Pharmacodynamic monitoring of calcineurin inhibitor therapy: Is there a clinical benefit?

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Keywords: calcineurin inhibitor; pharmacodynamic; renal transplantation; therapeutic drug monitoring

Introduction

Immunosuppressive therapy should provide a maximum of efficacy with a minimum of toxicity. The introduction of the immunosuppressive calcineurin inhibitors (CNI) cyclosporine A (CsA) in the early 1980s and tacrolimus (Tac) in the 1990s improved renal allograft survival, especially by a reduction of the acute rejection rate in the first year after transplantation [1,2]. With the increased experience of CNIs, the early and long-term side effects such as nephrotoxicity [3,4], post-transplant cancer [5,6] and metabolic deteriorations appeared [7]. Although the CNIs are one of the pivotal immunosuppressive drugs in organ transplantation, there is still no consensus on the optimal therapeutic drug level of CsA and Tac in terms of their safety and efficacy. Among patients treated with comparable CNI doses and trough levels, there is a broad range covering acute rejection episodes at one end and symptoms of CNI side effects at the other end. These findings have shown that CNIs are critical dose drugs with a narrow therapeutic window regarding adequate immunosuppression compared with adverse drug effects. Moreover, the differences between patients in pharmacokinetics (PK) and pharmacodynamics (PD) cause variability in the concentrations of effects of CNIs [8,9]. For this reason, making CNI therapy more individual should improve patient and transplant outcomes.

Pharmacokinetic (PK) monitoring of CNI therapy

Therapeutic drug monitoring (TDM) is an opportunity to reduce both toxicity and acute rejection through blood-concentration guided therapy. Monitoring is most effective when there is a measurement that is a good surrogate parameter for total drug exposure and when sampling is easy to perform. In general, CNI (CsA, Tac) treatment is monitored by C0 levels, although CNI C0 levels correlated poorly with the area under the concentration–time curve of 0–12 h (AUC0–12; \( r^2 = 0.3–0.6 \)) and monitoring of CsA by C0 levels is a controversial issue [10–14]. A number of studies have demonstrated that C0 levels did not differentiate those patients at risk of acute rejection [15,16]. In addition, long-term graft loss was associated with CsA C2 rather than C0 levels [17]. To achieve an even more accurate CsA exposure, the measurement of the AUC was introduced for various intervals (2–12 h). However, the most reliable PK parameter for CsA dosing is the assessment of the CsA AUC for 0–12 h (AUC0–12) [16], but its clinical implementation is impracticable, since it requires multiple blood sampling and is costly. CsA AUC for 0–4 h after administration (AUC0–4) provides precise information about the absorption of CsA microemulsion formulation [15]. PK studies demonstrated that CsA exposure during the first 4 h after a dose (AUC0–4) correlates well with exposure during the entire 12-h dosing interval (AUC0–12) [13]. In addition, the measurement of CsA exposure during the first 4 h post-dose improved clinical outcomes [15,18]. CsA blood concentration at 2 h after administration (C2) is reported to correlate closely with AUC0–12 and AUC0–4 as a simple monitoring method [14,15,19–21]. A large clinical trial using C2 monitoring (MO2ART study) demonstrated lower rates of acute rejection and reduced nephrotoxicity in 296 de novo renal transplant patients [22]. On the basis of these studies, the CONCERT group published a consensus statement on Neoral monitoring in transplant patients that concluded that C2 monitoring is the optimal method and they emphasized that C0 monitoring only poorly predicts clinical events [23]. A European consensus summarized that randomized, prospective, multi-centre studies and single-centre trials provide a robust evidence base for the benefits of this sensitive monitoring technique [24]. However, the hypothesis that C2 monitoring as a management tool for CNI therapy is a good predictor for the risk of rejection or CsA toxicity was not supported by the study results.
of Einecke et al. [23]. Another retrospective data analysis showed that in patients treated with basiliximab there is no correlation between C2 levels and clinical outcomes [25].

In a prospective single-centre trial, the authors did not observe any significant difference in renal transplant function between patients managed by C2 and C0 monitoring [26]. In addition, in liver transplant patients the strength of the evidence for C2 or other limited sampling strategies relative to C0 monitoring of CsA concerning clinical end points was reviewed. The authors pointed out that although there is a theoretical benefit for the monitoring of CsA exposure, the evidence for the use of limited sampling strategies rather than C0 to improve patient outcomes is weak [27].

