ABSTRACT

Patient variability in clinical response to the calcineurin inhibitors (CNIs) ciclosporin A and tacrolimus partly results from differences in CNI exposure. For tacrolimus drug interactions and genetic variability relate to tacrolimus exposure. Patients carrying the CYP3A5*1 allele have an increased tacrolimus metabolism, hence lower drug exposure. Adjusting the tacrolimus dose to this genotype is a tool to optimize therapy from a pharmacokinetic perspective. In contrast, no genetic variants have been found to clearly relate to ciclosporin A exposure. Despite therapeutic drug monitoring aimed at individualizing CNI therapy, patients still suffer from acute or chronic rejection and CNI toxicity. To further optimize CNI therapy future research may incorporate genetic polymorphisms in proteins involved in CNI pharmacodynamics (i.e. drug target). Proteins potentially relevant for drug response are calcineurin and the CNI binding proteins immunophilins. Moreover, since the expression of the nuclear factor of activated T-cells (NFAT) is reduced after calcineurin inhibition, genetic polymorphisms in the genes encoding NFAT may also be interesting candidates for studying inter-patient differences in CNI efficacy and toxicity. In addition, the existence of isoforms and differences in tissue distribution of the calcineurin protein could potentially explain variable drug response. At present, the focus has been on the metabolism of CNIs and not on variability in the drug target. Therefore, future improvements in CNI therapy are likely to occur from a systems pharmacology approach taking into account genetic markers for both CNI pharmacokinetics and pharmacodynamics.
Introduction

The use of tacrolimus (TRL) and ciclosporin A (CsA) in renal transplantation is associated with severe toxicity, such as acute and chronic nephrotoxicity, diabetes, liver- and neurotoxicity [1,2]. Toxicity is the main reason for the current trend to minimize and even withdraw the calcineurin inhibitors (CNIs) as early as possible after renal transplantation [3]. However, CNIs remain the primary immunosuppressant in most renal transplantation protocols since immunosuppressive therapy with these drugs has led to acute rejection rates as low as 10% at 1-year after transplantation [1]. This important clinical achievement has benefited from individualized dosing achieved by therapeutic drug monitoring (TDM), aiming at target CNI blood exposure in terms of area-under-the-blood-concentration-versus-time-curve (AUC), trough level or C2-level [4]. Indeed, TDM has become routine clinical practice in renal transplantation and is related to lower acute rejection rates as well as lower toxicity rates [5,6].

Dose individualization and reaching adequate drug exposure typically takes days to weeks due to factors such as co-administration of interacting drugs and changing physiology of the transplant recipient. Moreover, the genetic constitution of enzymes involved in the metabolism of CNIs, such as CYP3A5*1 allele, is associated with an increased time to achieve target exposure [7,8] and potentially impacts acute rejection rates. However, acute rejection episodes are observed in graft recipients with adequate CNI exposure and appropriate transplant related factors such as HLA-matching. In addition, patients that experience CNI toxicity do include individuals with adequate drug exposure and vice versa. Clearly, drug response to immunosuppressants is a complex trait and is determined by many factors. To optimize immunosuppressive therapy early after transplantation it is necessary to understand the factors that contribute to the variability in drug response. Pharmacokinetic information alone appears to be insufficient to explain the total variability of CNI response. To further optimize CNI therapy, clinical variability in pharmacodynamic drug targets may be interesting, but is yet marginally explored for CNIs in renal transplantation [9]. Additionally, (patient) characteristics such as demographics, co-administration of interacting drugs and diarrhea [7] may be taken into account in developing predictive models and representing a systems pharmacology approach.

This review describes the sources of variability in CNI therapy in renal transplantation with an emphasis on variability in genes encoding drug metabolizing enzymes and drug targets.

