefore, attempts have been made to measure CNIs in leukocytes or T-cells using LC-MS/MS [45,50]. Concentration measurements at the site of action may be of great benefit to optimization of CNI therapy. At least it would be a more sophisticated way of defining a patient's drug exposure than the present use of measurements in whole blood. With the current approach ciclosporin and tacrolimus concentrations actually reflect the amount in the red blood cell, with around 60% and 80% being bound in these cells respectively [11,12]. Besides, the effect of CNI-metabolites should be taken into account as well. Especially, since immunoassay and LC-MS/MS techniques are both used extensively at this time and as described above they differ in metabolite interference.

The second reason constitutes the target enzyme of CNIs, calcineurin. Ciclosporin A and tacrolimus exert their drug effect by inhibition of the calcineurin activity in T-cells. Dephosporylation of the nuclear factor of activated T-cells (NFAT) is inhibited resulting in decreased gene transcription of pro-inflammatory mediators or cytokines. CNIs inhibit calcineurin by a-specific binding of CNIs next to the active site of calcineurin. Since CNIs bind to immunophilins they are able to sterically hinder the active site of calcineurin. This non-competitive way of enzyme inhibition could be variable since the susceptibility of this system is likely to vary among transplant recipients. Patients could differ in the maximum effect or E_{max}, which provides variability among transplant recipients in the maximum inhibition of calcineurin. Another parameter which could be relevant is the potency or IC_{50}. Although CNIs act by a non-competitive way of inhibition of calcineurin, patients still could differ in the potency, due to genetic variability in the genes coding for calcineurin, leading to (conformational) changes in the structure of the enzyme and possibly altered affinity of the CNI for calcineurin. In addition, differences in the activity of P-glycoprotein on T-lymphocytes could cause variability in the concentration that reaches the active [51-53].

Variability in the susceptibility for ciclosporin A between patients was tested by developing a biomarker based on inhibition of calcineurin activity (Chapter 6). A clear concentra-

tion *versus* effect relationship was observed between ciclosporin A concentration in whole blood and calcineurin activity in leukocytes obtained from 98 renal transplant recipients followed for 6 months after transplantation. However, between patient variability in the biomarker was too small (13% *inter*-individual variability in E_{max}) to explain differences in susceptibility for ciclosporin A. Interestingly, within patient variability was high, 28%, which raised concerns regarding the methodology of calcineurin activity measurements in leukocytes. Clearly, the development of a clinical useful biomarker is complex. To be able to quantify calcineurin activity *in vitro*, concessions are being made. First of all the concentration is whole blood is correlated to calcineurin activity in leukocytes. The concentration in whole blood may not represent the concentration within the leukocytes and the active site of the enzyme. Furthermore, leukocytes consist of granulocytes, monocytes and lymphocytes with different calcineurin activity, which urges more specific measurement in for instance T-lymphocytes [54]. To be able to measure calcineurin phosphatase activity, the activity of other phosphatases has to be eliminated by using okadaic acid and EGTA. There is no guarantee that this is a successful approach and that all disturbing phosphatases are ruled out. Moreover, the calcineurin activity actually is a capacity measurement where the enzyme is maximally stimulated *in vitro* to attain maximum activity. This may not be a very reproducible approach. These assumptions may not be a problem if an adequate quality control exists for these measurements. In contrast to concentration measurements where the use of a quality control sample is common and essential, this is still in development for enzyme activity measurements. Of course it is much more complex to develop an adequate quality control since frozen storage is detrimental to the activity of a control sample. Whereas researchers worldwide are working already for over 15 years on the development of calcineurin activity as a biomarker, still no breakthrough in terms of clinical relationships has been reported. The study presented in Chapter 6 was the first to report on calcineurin activity in a population approach using data from multiple occasions. This provided insight in the behavior of this biomarker in transplant recipients in time, and explains the current lack of information on the association between *in vitro* enzyme activity and acute rejection or other clinical outcome measures. In fact, the high within patient variability presented in chapter 6 allows questioning previous reports on the relationship between calcineurin activity on a single time point with either nephrotoxicity/acute rejection after liver transplantation [55] or graft versus host disease after bone marrow transplantation [56,57]. Despite the high importance of demonstrating clinical relationships, chapter 6 illustrates the complexity of (immunological) biomarker development and underlines the importance to analyze repeated measurements of the biomarker in human material and to apply a population approach. A more precise and accurate technique for calcineurin activity measurement is necessary and the development of alternative biomarkers should be explored.