A recently performed systematic review of 10 prospective studies on clinical benefits of CsA C2 monitoring showed little evidence to support the theoretical benefits of C2 monitoring in de novo renal transplant patients [28]. All these data and results suggest that the optimal PK monitoring strategy for calcineurin inhibitor therapy remains controversial. Despite strict efforts to improve graft outcome by PK monitoring strategies, acute rejection episodes and side effects cannot be ruled out and it is presumed that long-term allograft loss is mainly due to CsA toxicity. Altogether, the monitoring of CNI therapy relies currently on different PK assays and methods including novel software programs for estimation of the individual AUC. These PK approaches include absorption, distribution, metabolism and excretion. Drug interaction in combination with various immunosuppressive regimens, polymorphism of drug targets and overall inter-individual differences limit the use of PK monitoring. In addition, all these methods provide only indirect information on the pharmacological activity of a drug and the biological effects of CNIs. Therefore, in addition to the conventional monitoring of blood concentration, a more informative therapeutic drug monitoring strategy is required.

Pharmacodynamic (PD) monitoring of CNI therapy

Non-specific assays for PD monitoring

In recent years, it has been realized that the assessment of biologically relevant effects provides a mean to improve immunosuppressive therapy. Pharmacodynamic monitoring is proposed as a new strategy to provide information about the biological effect of a drug and about the degree of immunosuppression. It has been recognized as a helpful tool to evaluate efficacy and to optimize drug dosing [29].

Non-specific PD biomarkers are general and reflect the overall activity of the immune system. One advantage of these non-drug-specific PD monitoring methods is that the effects of combination drug therapies can be evaluated. Most of the currently used immunosuppressive drugs inhibit lymphocyte proliferation. Several study groups investigated flow-cytometric or radio-nucleotide-based lymphocyte proliferation in stimulated whole blood cultures or peripheral blood mononuclear cells (PBMCs) isolated by Ficoll density centrifugation [30]. Other general PD monitoring strategies assess the lymphocyte surface antigens CD25, CD71 and CD154 by flow cytometry [31,32]. Increased expression of CD4+CD25+ by T cells correlated with the grade of rejections in heart transplant patients [33]. In bone marrow transplantation, high precursor frequencies of cytotoxic T lymphocytes were associated with prolonged leukaemia-free survival time [34], whereas in solid organ transplantation the benefits of monitoring of precursor frequencies of cytotoxic T lymphocytes have remained controversial [35,36]. Other studies confirmed that variations in the T-cell subset were associated with acute rejection episodes in transplant patients [37].

Development of donor-specific anti-human leukocyte antigen (HLA) antibodies is another general method to monitor immune function after successful transplantation, especially in highly sensitized transplant recipients [38]. Soluble CD30 has been supposed as a prognostic marker of acute rejection episodes prior to and after transplantation [39]. However, clinical studies have shown soluble CD30 to be a predictor of deterioration in chronic allograft nephropathy [40]. Recently, a commercially available whole blood assay measuring the adenosine triphosphate (ATP) levels in CD4+ cells has been introduced (ImmuKnow™) [41]. This assay is supposed to assess the overall immune function. This PD monitoring method has been evaluated in various study cohorts [42]. Data on risk of infection and rejection in Hispanic renal transplant patients are available, demonstrating that infectious disease and rejection episodes could be predicted by measuring ATP levels in CD4+ cells [43]. However, large prospective interventional studies have not yet been published. Moreover, ATP reflects the overall energy metabolism of cells and is not specific for immunosuppressive drugs.

Specific assays for PD monitoring

Knowledge about the pharmacological effect of a drug is essential to establish a drug-specific PD monitoring assay, and PD assays are only worthwhile if they provide reliable and valid results. Concerning CNIs, the biological consequences have been well known for several years. The PD action within the T lymphocytes is the inhibition of the phosphatase calcineurin. Calcineurin is a key component of T-cell activation and serves as a target for the CsA-cyclophilin and Tac-FK506-binding protein 12 complex [44]. The inhibition of calcineurin downregulates the dephosphorylation of the nuclear factor of activated T cells (NFAT). Following the transcription and, therefore, the production of NFAT-regulated genes, such as interleukin 2 (IL-2) or interferon-γ (IFNγ), tumour necrosis factor α (TNFα) is inhibited (Figure 1).