Variability in CNI pharmacokinetics

CNIs display a large variability in exposure upon standard oral dosing. Naturally, variability in drug bioavailability and clearance are the reasons for the variable exposure which originates in the intestine and the liver [7]. For TRL a range in apparent clearance (CL/F) of 15-70 L/h [10,11] has been described and for bioavailability (F) a range from 5 to 93% with a median of 25% [7,12]. For CsA (Neoral®) a median apparent clearance of 29 L/h with a range from 20-50 L/h [13-15] and a bioavailability ranging from 16 to 55% [16-18] has been reported. This variability in apparent clearance after oral administration of CNIs is
multi-causal and relates first of all to intestinal motility. An uremic intestine in dialysis patients or delayed emptying of the stomach in diabetics is likely to affect drug absorption [7,19-21]. Food is thought to reduce the uptake of CNIs, while diarrhea increases exposure to TRL [22-24]. Subsequently, metabolic or efflux proteins in cells lining the intestinal wall and/or in the liver are present to eliminate CNIs out of the body [7,25]. This first-pass metabolism is the main reason that CNIs have a relatively low and highly variable bioavailability. The cytochromic enzymes CYP3A4 and CYP3A5 are responsible for metabolism of CNIs in the intestine and the liver [26-28] (Figure 1). Variable metabolic activity originates from inherited differences in metabolic capacity of specific isozymes in the liver accompanied by acquired differences due to co-medication of drugs, hepatitis or other sources of liver dysfunction or failure [7,26,29-35]. Moreover, the efflux transporter P-glycoprotein (ABCB1, MDR1) is responsible for transporting the drug out of the intestinal cell or removing it from liver cells into the bile [26-28].

Figure 1. The variability in absorption and elimination of CNIs upon oral administration. The drug efflux pump P-glycoprotein is present in the intestine and acts together with the cytochrome enzyme subfamily CYP3A, present in the intestine and liver, to eliminate the CNIs from the body. The reflection of this process is represented by the typical CNI blood concentration versus time curve in the right part of the plot, with the trough level, Cmax and tmax indicated. The dotted circle inlay in the upper right part depicts the variability in the absorption process and demonstrates the variability in trough level as well as Cmax in relation to total exposure (AUC). The three curves reflect the absorption of the typical patient (solid line), a slow absorption profile (dotted line, i.e. diabetic), a low absorption profile (small striped line) and a flat absorption profile (wide striped line). P-glycoprotein (P-gp), cytochrome CYP3A-enzymes (CYP), maximum concentration after dosing (Cmax), time after dose when maximum concentration occurs (tmax), calcineurin inhibitor (CNI).
As soon as CNIs have passed the metabolizing organs they distribute within the vasculature and throughout the body while bound to serum albumin, lipoproteins, α-acid-glycoprotein and binding proteins in the red blood cells [7,25,36-40]. Especially, early after transplantation these factors tend to be highly variable and are a major source of variability in CNI pharmacokinetics [41,42]. Ultimately these drugs bind in tissues to binding proteins such as FK-BP and cyclophilin [43]. Distribution throughout the body is influenced by drug transporters such as P-glycoprotein at the blood brain barrier, kidneys and T-cells. Tissue specific expression of P-glycoprotein is likely to be responsible for selective tissue distribution of CNIs [44]. This could potentially explain variability in drug effects (T-cell) and toxicity such as nephro- and neurotoxicity.

In the present situation, CNI therapy is guided by measuring drug concentrations in blood. These measurements have an emphasis on the absorption phase which typically covers the first 4-hours after dose intake. This absorption profiling is performed to deal with the variability as discussed above and is a reflection of the systemic exposure after oral drug administration [45-47] (Figure 1). Whole blood is selected for routine clinical practice due to the highly variable measurements in plasma [36,37,40,48-50]. The measurement of the unbound concentration of these drugs would be the preferred method as it theoretically reflects best the concentration available for the target cell, but this remains a time-consuming and difficult technique [38,40,51]. The AUC has been demonstrated to relate to clinical outcome in renal transplantation in terms of nephrotoxicity and acute rejection [5,6]. Despite the fact that it has been shown that TRL and CsA trough levels relate poorly to the AUC they are still widely used in routine clinical practice, because this is a more practical way of monitoring for most centers [45,46]. Also CsA C2 levels have been used to estimate CsA exposure. The C2 reflects the concentration 2 hours after dosing and is thought to be equal to the maximum blood-concentration (Cmax). However, due to high variability in the time after drug administration to reach the maximum blood-concentration (tmax), this method is not considered first choice [52-57] (Figure 1). A mini-AUC obtained by a limited sampling Bayesian model is the best reflection of the total exposure, hence the preferred approach for routine TDM [15,45,46].