(Sub-)clinical relationships with CNI exposure and pharmacogenetics

The quadruple immunosuppressive regimen that currently is used throughout the world is capable of decreasing the occurrence of acute rejection episodes to around 10% (Chapter 7). CNIs are part of this regimen and are the first choice to taper or withdraw as soon as possible after transplantation. To be able to do this safely, information should

be obtained on the risk of rejection after decreasing the level of immunosuppression. In fact individualized tapering regimes are required. In this respect adequate biomarkers are important [58]. By means of biomarkers the choice of the most effective and least toxic combination of immunosuppressive drugs, and their doses, could be determined. Cur-

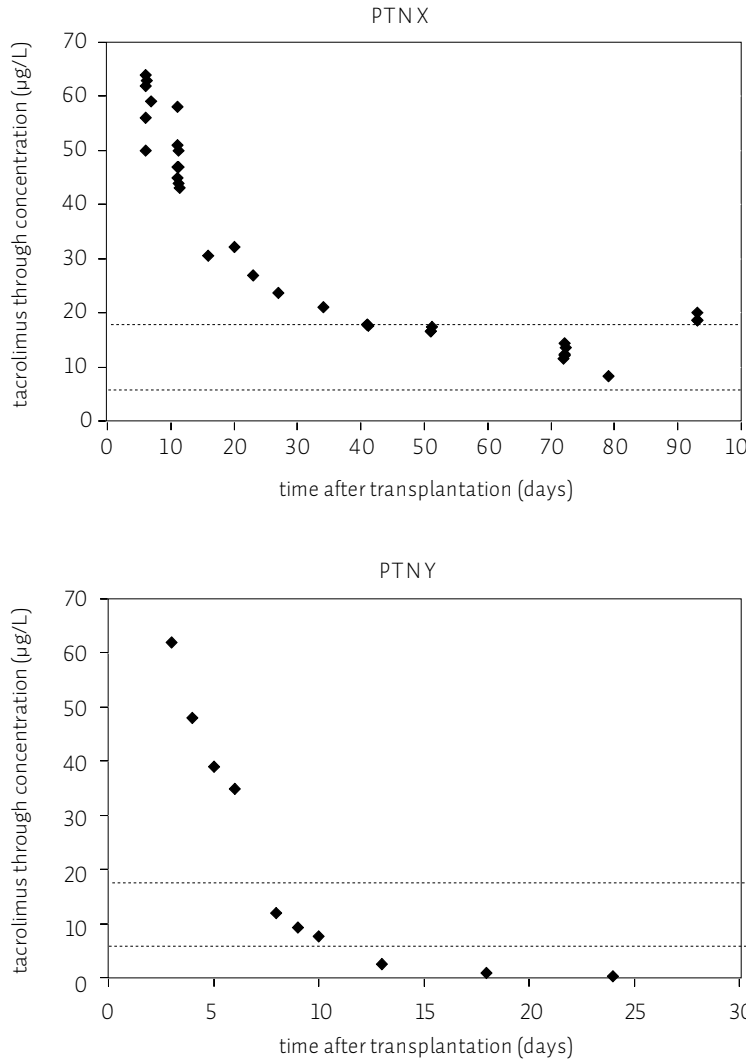

Figure 1. Tacrolimus concentration *versus* time after transplantation, for two renal transplant recipients. These figures illustrate excessive exposure for transplant recipients in the first weeks after transplantation. No interactions or other conditions were present that could explain the high exposure after an initial (lean) body weight based tacrolimus doses of 8 and 5 mg b.i.d. respectively. Target trough exposure is indicated with the solid line (between 5 and 18 µg/L). Patient X was a 34 years old male and weighed 100 kg when transplanted in 2005 and patient Y was a 67 years old female renal transplant recipient and weighed 55 kg when transplanted in 2010.

rently, for this purpose non-invasive biomarkers are not available, the only reliable marker is a renal biopsy. To safely withdraw immunosuppressive drugs a biopsy should show no signs of acute rejection, also not in the absence of functional kidney deterioration, so called subclinical rejection (SCR). But, more importantly SCR may be related to chronic damage to the kidney, so called interstitial fibrosis/tubular atrophy. Therefore insight into the factors determining SCR should be obtained and was the object of study in Chapter 7. In a multicenter study, 361 renal transplant recipients were followed for 6 months after transplantation and a renal biopsy was obtained at 6 months. Covariates were selected that could theoretically be related to this outcome measure and concerned besides demographic (age) and transplant related factors (donor information, HLA-matching, transplant type), exposure data ($AUC_{0\text{-}12h}$) and pharmacogenetic information. Of interest were variability in genes (possibly) related to metabolism and transport of ciclosporin A: *ABCB1*, *CYP3A5*, *CYP2C8*, *NR1I2*.