Several approaches have been undertaken to assess the pharmacodynamic consequences of CNI-based immunosuppression, and several PD parameters have been identified for the monitoring of immunosuppressive therapy. These include specific parameters as well as non-drug-specific parameters, which reflect the activity of particular immune responses (Table 1).

Previous studies have attempted to use calcineurin inhibition and cytokine production, especially IL-2 production, by means of an enzyme-linked immunosorbbent assay (ELISA) quantification from serum, by intracellular cytometric analysis of whole blood or by detection at the messenger RNA
Table 1. Non-specific and calcineurin inhibitor-specific pharmacodynamic assays to monitor calcineurin inhibitor therapy

<table>
<thead>
<tr>
<th>Non-specific pharmacodynamic assay</th>
<th>Specific pharmacodynamic assay</th>
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<tbody>
<tr>
<td>T-cell proliferation</td>
<td>Calcineurin phosphatase activity (PBMC/whole blood)</td>
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<tr>
<td>T-cell surface antigen</td>
<td>Cytokine production (ELISA/flow cytometric assay)</td>
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<tr>
<td>T-cell subsets</td>
<td>Cytokine expression (rt-PCR)</td>
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<tr>
<td>Anti-HLA antibodies</td>
<td>ATP levels in CD4+ T cells</td>
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<tr>
<td>Soluble CD30</td>
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</table>

ATP, adenosine triphosphate; ELISA, enzyme-linked immunosorbent; HLA, human leukocyte antibody; PBMC, peripheral blood mononuclear cells; rt-PCR, reverse transcription polymerase chain reaction.

(mRNA) level as a marker of the degree of calcineurin inhibition [45–48]. Calcineurin phosphatase activity as a CsA-and Tac-specific PD parameter has been evaluated by several approaches [45,49–51]. They demonstrated that the inhibition of calcineurin in lymphocytes correlates inversely with blood CsA concentrations, with a maximum inhibition of calcineurin 2 h after oral CsA uptake in 90% of the patients. An extensive inter- and intra-individual variability in calcineurin activity for CsA and Tac has been reported. It could also be observed that calcineurin activity is not inhibited completely by calcineurin inhibitors. Renal transplant patients exhibited a calcineurin activity of 50% of that of healthy volunteers [52]. The peak CsA blood concentrations were followed by a reduction in calcineurin activity of 70–96% with an high inter-patient variability [45,53]. In Tac-treated patients, the maximal inhibitory effect on calcineurin activity was ~60%, correlating with the highest Tac concentration [45,51]. In another study, calcineurin activity did not correlate significantly with drug dosages or PK parameters [54]. Variations in correlation between PD effects and PK concentrations confirm that immunosuppressive drugs induce a variety of responses in patients even when drug dosages and drug levels are similar. For this reason, a PD evaluation of CNIs may be useful for gaining an understanding of individual sensitivities towards CsA and Tac. Different methods to assess calcineurin activity have been evaluated: high performance liquid chromatography (HPLC)-ultraviolet measurement of dephosphorylated peptide [55], the radioactive measurement of 32P-labelled phosphate [56] and a spectrophotometric method [57]. Calcineurin activity is measured in various blood fractions as whole blood [54], PBMCs [58] and leukocyte subsets [59]. A cell-specific activity has been recognized and, for this reason, inter- and intra-individual variations in leucocyte
subset cell counts may influence the measured calcineurin activity [59].

Other studies focused on T-cell cytokine production as PD parameters as an intermediate step in the mode of calcineurin inhibitor action. Stein et al. [46] proposed measuring IL-2 protein in mitogen-activated whole blood. With ELISA and flow cytometric techniques, the concentrations of cytokines or chemokines have been measured in serum and stimulated PBMCs derived from patients post-transplantation [32,60–62]. Millan et al. [58] showed that the addition of mycophenolic acid to CsA or Tac-based immunosuppression reduced IL-2 production. The authors argued that the inhibition of clonal expression of activated T cells lowers the number of T cells ready to produce IL-2. Any correlation between cytokine production and PK parameters is still a controversial issue, however. There are data available demonstrating a tight correlation between CsA C0 or C2 levels and IL-2 production [46,62], yet other studies have not confirmed this strong PK and PD association [31]. Grinyo et al. [53] showed that IFN-γ is a more specific marker of CsA in pharmacodynamics. However, monitoring of cytokine production is a problematic issue as a result of restrictions to certain cell-cycle phases, different half-lives for the circulation of cytokines and changes in the up and downregulation of cytokine expression [63].