TDM is an adaptive strategy after administration of a starting dose based only upon the patient’s bodyweight. In order to reach the CNI target level as early as possible after transplantation and to limit the number of monitoring visits, additional markers for initial CNI dosing are necessary. In the previous section it has been described that CYP3A4, CYP3A5 and P-glycoprotein have a role in the absorption and metabolism of CNIs, and therefore genetic variants in the genes encoding these enzymes may be useful in individualizing CNI therapy. Moreover, the nuclear pregnane X receptor (PXR) is involved in the regulation of gene expression of the CYP3A and MDR-1 genes and may thus play an indirect role in CNI disposition and metabolism [58-64]. The genes encoding these enzymes are highly polymorphic and genetic variability may therefore be the cause of inter-individual variability in pharmacokinetics.

**CYP3A4 and CYP3A5**

*In vitro* experiments have demonstrated that CYP3A5 is involved in the metabolism of TRL [65,66]. In fact, the variability in TRL exposure between renal transplant recipients
is shown to be related to a genetic polymorphism in CYP3A5. Specifically, carriers of the CYP3A5*1 allele have a lower concentration-to-dose ratio [67-71] or a lower AUC [71-73] as compared to CYP3A5*3 allele carriers. However, whether this affects clinical outcome, such as acute rejection, remains uncertain since no conclusive study has been presented so far mainly due to intensive TDM protocols, limited sample size and the absence of measurements of total exposure [74,75]. An informative study would be to identify the effect of CYP3A5 on subclinical rejection, by obtaining protocol biopsies 6 months after transplantation in a large cohort of renal transplant patients, using a sensitive measure for drug exposure (AUC) and accounting for other factors known to influence the exposure of TRL [76]. Interestingly, CYP3A5 genotype is related to the time to achieve target concentration of TRL. In clinical practice it could take days to weeks to achieve target TRL exposure in carriers of the CYP3A5*1 allele, whereas in CYP3A5*3 allele carriers target TRL exposure was observed directly or in the first days after transplantation [8,77]. In contrast to CYP3A5, contradictory reports are published with regard to CYP3A4*1B as a marker for TRL exposure [68,77]. Hesselink et al. reported a lower TRL dose-adjusted trough level for CYP3A4*1B carriers compared to the wild type (*1/*1) genotype [68], while Roy et al. did not find a relationship between CYP3A4 polymorphisms and TRL pharmacokinetics [77]. These findings indicate that the CYP3A5 marker for TRL exposure has greater potential clinical relevance than the CYP3A4 genotype.

Despite the fact that CsA is mainly metabolized by CYP3A4 in the liver [27], genetic variants in the CYP3A4 gene were found not associated with CsA exposure in kidney transplant recipients [68]. The CYP3A4*1B genotype overall was not related to a pharmacokinetic parameter for CsA [78]. However, a small effect was reported which consisted of a 9% higher apparent clearance in carriers of the CYP3A4*1B allele [14]. This study was performed in a mixed transplant population (heart, kidney) without incorporation of the effect of a concomitant prednisolone dose in the model. In addition, no association was found of the CYP3A5*1 allele with CsA exposure, which is in line with the observation that CYP3A5 is thought to play only a minor role in CsA metabolism [78-81].

P-glycoprotein

The drug transporter P-glycoprotein is encoded by the ATP-binding cassette B1 gene (ABCB1). Several genetic variants in the ABCB1 gene, such as C3435T, C1236T, G2677T, T-129C have been described in relation to TRL exposure [68,70,72,73,81-84]. Recent reports suggest that these genetic variants of ABCB1 are not related to TRL exposure [70-73,85], while Roy et al. showed that less than three copies or SNPs of ABCB1 polymorphisms (T-129C, C3435T and G2677T) are associated with lower TRL blood levels [77], hence requiring higher TRL doses. In contrast, a study by Anglicheau et al. identified the SNP ABCB1 G2677T to be related to higher TRL concentration/dose ratio, hence lower dose requirement [86]. In conclusion, genetic markers in the ABCB1 gene do not seem to relate (clinically relevant) to TRL exposure.