Besides, genetic variability in the genes encoding the target protein calcineurin were of interest as well. Three isoforms for calcineurin have been described: alpha, beta en gamma [59,60]. There is evidence that the calcineurin alpha form, coded for by the *PPP3CA* gene, is highly expressed in renal tubular cells, while the beta form coded for by *PPP3CB* is primarily expressed in immune cells (lymphocytes). *PPP3CC,* coding for the gamma variant is predominantly in the testis. The clinical relevance of these different isoforms as determinants of inter-individual variation in immune suppression has not been demonstrated yet, but is illustrated by genetic differences between renal transplant recipient and donor. Whereas the kidney originates from the donor with its genetic constitution of the *PPP3CA* gene, the immune system consequences are related to the recipient with is genetic code for the *PPP3CB* gene. To test this hypothesis genetic variability in the *PPP3CB* gene in renal transplant recipients was studied in Chapter 7 by selecting polymorphisms to create a haploblock. A haploblock consisting of 3 polymorphisms was found to reflect genetic variability in the *PPP3CB* gene. To check the assumption regarding the isoforms and variable tissue distribution, a haploblock for the larger *PPP3CA* gene was identified as well and consisted of 5 polymorphisms.

The binary outcome measure SCR was analyzed with an integrated approach, including the number of patients that drop-out during the study and including all covariate information. A biopsy was obtained from 275 patients, of which 18% contained signs of SCR. However, only the experience of a previous acute rejection episode and receiving a cadaveric donor were related to a SCR incidence of 52% versus an incidence of 11% for living donations in the absence of an acute rejection episode. This powerful approach on an AUC targeted population did not identify genetic factors as relevant covariates for SCR. Despite the absence of relationships between the selected genetic factors and SCR, a powerful analysis tool was used. It would be too simple to relate the susceptibility for CNIs solely to genetic variability in calcineurin isoforms, what is often done for other genetic association studies, disregarding the effect of other important factors. Therefore, as reported in Chapter 7 an array of demographic, transplantation related factors and exposure measurements should be taken into account as well, a more systems pharmacology approach. But, as discussed in Chapter 2 one should also study genetic variability in immunophillins and in the nuclear factor of activated T-cells (NFAT) itself. This could be the focus in future projects.

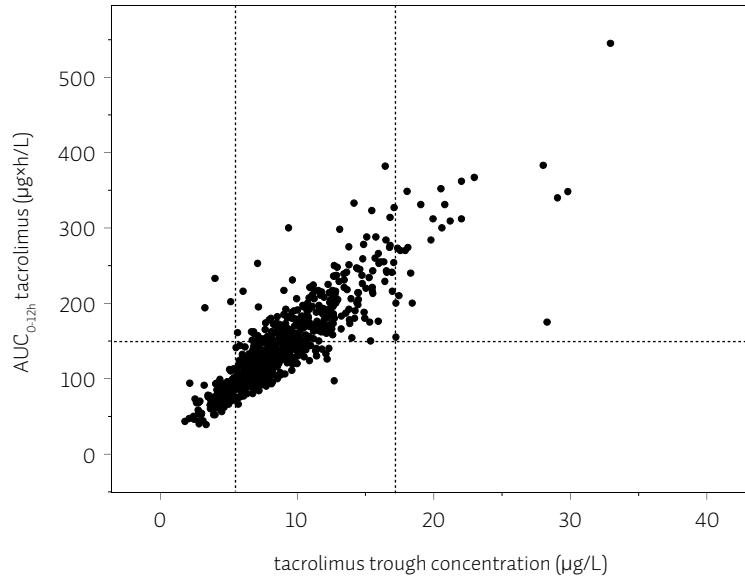

Figure 2. AUC_{0-12h} *versus* trough concentration (n = 734 data couples) for tacrolimus obtained from 343 transplant recipients. Tacrolimus concentrations were determined with LC-MS/MS in the LUMC in the period March 2009 to May 2010.

Perspectives

The one thing we need to achieve is getting the right dose of CNIs, to the right patient, at the right time. In this sentence lies the entire foundation for this thesis. It is not a new approach, but as old as Paracelsus (1493-1541) [61,62]. The attempts made and described in this thesis aimed to achieve this in renal transplantation. Especially since transplantation medicine is pre-eminently the specialism to optimize drug treatment. Balancing between the risk for acute or subclinical rejection on one side and acute toxicity, infection, malignancies, vascular damage and chronic allograft nephropathy (CAN) on the other side, is a great challenge in which important progress has been made in recent years. The biggest challenge in renal transplantation will be to prolong graft survival along with low comorbidity for the recipient.

The key to such improved outcome from a pharmacological perspective will be determined by three factors: the development of biomarkers for immunological risk and biomarkers that reflect the response to the combination of the various immunosuppressive drugs, pharmacometric analysis of the available data and enlargement of the amount of data by co-operations of transplant centers.