Another specific PD monitoring method has been introduced with the assessment of cytokine mRNA expression. Härtel et al. [47] described a human whole blood assay based on quantitative real-time cytokine reverse transcription polymerase chain reaction (rt-PCR) for the PD assessment of immunosuppressive drug effects. The authors observed a decreased basal mRNA expression of TNFα in patients on CsA therapy and a delayed cytokine mRNA expression kinetics during T-cell co-stimulation. These data suggested that distinct shifts in peak cytokine mRNA expression might represent a sensitive PD marker of individual CsA response. For prospective studies on cytokine mRNA concentrations, the parameter ‘area of cytokine mRNA expression over time’ was suggested as PD monitoring tool, which should include absolute cytokine mRNA concentrations measured at two different time points. The same study group investigated the potential PD effects of tacrolimus on IL-2 mRNA expression in an in vitro human whole blood assay [48]. IL-2 mRNA profiles revealed variable Tac sensitivity. Kinetic profiles of IL-2 mRNA expression demonstrated individually distinct degrees of CNI sensitivity in patients undergoing Tac monotherapy before living-donor kidney transplantation. Individuals with unaffected IL-2 mRNA expression may be at increased risk of transplant rejection. Other studies have investigated the expression of cytokines in kidney-transplant biopsies [64]. The recently available technique of rt-PCR provides a fast, highly reproducible and sensitive tool for the quantitative analysis of gene expression. This method was employed to measure directly the functional effects of calcineurin inhibition, namely the inhibition of the transcriptional activities of NFAT-regulated genes in the peripheral blood [65–67]. This assay is based on the quantitative analysis of IL-2, IFN-γ and GM-CSF gene expression in whole blood samples at CsA C0 and C2 or Tac C0 and C1.5.

Despite the increasing numbers of established assays for the monitoring of CNI pharmacodynamics, important questions remain to be answered: How can these assays be standardized; when are they applied; and finally, are there any clinical benefits?

**Clinical data of specific PD monitoring**

Despite the various methods described for the pharmacodynamic monitoring of CNIs, until now none of these assays have been implemented in routine clinical practice. Only a few data are available on PD monitoring of calcineurin inhibitors and clinical outcomes (Table 2). Although clear relationships between drug concentration and calcineurin activity have been observed, no clear

<table>
<thead>
<tr>
<th>Authors (reference)</th>
<th>Calcineurin inhibitor</th>
<th>Year</th>
<th>Transplant type</th>
<th>Pharmacodynamic assay</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pai et al. [69]</td>
<td>CsA</td>
<td>1994</td>
<td>Allogeneic stem cells transplantation (n = 33)</td>
<td>Calcineurin phosphatase activity</td>
<td>Acute graft versus host disease</td>
</tr>
<tr>
<td>Sanquer et al. [70]</td>
<td>CsA</td>
<td>2004</td>
<td>Allogeneic stem cells transplantation (n = 31)</td>
<td>Calcineurin phosphatase activity</td>
<td>Acute graft versus host disease</td>
</tr>
<tr>
<td>Fukudo et al. [71]</td>
<td>CsA/Tac</td>
<td>2005</td>
<td>Liver transplantation (n = 40)</td>
<td>Calcineurin phosphatase activity</td>
<td>Acute rejection, nephrotoxicity</td>
</tr>
<tr>
<td>Härtel et al. [48]</td>
<td>Tac</td>
<td>2004</td>
<td>Renal transplantation (n = 8)</td>
<td>IL-2 production</td>
<td>Acute rejection</td>
</tr>
<tr>
<td>Sommerer et al. [67]</td>
<td>CsA</td>
<td>2006</td>
<td>Renal transplantation (n = 133)</td>
<td>NFAT-regulated gene expression</td>
<td>Malignancy, infection</td>
</tr>
<tr>
<td>Konstandin et al. [72]</td>
<td>CsA</td>
<td>2007</td>
<td>Heart transplantation (n = 53)</td>
<td>NFAT-regulated gene expression</td>
<td>Infection</td>
</tr>
<tr>
<td>Sommerer et al. [73]</td>
<td>CsA</td>
<td>2008</td>
<td>Renal transplantation (n = 55)</td>
<td>NFAT-regulated gene expression</td>
<td>Non-melanoma skin cancer</td>
</tr>
<tr>
<td>Sommerer et al. [74]</td>
<td>CsA</td>
<td>2008</td>
<td>Renal transplantation (n = 40)</td>
<td>NFAT-regulated gene expression</td>
<td>Allograft function</td>
</tr>
</tbody>
</table>