P-glycoprotein in the small intestine plays a role in the absorption of CsA [27]. However, several studies have shown no relationship between genetic polymorphisms in ABCB1 and CsA exposure in kidney transplant recipients [16,81,84]. However, in a recent study by Fanta et al. a higher pre-hepatic extraction ratio for carriers of the ABCB1 2677 G-allele
was found in pre-transplant pediatric patients on dialysis. This study used a population pharmacokinetic approach and identified factors relevant for the variability in drug exposure, while accounting for other factors such as bodyweight. An explanation for the discordant findings is that in the study of Fanta et al., the analysis was performed in a different population including steroid free pediatric patients waiting for dialysis and is therefore not comparable to the adult kidney transplant population [87].

**Pregnane X receptor**

The pregnane X receptor (PXR), coded by the NR1I2 gene is a nuclear factor receptor involved in the induction of various genes, such as CYP3A4 and ABCB1 [88]. Rifampicin and prednisolone are ligands for this receptor and can stimulate indirectly the expression of CYP3A4 and ABCB1 [58]. Especially, prednisolone is of interest since the majority of regimens in renal transplantation include this immunosuppressant. Prednisolone therapy is often started in high doses as part of the induction therapy and gradually tapered towards low maintenance doses. Steroids are finally withdrawn in a limited number of centers [89].

High levels of cortisol or exogenous steroids saturate the glucocorticoid receptor and these steroids then activate PXR as a low affinity high capacity receptor in order to induce its own metabolism [58]. At that point, the use of prednisolone may affect the pharmacokinetics of other substrates for CYP3A4 or ABCB1, such as the CNIs. This could potentially be the mechanism behind the interactions between prednisolone and the CNIs [90-92]. Specifically, inter-individual variability in this interaction could be explained by genetic variability in the gene encoding this receptor. To date, no genetic variants in NR1I2 have been identified that influence CsA or TRL pharmacokinetics. However, a recent study by Miura et al. demonstrated a significantly lower prednisolone AUC in PXR 7635 G-allele carriers [93]. The A+7635G SNP as well as other SNPs in the gene encoding PXR could be of interest regarding a relationship with TRL clearance.

**Intracellular CNI exposure**

Clinical impact of pharmacogenetics in genes encoding metabolic enzymes related to CNIs may be limited due to the fact that whole blood concentrations are not very representative for intracellular drug concentrations in the target T-cell. In fact, it has been reported that intracellular CsA concentrations correlate poorly to blood concentrations [94]. The concentrations in the target T-cell can differ from the concentration in blood due to the presence of transporters such as P-glycoprotein (ABCB1) on the cell surface of T-cells [95] (Figure 2). Efficacy of CNIs, especially CsA, could be lowered by an active P-glycoprotein pump eliminating CNIs out of the cell [96]. Falck et al. demonstrated declined CsA T-lymphocyte concentrations 3 days prior to a rejection episode, while genetic variants of ABCB1 could not be related to this decline [96]. However, Crettol et al. reported a 1.7 fold increased intracellular concentrations for patients carrying the ABCB1 3435T-allele [94]. Similar to variable drug concentrations in the target cell is the potential variable drug exposure in organ tissue prone to toxicity. Nephrotoxicity is an important and major clinical problem during CNI treatment after renal transplantation [1,2]. Nephrotoxicity could potentially be related to polymorphisms in ABCB1 as P-glycoprotein is expressed in the kidney and is likely to be involved in transporting CsA into renal tubules [97,98].
Hauser et al. published a study relating a polymorphism in \textit{ABCB1} to nephrotoxicity [99]. An important conclusion from this study was that the donor’s \textit{ABCB1} 3435 TT genotype appeared to be strongly associated with CsA related nephrotoxicity as demonstrated by an odds ratio of 13 (CI: 1.2 to 148). Moreover, tissue specificity of CYP3A enzymes has also been described with a specific role for CYP3A5 in the kidney and the absence of CYP3A enzyme expression in peripheral blood lymphocytes [100]. Finally, a study by Kuypers et al. demonstrated the development of biopsy proven TRL related nephrotoxicity in renal transplant recipients carrying CYP3A4*1 or *1B and CYP3A5*1 alleles [101].