The initial immunological risk depends highly on an individuals transplantation characteristics and the extent to which one's immunosuppressive therapy is individualized [63,64]. Immunological risk results from transplant characteristics, such as type of transplantation, HLA-DR mismatch, cold-ischemic time, donor age etc. To attain the optimal

level of immunosuppression, therapy should be adjusted to the level of his or her individual immunologic risk. In clinical practice this is not applied universally, due to the absence of appropriate biomarkers [64]. But, in case organs are transplanted between twins or donor-acceptor couples without HLA-mismatches, immunosuppression will generally be lower compared to patients with higher immunologic risk. Besides the initial immunological risk one should also take the dynamic interplay between the immune system of the transplant recipient, the donor organ and the immunosuppressive drugs into account that develops after transplantation. A distinction between high and low risk patients may be made, ultimately resulting in chronic rejection and tolerance respectively [65]. Generally immunosuppression is stepwise reduced after transplantation towards minimal immunosuppression with two drugs or in certain instances even one immunosuppressant. In the latter cases an almost tolerant state is achieved. The ultimate goal is operational tolerance, where transplant recipients maintain graft function without using immunosuppressive drugs. Currently this is primarily observed in non-compliant patients and is still exceptional in renal transplantation [64,66-68]. In clinical practice the biomarker used to determine the possibility of minimizing immunosuppression is an invasive one, the kidney biopsy. In case no immune cell infiltrates are observed, SCR is absent, and kidney function is stable, one could decide to further reduce maintenance immunosuppression several months after transplantation. Clearly, the need for adequate biomarkers to give insight in the activity of the host's immune response against the foreign organ is warranted.

Besides tailoring the number of immunosuppressants to ones immunological risk the immunosuppressive effect is not solely determined by the dose of the different drugs. As previously discussed, between patient variability in pharmacokinetics and pharmacodynamics are responsible for this. In this respect we now placed biomarkers and finally clinical outcome central in this thesis to elucidate which dose should be administrated to which patient. Individualization of the CNI dose clearly is irrefutable, but has some big challenges to overcome. Obvious it is a complex trait to optimize treatment with 3 or 4 drugs with a different mechanism of action. A combination of biomarkers, each reflecting a drug's action, could assist clinicians. Considering the low number of rejection events with the quadruple therapy, biomarkers could be used to further reduce or individualize immunosuppression.

But, major efforts are expected from employing biomarkers in toxicity control. As soon as susceptibility differences between patients for acute and chronic CNI toxicity are explained, individualization of CNI therapy can truly be applied. With this in mind, the next year's great effort should be put into the development of biomarkers, either related to concentration of the parent compound and its metabolites (at the site of action), target enzyme activity and/or pharmacogenetics (of the target proteins) [58,69]. Emphasis should be placed on methodology of measurements in cells, especially on quality control samples. Furthermore, biomarker research in transplantation should increasingly focus on obtaining more data within the same individual. Until now often limited data obtained from single study visits were obtained.

Important development should be made in the type of analysis and patient numbers. Up to this moment in most cases renal transplant populations of up to roughly 100 patients are studied, mostly aiming at identifying a single relationship. In case multi-factorial

analyses were performed these were highly simplified. The next step would be to study the whole system defined as the donor-recipient combination with its specific treatment, a *systems pharmacology approach*. That would be a more integrative and quantitative analysis, including pharmacokinetic, pharmacodynamic or biomarker and clinical information, such as performed in Chapter 7. Here pharmacokinetic information and clinical outcome are analyzed together to identify relationships with a series of genetic and non-genetic covariates that have a relationship with either pharmacokinetics or pharmacodynamics of a single drug. Between patient and within patient variability can be described and systematically explained by covariate relationships. Including biomarkers that indicate specific activity of the immune system or the kidney as well as biomarkers reflecting the response to the different immunosuppressive drugs in the analysis, would improve this approach even more. The emphasis should be on the development of non-invasive biomarkers, observed in easily obtainable body fluids, such as blood and urine. Especially the latter could cover new ways to identify renal damage or alterations. The response to a combination of immunosuppressive drugs and/or their metabolites should be analyzed together using 3-dimensional response surfaces as has been demonstrated for anesthetic and antiviral drugs [70,71]. The theoretical concepts of the modeling of pharmacodynamic drug-drug interactions have recently been reviewed [72]. Furthermore, after transplantation the immune response alters, it adapts to the altered situation as a result of drug action. One could view that as a form of disease-progression as has been studied in for instance Alzheimer's disease [73,74]. A systems pharmacology approach [75], which should use pharmacometrics to its full extent, should be employed. Pharmacometrics concerns the comprehensive mathematical-statistical analysis of drug action. This thesis embodies this approach with the application of population analysis of pharmacokinetics, pharmacodynamics and clinical events. In future analysis these models should be extended incorporating changes in the immune system by including an immune system ('disease') progression model and 3-dimensional response surface analysis to account for interaction between the 2 or more immunosuppressive drugs that are typically used. Herewith, therapy with multiple drugs could be optimized, especially when appropriate biomarkers have become available.