CsA, cyclosporine A; Tac, tacrolimus; IL-2, interleukin 2; n, number of patients; NFAT, nuclear factor of activated T cells.
relationship between clinical outcomes and calcineurin activity has been reported [68]. The clinical usefulness of PD monitoring has been supported by the observation of Pai et al. [69] who found that a lower calcineurin activity results in a higher rate of graft versus host diseases in bone marrow recipients. This is supported by Sanquer et al. who demonstrated that calcineurin activity might be a predictive marker for graft-versus-host disease in allogeneic stem cell transplantation [70]. In organ transplant patients, calcineurin activity has been related to clinical response after liver transplantation [71]. Concerning cytokine production, an association between pre-transplant IL-2 production and the risk of acute rejection has been shown in a small study cohort (n = 8, tacrolimus) [48]. Recently, it could be demonstrated that specific PD monitoring of CNIs by the assessment of NFAT-regulated gene expression in stable transplant patients may be a useful tool to personalize CNI therapy. The expression of NFAT-regulated genes correlated inversely with CsA and Tac levels [65]. However, there is a high inter-individual variability of NFAT-regulated gene expression in different patients with corresponding CsA or Tac doses. Even in long-term renal transplant patients, there was a high variability in residual NFAT-regulated gene expression varying from 2% to 50% that shows various degrees of immunosuppression and T-cell activation [67]. On the other side, intra-individual variability of residual NFAT-regulated gene expression was very low in repetitive measurements in one single patient with a constant CNI dose [67]. CsA-treated patients with low residual expression of NFAT-regulated genes were more likely to get recurrent infections or tumour lesions [67,72], and patients with a low residual NFAT-regulated gene expression have an increased risk of non-melanoma skin cancer [73]. The monitoring of residual NFAT-regulated gene expression has been proven as a useful and safe tool to reduce CsA therapy [74]. In a biopsy-controlled study, the reduction of CsA dose and the increase of residual NFAT-regulated gene expression at CsA C2 proceeded without adverse effects, e.g. acute rejections, when the residual gene expression was below 30%. One patient had an acute interstitial rejection with a residual NFAT activity of over 40% after the tapering of the CsA dose. In this study cohort, the reduction of the CsA dose also resulted in a decrease of systolic and pulse pressure [74]. Altogether, a clinical benefit has been demonstrated concerning the PD monitoring of CNI therapy by calcineurin phosphatase activity in allogeneic stem cell and liver transplantation, IL-2 production in renal transplantation, as well as NFAT-regulated gene expression in renal and heart transplant patient. These methods might be promising tools to optimize CNI therapy. Additional studies are needed to support this hypothesis of an individualized CNI therapy in the early post-transplant phase by monitoring NFAT-regulated gene expression and applying this practice to daily clinical routine.

**Conclusions**

The best way to individualize calcineurin inhibitor therapy is still a controversial issue. Despite strict efforts to improve graft outcome by PK monitoring strategies, acute rejection episodes and side effects cannot be ruled out. PD assessment to determine biological drug efficacy *in vivo* is a promising tool supporting clinical decisions on the dose and type of immunosuppressive drug. PD monitoring is not supposed to replace PK monitoring; rather a combination of the complementary PK and PD monitoring methods could help to improve therapeutic drug dosages for more effective and safe results in individual patients. Several PD monitoring methods have been established, and most of these correlate well with PK data. However, there are only a few studies that investigate PD monitoring strategies and clinical outcomes, but all of these could demonstrate a clinical benefit in PD monitoring. Further studies are necessary to provide clear answers to the question as to which approach to PD monitoring is the most efficient and able to improve clinical outcomes by making immunosuppressive therapies more individual.

**Conflict of interest statement.** None declared.

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Usefulness of pharmacodynamic monitoring

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Received for publication: 23.1.08
Accepted in revised form: 12.9.08