**Variability in CNI pharmacodynamics**

Recent years focused on biomarkers originating from the pharmacokinetic pathway in order to predict exposure, as was described in the previous section. The second part of this review will elaborate on the (future) identification of markers derived from the pharmacodynamic pathway.

In transplant immunology a T-cell encounters donor-antigen presented on a \textit{HLA} molecule by antigen presenting cells (APC) originating from the donor or the recipient (Figure 2).
The HLA/antigen domain interacts with T-cells through the T-cell receptor (CD3) upon which it activates the T-cell and initializes a calcium influx into the cell [102,103]. Intracellular calcium then forms a complex with the calcium binding protein calmodulin. This calcium/calmodulin complex is able to bind to the enzyme calcineurin A (CNA), facilitates a conformational change and causes the autoinhibitory domain of CNA to free the catalytic site upon which CNA is activated [104,105]. Activated calcineurin de-phosphorylates members of the transcription factor family of nuclear factor of activated T-cells (NF-AT) within the cell cytoplasm. De-phosphorylated NFAT transports to the nucleus of the cell and act as a transcription factor initiating gene expression of cytokines and receptors (i.e. expression of interleukin-2) [104,106].

This pathway can pharmacologically be disrupted by the use of CNIs [107,108] after the drug has entered the target T-cell. TRL and CsA bind to FK506-binding protein (FK-BP) and cyclophilin A respectively, which are intracellular immunophilins acting as binding proteins [109]. The immunophilin/CNI complex binds to calcineurin with the CNI side sterically hindering the active site of calcineurin with the large immunophilin tale [105,110]. NFAT can then no longer be de-phosphorylated and gene expression is reduced with immunosuppression as the clinical result (Figure 2). Measurement of calcineurin activity and calcineurin inhibition upon CNI administration is interesting as a potential biomarker for CNI therapy [111-114]. Measuring calcineurin activity after renal transplantation could add to the current CNI monitoring strategy which mainly concerns whole blood level monitoring [115-118]. However, the role of measuring calcineurin inhibition has to be determined.

Genetic variants in proteins involved in the calcineurin inhibition pathway are of potential interest for understanding variable CNI drug response [119]. The calcineurin inhibition pathway provides three main protein groups: calcineurin, immunophilins and NFAT.

**Calcineurin**

Calcineurin, a serine/threonine phosphatase also known as protein phosphatase 2B (PP2B) or protein phosphatase 3 (PPP3), is a heterodimer of the catalytic subunit A and the regulatory subunit B [104,107,120]. Its structure is highly conserved from yeast to man and essential for activity [121]. Calcineurin has a relatively narrow substrate specificity including phosphoproteins such as DARPP32 and inhibitor-1 and for this paper more importantly NFATs [121,122].

**Calcineurin A** (CNA) is composed of a catalytic domain and three regulatory domains: a CNB binding domain, a calmodulin binding domain and an auto-inhibitory domain [105,123]. The C-terminus of calcineurin contains the auto-inhibitory domain which relieves its activity upon calmodulin binding. Adjacent to this area the calmodulin binding domain is situated followed by the CNB binding domain [122] (Figure 3).