Large collaborations of nephrologists should work together with clinical pharmacologists or hospital pharmacists to create large patient cohorts adding up to large databases, which can be employed to quantitatively analyze the data of multiple drugs simultaneously with a pharmacometric approach.

Clearly data collection, the development of biomarkers and models reflect an enormous amount of work and a lot of effort will have to put in to it, as we are only at the very beginning of understanding the immune system and the intervention of immunosuppressive drugs. Yet, the rate limiting step will be the development of adequate biomarkers. Although it is a magnificent challenge to describe such a complex system and to explain variability in treatment response with success not being guaranteed, this is the only successful approach to really individualize immunosuppressive therapy in renal transplantation.

Conclusions

Therapy should be optimized for drugs with a small therapeutic window and high between-patient variability in drug response, such as the calcineurin inhibitors (CNI). The initial body weight based dosed must be adjusted in renal transplant recipients to a predefined target blood-exposure to balance between acute rejection and (nephro)toxicity. With this so called therapeutic drug monitoring approach a beginning has been made to individualize CNI therapy. This thesis demonstrates that pharmacometric approaches allow us to identify factors responsible for variability in exposure and response to CNIs and to discriminate between these effects and their weight. The CYP3A5*1 allele is predictive for at least a 50% higher clearance of tacrolimus. This factor can be used to individualize the tacrolimus starting dose. Besides, the interactive effect of co-administered prednisolone should be accounted for as well. To date, AUC-monitoring combined with an early trough concentration measurement remains state of the art monitoring for CNIs in transplant medicine. This thesis demonstrates that effect biomarkers such as calcineurin activity are still in their infancy due to technical and methodological issues. Optimization and validation of these assays with human material and developing an appropriate quality control sample are essential. Furthermore, repeated measurements should be analyzed early in assay development, preferably in combination with a population approach. Finally, clinical events should be analyzed in an integrative approach as performed in the final chapter of this thesis. Especially when large data sets are analyzed with a sophisticated pharmacometric approach major developments are expected. In future analysis response biomarkers should be included that reflect the action of the combination of drugs used. When this information is combined with markers that reflect the activity of the immune system against the transplanted organ understanding of the pharmacological approach will improve substantially with true individualization of immunosuppressive therapy as a result.

1. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; 351(26):2715-2729.
2. Solez K, Vincenti F, Filo RS. Histopathologic findings from 2-year protocol biopsies from a U.S. multicenter kidney transplant trial comparing tarolimus versus cyclosporine: a report of the FK506 Kidney Transplant Study Group. *Transplantation* 1998; 66(12):1736-1740.
3. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 2002; 346(8):580-590.
4. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *Am J Transplant* 2004; 4(3):378-383.
5. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Chapman JR, Allen RD. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. *Transplantation* 2004; 78(4):557-565.
6. Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gurkan A *et al.* Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med* 2007; 357(25):2562-2575.
7. Ekberg H. Calcineurin inhibitor sparing in renal transplantation. *Transplantation* 2008; 86(6):761-767.
8. Fishman JA. Infection in solid-organ transplant recipients. *N Engl J Med* 2007; 357(25):2601-2614.
9. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ *et al.* Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med* 2002; 347(7):488-496.
10. Manitpisitkul W, Drachenberg C, Ramos E, Munivenkatappa R, Philosophe B, Klassen D *et al.* Maintenance immunosuppressive agents as risk factors for BK virus nephropathy: a case-control study. *Transplantation* 2009; 88(1):83-88.
11. McMillan MA. Clinical pharmacokinetics of cyclosporin. *Pharmacol Ther* 1989; 42(1):135-156.
12. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clin Pharmacokinet* 2004; 43(10):623-653.
13. Oellerich M, Armstrong VW, Schutz E, Shaw LM. Therapeutic drug monitoring of cyclosporine and tacrolimus. Update on Lake Louise Consensus Conference on cyclosporin and tacrolimus. *Clin Biochem* 1998; 31(5):309-316.
14. Clase CM, Mahalati K, Kiberd BA, Lawen JG, West KA, Fraser AD *et al.* Adequate early cyclosporin exposure is critical to prevent renal allograft rejection: patients monitored by absorption profiling. *Am J Transplant* 2002; 2(8):789-795.
15. Lindholm A, Kahan BD. Influence of cyclosporine pharmacokinetics, trough concentrations, and AUC monitoring on outcome after kidney transplantation. *Clin Pharmacol Ther* 1993; 54(2):205-218.
16. Mahalati K, Belitsky P, Sketris I, West K, Panek R. Neoral monitoring by simplified sparse sampling area under the concentration-time curve: its relationship to acute rejection and cyclosporine nephrotoxicity early after kidney transplantation. *Transplantation* 1999; 68(1):55-62.
17. MacPhee IA, Fredericks S, Tai T, Syrris P, Carter ND, Johnston A *et al.* The influence of pharmacogenetics on the time to achieve target tacrolimus concentrations after kidney transplantation. *Am J Transplant* 2004; 4(6):914-919.
18. Rosano TG. Effect of hematocrit on cyclosporine (cyclosporin A) in whole blood and plasma of renal-transplant patients. *Clin Chem* 1985; 31(3):410-412.
19. Vine W, Flye MW, Jatlow P. Relationships of cyclosporine concentrations in serum, whole blood, and bile after renal and hepatic transplantation. *Clin Chem* 1986; 32(10):1828-1831.
20. Beal SL, Sheiner L.B., Boeckmann AJ. NONMEM Users Guide (1989-2006). 2006. Icon Development Solutions. Ref Type: Computer Program
21. Press RR, Ploeger BA, den Hartigh J, van der Sraaten T, van Pelt J, Danhof M *et al.* Explaining variability in tacrolimus pharmacokinetics to optimize early exposure in adult kidney transplant recipients. *Ther Drug Monit* 2009; 31(2):187-197.
22. Press RR, Ploeger BA, den Hartigh J, van der Sraaten T, van Pelt H, Danhof M *et al.* Explaining variability in ciclosporin exposure in adult kidney transplant recipients. *Eur J Clin Pharmacol* 2010.
23. van Schaik RH, van der Heiden, I, van den Anker JN, Lindemans J. CYP3A5 variant allele frequencies in Dutch Caucasians. *Clin Chem* 2002; 48(10):1668-1671.
24. Haufroid V, Wallemacq P, VanKerckhove V, Elens L, De MM, Eddour DC *et al.* CYP3A5 and ABCB1 polymorphisms and tacrolimus pharmacokinetics in renal transplant candidates: guidelines from an experimental study. *Am J Transplant* 2006; 6(11):2706-2713.
25. Daly AK. Significance of the minor cytochrome P450 3A isoforms. *Clin Pharmacokinet* 2006; 45(1):13-31.
26. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J *et al.* Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 2001; 27(4):383-391.
27. Ng FL, Holt DW, Chang RW, MacPhee IA. Black renal transplant recipients have poorer long-term graft survival than CYP3A5 expressers from other ethnic groups. *Nephrol Dial Transplant* 2010; 25(2):628-634.
28. Malat GE, Culkin C, Palya A, Ranganna K, Kumar MS. African American kidney transplantation survival: the ability of immunosuppression to balance the inherent pre- and post-transplant risk factors. *Drugs* 2009; 69(15):2045-2062.
29. Christians U, Jacobsen W, Benet LZ, Lampen A. Mechanisms of clinically relevant drug interactions associated with tacrolimus. *Clin Pharmacokinet* 2002; 41(11):813-851.
30. Dunn CJ, Wagstaff AJ, Perry CM, Plosker GL, Goa KL. Cyclosporin: an updated review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (neoral)1 in organ transplantation. *Drugs* 2001; 61(13):1957-2016.
31. Campana C, Regazzi MB, Buggia I, Molinaro M. Clinically significant drug interactions with cyclosporin. An update. *Clin Pharmacokinet* 1996; 30(2):141-179.
32. Anglicheau D, Flamant M, Schlageter MH, Martinez F, Cassinat B, Beaune P *et al.* Pharmacokinetic interaction between corticosteroids and tacrolimus after renal transplantation. *Nephrol Dial Transplant* 2003; 18(11):2409-2414.
33. Hesselink DA, Ngyuen H, Wabbijn M, Gregoor PJ, Steyerberg EW, van R, I *et al.* Tacrolimus dose requirement in renal transplant recipients is significantly higher when used in combination with corticosteroids. *Br J Clin Pharmacol* 2003; 56(3):327-330.
34. Lam S, Partovi N, Ting LS, Ensom MH. Corticosteroid interactions with cyclosporine, tacrolimus, mycophenolate, and sirolimus: fact or fiction? *Ann Pharmacother* 2008; 42(7):1037-1047.
35. Falck P, Midtvedt K, Van Le TT, Storehagen L, Holdaas H, Hartmann A *et al.* A population pharmacokinetic model of ciclosporin applicable for assisting dose management of kidney transplant recipients. *Clin Pharmacokinet* 2009; 48(9):615-623.
36. Fanta S, Jonsson S, Karlsson MO, Niemi M, Holmberg C, Hoppu K *et al.* Long-term changes in cyclosporine pharmacokinetics after renal transplantation in children: evidence for saturable presystemic metabolism and effect of NR1I2 polymorphism. *J Clin Pharmacol* 2010; 50(5):581-597.
37. Saint-Marcoux F, Marquet P, Jacqz-Aigrain E, Bernard N, Thiry P, Le MY *et al.* Patient characteristics influencing ciclosporin pharmacokinetics and accurate Bayesian estimation of ciclosporin exposure in heart, lung and kidney transplant patients. *Clin Pharmacokinet* 2006; 45(9):905-922.
38. Lamba J, Lamba V, Schuetz E. Genetic variants of PXR (NR1I2) and CAR (NR1I3) and their implications in drug metabolism and pharmacogenetics. *Curr Drug Metab* 2005; 6(4):369-383.
39. Chen YL, Hirabayashi H, Akhtar S, Pelzer M, Kobayashi M. Simultaneous determination of three isomeric metabolites of tacrolimus (FK506) in human whole blood and plasma using high performance liquid chromatography-

tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; 830(2):330-341.
40. Gonschior AK, Christians U, Winkler M, Linck A, Baumann J, Sewing KF. Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients. *Clin Chem* 1996; 42(9):1426-1432.
41. Iwasaki K, Shiraga T, Matsuda H, Nagase K, Tokuma Y, Hata T *et al.* Further metabolism of FK506 (tacrolimus). Identification and biological activities of the metabolites oxidized at multiple sites of FK506. *Drug Metab Dispos* 1995; 23(1):28-34.
42. Iwasaki K, Shiraga T, Nagase K, Tozuka Z, Noda K, Sakuma S *et al.* Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. *Drug Metab Dispos* 1993; 21(6):971-977.
43. Tamura K, Fujimura T, Iwasaki K, Sakuma S, Fujitsu T, Nakamura K *et al.* Interaction of tacrolimus(FK506) and its metabolites with FKBP and calcineurin. *Biochem Biophys Res Commun* 1994; 202(1):437-443.
44. Oellerich M, Armstrong VW. The role of therapeutic drug monitoring in individualizing immunosuppressive drug therapy: recent developments. *Ther Drug Monit* 2006; 28(6):720-725.
45. Ansermot N, Fathi M, Veuthey JL, Desmeules J, Hochstrasser D, Rudaz S. Quantification of cyclosporine A in peripheral blood mononuclear cells by liquid chromatography-electrospray mass spectrometry using a column-switching approach. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 857(1):92-99.
46. Wallemacq PE, Firdaous I, Hassoun A. Improvement and assessment of enzyme-linked immunosorbent assay to detect low FK506 concentrations in plasma or whole blood within 6 hours. *Clin Chem* 1993; 39(6):1045-1049.
47. Lensmeyer GL, Wiebe DA, Carlson IH. Distribution of cyclosporin A metabolites among plasma and cells in whole blood: effect of temperature, hematocrit, and metabolite concentration. *Clin Chem* 1989; 35(1):56-63.
48. Crettol S, Venetz JP, Fontana M, Aubert JD, Ansermot N, Fathi M *et al.* Influence of ABCB1 genetic polymorphisms on cyclosporine intracellular concentration in transplant recipients. *Pharmacogenet Genomics* 2008; 18(4):307-315.
49. Elens L, Capron A, Kerckhove VV, Lerut J, Mourad M, Lison D *et al.* 1199G>A and 2677G>T/A polymorphisms of ABCB1 independently affect tacrolimus concentration in hepatic tissue after liver transplantation. *Pharmacogenet Genomics* 2007; 17(10):873-883.
50. Capron A, Musuamba F, Latinne D, Mourad M, Lerut J, Haufroid V *et al.* Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in peripheral blood mononuclear cells. *Ther Drug Monit* 2009; 31(2):178-186.
51. Hemenway CS, Heitman J. Calcineurin. Structure, function, and inhibition. *Cell Biochem Biophys* 1999; 30(1):115-151.
52. Kissinger CR, Parge HE, Knighton DR, Lewis CT, Pelletier LA, Tempczyk A *et al.* Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* 1995; 378(6557):641-644.
53. Etzkorn FA, Chang ZY, Stolz LA, Walsh CT. Cyclophilin residues that affect noncompetitive inhibition of the protein serine phosphatase activity of calcineurin by the cyclophilin.cyclosporin A complex. *Biochemistry* 1994; 33(9):2380-2388.
54. van Rossum HH, Romijn FP, Sellar KJ, Smit NP, van der Boog PJ, de Fijter JW *et al.* Variation in leukocyte subset concentrations affects calcineurin activity measurement: implications for pharmacodynamic monitoring strategies. *Clin Chem* 2008; 54(3):517-524.
55. Fukudo M, Yano I, Masuda S, Fukatsu S, Katsura T, Ogura Y *et al.* Pharmacodynamic analysis of tacrolimus and cyclosporine in living-donor liver transplant patients. *Clin Pharmacol Ther* 2005; 78(2):168-181.