Three genes code for three specific isoforms of CNA: CNA α (PPP3CA: neural, kidney), CNA β (PPP3CB: widely, T-cell, B-cell), CNA γ (PPP3CC: testis) [120,121,124-126]. The predominant isoform contributing to calcineurin activity in most tissues is CNA α [124]. CNA α and CNA β were localized on human chromosomes 4 and 10 respectively [127]. The isoforms have large overlap in structure. CNA α compares for 84% to CNA β and for 81% to CNA γ with greatest similarities in the catalytic domain [125].
Calcineurin B (CNB), also called PPP3R, belongs to the so called ‘EF-hand calcium binding protein family’ [105,121]. CNB is essential for calcineurin activity and like calmodulin it is activated by calcium. In fact it has a similar structure to calmodulin [121,128], but a different activity. The calcium/CNB complex increases affinity of calcineurin for its substrate in contrast to calcium/calmodulin which displaces the auto-inhibitory domain and increases the enzymatic rate. CNA alone has a low activity which becomes a very high specific activity upon binding to CNB [105,121,128]. Two mammalian isoforms are known, CNB1 and CNB2, of which the first is associated with CNAα and CNAβ, while CNB2 relates to the γ variant and is only expressed in testis [121]. The genes coding for CNB1 and CNB2 are PPP3R1 and PPP3R2 respectively [127].

The interface between the CNB binding domain in CNA and CNB itself is the target area for the immunophilin/CNI complex. A large part of the contact area for the FK-BP/tacrolimus complex origins as the ‘latch region’ where CNB and the cyclophilin/CsA complex binds and which consists of residues 118-125 in the loop of CNB [129]. Interestingly, this is the principle reason that these two complexes display competitive binding to calcineurin [121].

To date, limited studies are published investigating polymorphisms in the gene encoding calcineurin and no studies are known relating them to clinical effect. Potentially, three regions are of interest for explaining variability in CNI response. First, the latch region between CNA and CNB is the principle region for binding the immunophilin/CNI complex. Genetic polymorphisms resulting in aminoacid changes in this region may affect the ability of the complex to bind to calcineurin. Indeed, Wang et al. demonstrated that the CNB binding domain is important for the inhibition of calcineurin [123]. Substitutions in the latch region are shown to result in resistance to CNI inhibition. This is also true for aminoacid changes in the CNB binding domain (Thr352, Leu354, Lys360) [129]. As an extension to this, the so called Loop7 has been investigated. This is a β-hairpin within CNA which contacts immunophilin/CNI complexes as well as the auto-inhibitory domain. The connections made by Loop7 are important for CNI. Inhibition by TRL appeared to be increased by single deletions of Val314 or surrounding residues, while it reduced CsA mediated inhibition [129]. Second, basal phosphatase activity of calcineurin could theoretically be altered by changes in the calmodulin binding region with a lower basal activity of calcineurin as

Figure 3. The structural domains of calcineurin A. Calcineurin A consists of a catalytic domain and a regulatory domain. The latter consists of three important parts, a calcineurin B binding domain, a calmodulin binding domain and an auto-inhibitory domain.

Calcineurin A consists of a catalytic domain and a regulatory domain. The regulatory domain consists of three important parts, a calcineurin B binding domain, a calmodulin binding domain and an auto-inhibitory domain.
a result. This is likely to have its consequences for baseline calcineurin activity. Potentially this could affect the enzymatic activity of calcineurin by altering an essential factor in the motor of the system. Third, conformational changes in the catalytic site of calcineurin are likely to change the affinity of calcineurin for its substrates, the phosphorylated NFATs. One can imagine that these changes are able to lead to a different basal enzymatic activity, which could explain inter-individual changes in baseline activity and probably the potential for CNIs to inhibit calcineurin activity.

**Immunophilins**
The immunophilins cyclophilin and FK-BP belong to the protein families collectively referred to as peptidyl-prolyl isomerases (PPIases) [130-132]. Besides these proteins parvulins are considered the third member of this group [132]. PPIases are cis-trans peptidyl-prolyl isomerases (rotamases) which catalyze the cis-trans interconversion of peptide bonds N-terminal to proline with a physiological role in protein folding and conformational stability. But their role in inhibition of calcineurin is different from their physiological role [132,133]. The function of immunophilins in calcineurin inhibition is related to its size, as binding of cyclophilin A and CsA leads to a large complex which is able to sterically hinder the catalytic site of calcineurin to phosphorylate NFAT. CsA binds on one side to a groove on cyclophilin A, while the other side remains available for binding calcineurin [132] (Figure 2).

Immunophilins contain CNI binding properties and were thought to be abundant in cells [130]. This abundancy is currently under debate [115,134] and one can easily hypothesize that these proteins are expressed in different amounts between or even within individuals. Therefore, genetic variability in the genes encoding the immunophilin may lead to differences in response to CNIs. Other possible sources of genetic variability that could result in therapeutic differences are changes in the binding domain for CNIs. A lower or higher affinity could change the potency of CNIs to inhibit calcineurin.

**Cyclophilines (Cyp)** are a group of 15 different members of which cyclophilin A (CypA) is thought to be the most important in the context of calcineurin inhibition. The residues in CypA that are involved in CsA binding are highly conserved in other members of the group indicating a potential role for other Cyps (i.e. Cyp B, C and F) as a target for CNIs. PPIA is the gene coding for CypA [132,135] and CsA binds to CypA through contact on its hydrophobic pocket [132,136]. Only one study in renal transplant recipients is reported in literature [137] where a CypA polymorphisms in a coding region (exon: +36 G/A) and one in the promoter region (-11 G/C) were identified. The promotor polymorphism was associated with clinical nephrotoxicity. In this study DNA was not obtained from the donor but from the recipient, which makes the interpretation of this result difficult. Moreover, nephrotoxicity was not confirmed by a biopsy, but only related to an increase in serum creatinine values. In an earlier study this polymorphism was related to rapid CD4+ T-cell loss and progression in patients diagnosed with Auto-Immune Deficiency Syndrome (G vs. C allele) [135].

**FK506-binding proteins (FKBP)** constitute a group of 16 human proteins of which FKBP12 and FKBP12.6 are related to the inhibition of calcineurin phosphatase activity [138]. Immunosuppressive properties are primarily thought to be regulated by FKBP12. However,
one cannot exclude the role of other PPIases with abundancy in human, such as FKBP51 [138]. The FKBP family members are structurally variable, but the PPIase domains of FKBP proteins do display overlap in parts of the protein sequence. The FK506 binding site consists of a hydrophobic pocket of at least 12 amino acid residues [138]. The positions K34, H87 and I90 appeared to be the most variable positions and were thought to be important for TRL-FKBP interaction [138]. Indeed, it was shown that a change at the position of K34 from a lysine to a threonine residue lowers the affinity for calcineurin [138].

**Nuclear Factor of Activated T-cells (NFAT)**

NFAT is a family of transcription factors inducing gene transcription in T-cells and other immune system related cells [139,140]. NFAT proteins are present in the cell cytoplasm in the phosphorylated form (Figure 2). NFAT is a substrate for the phosphatase calcineurin which is capable of de-phosphorylating NFAT. When NFAT is de-phosphorylated it translocates to the nucleus where it becomes transcriptionally active and regulates the transcription of a large number of genes [106,141]. Calcineurin binds NFAT at its regulatory domain at two specific calcineurin binding regions which are called binding region A and B. Region A is called PxIxIT or SRIEIT found in all NFAT proteins, while region B is dedicated to NFAT 2 and 4 [106]. Polymorphisms resulting in changes in the calcineurin binding regions are likely to result in altered activity of NFAT, while changes in the DNA binding domain (Rel homology domain) are also likely to result in altered gene expression. Besides a potential alteration in the catalytic site of calcineurin a change in the PxIxIT recognition region of calcineurin is a potential source for differences in response [104,106,107,142,143].

The NFAT family constitutes of 5 proteins NFAT1-5 [106,140] and is constructed out of an activation domain, a regulatory domain, a DNA binding domain and the C-terminal domain [142]. A highly variable tissue distribution is observed for NFAT. Protein and mRNA of NFAT1 and 2 are present in T-cells and other immune cells, but not in kidney cells, while mRNA of NFAT3 and 4 is found in kidney cells [139].

**Systems pharmacology approach**

Variability in response to CNI treatment may be related to several factors originating in the pharmacokinetic and pharmacodynamic pathway [7].