56. Pai SY, Fruman DA, Leong T, Neuberg D, Rosano TG, McGarigle C *et al.* Inhibition of calcineurin phosphatase activity in adult bone marrow transplant patients treated with cyclosporine A. *Blood* 1994; 84(11):3974-3979.
57. Sanquer S, Schwarzinger M, Maury S, Yakouben K, Rafi H, Pautas C *et al.* Calcineurin activity as a functional index of immunosuppression after allogeneic stem-cell transplantation. *Transplantation* 2004; 77(6):854-858.
58. Danhof M, Alvan G, Dahl SG, Kuhlmann J, Paintaud G. Mechanism-based pharmacokinetic-pharmacodynamic modeling-a new classification of biomarkers. *Pharm Res* 2005; 22(9):1432-1437.
59. Jiang H, Xiong F, Kong S, Ogawa T, Kobayashi M, Liu JO. Distinct tissue and cellular distribution of two major isoforms of calcineurin. *Mol Immunol* 1997; 34(8-9):663-669.
60. Kung L, Batiuk TD, Palomo-Pinon S, Noujaim J, Helms LM, Halloran PF. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. *Am J Transplant* 2001; 1(4):325-333.
61. Peck CC, Cross JT. "Getting the dose right": facts, a blueprint, and encouragements. *Clin Pharmacol Ther* 2007; 82(1):12-14.
62. Stanski DR, Rowland M, Sheiner LB. Getting the Dose Right: report from the Tenth European Federation of Pharmaceutical Sciences (EUFEPS) conference on optimizing drug development. *J Pharmacokinet Pharmacodyn* 2005; 32(2):199-211.
63. Kahan BD. Individuality: the barrier to optimal immunosuppression. *Nat Rev Immunol* 2003; 3(10):831-838.
64. Sawitzki B, Pascher A, Babel N, Reinke P, Volk HD. Can we use biomarkers and functional assays to implement personalized therapies in transplantation? *Transplantation* 2009; 87(11):1595-1601.
65. shton-Chess J, Giral M, Soulillou JP, Brouard S. Using biomarkers of tolerance and rejection to identify high- and low-risk patients following kidney transplantation. *Transplantation* 2009; 87(9 Suppl):S95-S99.
66. Roussey-Kesler G, Giral M, Moreau A, Subra JF, Legendre C, Noel C *et al.* Clinical operational tolerance after kidney transplantation. *Am J Transplant* 2006; 6(4):736-746.
67. Golshayan D, Pascual M. Tolerance-inducing immunosuppressive strategies in clinical transplantation: an overview. *Drugs* 2008; 68(15):2113-2130.
68. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation--how much of the promise has been realized? *Nat Med* 2005; 11(6):605-613.
69. Rolan P, Atkinson AJ, Jr., Lesko LJ. Use of biomarkers from drug discovery through clinical practice: report of the Ninth European Federation of Pharmaceutical Sciences Conference on Optimizing Drug Development. *Clin Pharmacol Ther* 2003; 73(4):284-291.
70. Minto CF, Schnider TW, Short TG, Gregg KM, Gentilini A, Shafer SL. Response surface model for anesthetic drug interactions. *Anesthesiology* 2000; 92(6):1603-1616.
71. Prichard MN, Shipman C, Jr. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res* 1990; 14(4-5):181-205.
72. Jonker DM, Visser SA, van der Graaf PH, Voskuyl RA, Danhof M. Towards a mechanism-based analysis of pharmacodynamic drug-drug interactions in vivo. *Pharmacol Ther* 2005; 106(1):1-18.
73. Holford NH, Peace KE. Methodologic aspects of a population pharmacodynamic model for cognitive effects in Alzheimer patients treated with tacrine. *Proc Natl Acad Sci U S A* 1992; 89(23):11466-11470.
74. Chan PL, Holford NH. Drug treatment effects on disease progression. *Annu Rev Pharmacol Toxicol* 2001; 41:625-659.
75. Post TM, Freijer JI, DeJongh J, Danhof M. Disease system analysis: basic disease progression models in degenerative disease. *Pharm Res* 2005; 22(7):1038-1049.