The past years the focus was mainly on variability in the pharmacokinetics of CNIs and this has lead to a practice of measuring blood concentrations as a monitoring tool for CNI therapy. More recently, pharmacogenetics has been introduced and related to variable pharmacokinetics. Genetic variants were explored as potential markers to optimize CNI therapy as early as possible after transplantation. There appears to be a role for CYP3A5*1 in TRL but not in CsA dosing. However, conclusive evidence regarding the impact of genotyping in routine clinical practice is still warranted. Especially, the impact of genotype based dosing on clinical outcome remains unclear [74,144]. A limitation of pharmacogenetic studies in renal transplantation done so far is that the majority did not take other important factors contributing to CNI disposition and metabolism, such as motility problems and co-administration of interacting drugs, into account. Moreover, often an
insensitive marker for exposure, such as the dose-adjusted trough level, was used. Optimizing CNI therapy from a pharmacokinetic perspective is limited by the fact that whole blood concentrations are measured, while plasma-, free-drug-or even T-cell-concentrations would be more informative. Technical advancement is necessary to routinely estimate drug concentrations at the site of action.

Future optimization of CNI therapy could be the use of pharmacodynamic measurements. Several groups have reported assays to determine calcineurin activity and its inhibition by CNIs [111-113,145-148]. Technical difficulties with regard to defining and obtaining the intended blood fraction have been discussed as well as the preservation of the phosphatase activity and the blockade of other phosphatases. Yet, the interpretation of these findings in relation to clinical usefulness is unclear [145,149,150]. More data should be generated to establish the usefulness and feasibility of this marker in routine clinical practice. Moreover, genetic variability in the genes encoding the proteins involved in the calcineurin inhibition pathway such as immunophilins, calcineurin and NFAT are likely to be related to differences in potency, efficacy and toxicity of CNIs. Genetic variants in genes encoding these proteins could be explored as potential markers for CNI therapy. One should be aware that specific isoforms of these proteins are known to exist which are likely to result in differences in tissue sensitivity [124].

Besides pharmacogenetics in PK and PD, also non-genetic determinants are related to the response of CNIs. These factors include co-morbidity (diabetes, cardiovascular), demographics (age, sex, race and bodyweight), organ functioning (intestine, liver, kidney, cardiovascular system), the administration of interactive drugs (prednisolone, fluconazol) and food [7] and finally compliance to drug therapy [151-154].

Obviously, response to CNIs is a complex trait and the result of interactions of a complex system. Therefore, it is unlikely that variability of CNI response can be captured by the variability in a single determinant. Instead, a systems pharmacology approach should be used incorporating the most important sources of genetic and non-genetic variability in terms of pharmacokinetics and pharmacodynamics. Several population pharmacokinetic approaches are published for CNIs used in a kidney transplant population [10,11,13,14,155], but only few used an integrative genetic-non-genetic approach [14,156]. Currently, no studies are published applying a population pharmacodynamic or combined PK/PD analyses in renal transplant recipients. In liver transplant recipients two such studies have been published [157,158], but these were of limited sample size with respects to patients as well as blood sampling and therefore had low power to identify factors associated with variability in drug response. Moreover, these studies did not incorporate pharmacogenetic markers.

Interestingly, novel approaches could also include the genotype of the donor next to the genotype of the recipient. Different genetic profiles between donor and recipient can potentially explain differences in nephrotoxicity in kidney transplant recipients [99], which is in agreement with liver transplant recipients displaying different metabolic capacity between intestine and liver [69]. Besides the inherited profiles, the body could also be responsible for different activities in organs due to tissue specific gene expression [58].
Conclusion

The high variability in response to CNI therapy has a multi-factorial origin and is likely to be related to genetic variability in genes encoding proteins in the pharmacokinetic and pharmacodynamic pathways of the CNIs. The complexity of response to CNIs requires a sophisticated approach in order to individualize CNI therapy. Specifically, a systems pharmacology approach integrating genetic information with other non-genetic determinants of pharmacokinetics and pharmacodynamics is likely to result in predictive models useful for optimal CNI mediated immunosuppression, further reducing toxicity and rejection episodes.
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