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REVIEW

Pharmacokinetic considerations related to therapeutic drug monitoring of tacrolimus in kidney transplant patients

Louise M. Andrews[a,*], Yi Li[a,b,*], Brenda C. M. De Winter[a], Yun-Ying Shi[c], Carla C. Baan[d], Teun Van Gelder[a,d] and Dennis A. Hesselink[d]

[a]Department of Hospital Pharmacy, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands; [b]Department of Laboratory Medicine, West China Hospital of Sichuan University, Chengdu, China; [c]Department of Nephrology, West China Hospital of Sichuan University, Chengdu, China; [d]Department of Internal Medicine, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

ABSTRACT

Introduction: Tacrolimus (Tac) is the cornerstone of immunosuppressive therapy after solid organ transplantation and will probably remain so. Excluding belatacept, no new immunosuppressive drugs were registered for the prevention of acute rejection during the last decade. For several immunosuppressive drugs, clinical development halted because they weren't sufficiently effective or more toxic.

Areas covered: Current methods of monitoring Tac treatment, focusing on traditional therapeutic drug monitoring (TDM), controversies surrounding TDM, novel matrices, pharmacogenetic and pharmacodynamic monitoring are discussed.

Expert opinion: Due to a narrow therapeutic index and large interpatient pharmacokinetic variability, TDM has been implemented for individualization of Tac dose to maintain drug efficacy and minimize the consequences of overexposure. The relationship between predose concentrations and the occurrence of rejection or toxicity is controversial. Acute cellular rejection also occurs when the Tac concentration is within the target range, suggesting that Tac whole blood concentrations don't necessarily correlate with pharmacological effect. Intracellular Tac, the unbound fraction of Tac or pharmacodynamic monitoring could be better biomarkers/tools for adequate Tac exposure – research into this has been promising. Traditional TDM, perhaps following pre-emptive genotyping for Tac-metabolizing enzymes, must suffice for a few years before these strategies can be implemented in clinical practice.

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1. Introduction

Treatment with low-dose tacrolimus (Tac) combined with the antimetabolite mycophenolate and glucocorticoids seems to offer the best outcomes after kidney transplantation in terms of renal function, allograft survival, and acute rejection rates, as compared with ciclosporin (CsA)-based regimens [1]. More than a decade ago, Tac largely replaced CsA as the calcineurin inhibitor (CNI) of choice and has remained so ever since [2]. Nonetheless, although the introduction of modern immunosuppressive drugs has improved the short-term outcome after transplantation, long-term allograft failure remains an important problem with 3–5% of kidney allografts being lost annually after the first transplant year [3,4]. Although the causes of long-term kidney allograft failure are multifactorial, chronic CNI-associated nephrotoxicity is considered an important cause [5,6]. Tac exerts its immunosuppressive properties by inhibiting the phosphatase activity of calcineurin (CN) after binding to the intracellular FK-binding protein 12 (FKBP12) [7]. This inhibition subsequently leads to decreased de-phosphorylation and activation of the nuclear factor of activated T cells (NFAT), which activates the transcription of genes important for T cell activation including interleukin (IL)-2 and interferon (IFN)-γ. This eventually results in a diminished inflammatory alloreactive response [8,9].

Belatacept is a novel, non-nephrotoxic immunosuppressive agent which blocks the CD80/86–CD28 co-stimulatory signal necessary for T cell activation [10]. Belatacept-based immunosuppression may result in improved long-term patient and graft survival but it is less effective than Tac in preventing acute rejection [11,12]. It thus remains to be seen whether belatacept will replace Tac as the first-line immunosuppressive drug anytime soon [13–15]. Multicenter, randomized clinical studies also showed higher incidences of acute rejection and dnDSA development in the Tac withdrawal or rapamycin-based groups compared with Tac-based regimen [1,3,16,17] In the foreseeable future, no other novel immunosuppressants are likely to emerge that can replace Tac.

2. Therapeutic drug monitoring

Due to a narrow therapeutic index and its large interpatient and intrapatient pharmacokinetic variability, therapeutic drug monitoring (TDM) is routinely performed for individualization of the Tac dose to maintain drug efficacy and minimize the consequences of overexposure [18]. As allografts are nowadays rarely lost as a consequence of acute rejection, adverse events

CONTACT Dennis A. Hesselink d.a.hesselink@erasmusmc.nl Erasmus MC University Medical Center Rotterdam, Dept. of Internal Medicine Division of Nephrology and Renal Transplantation, Room D-427, P.O. Box 2040 3000 CA Rotterdam, The Netherlands
*These authors contributed equally to this work.

Article highlights

- Acute cellular rejection can occur when the tacrolimus concentration is within the target concentration range, demonstrating that tacrolimus whole blood concentrations do not always fully reflect its pharmacological effect.
- Immunoassays remain the backbone of assay services for tacrolimus now, so we must ensure that they are being used correctly.
- A high tacrolimus intra-patient variability is considered a risk factor for poor long-term transplantation outcomes.
- Other strategies for TDM, including pharmacodynamic monitoring, are promising clinical tools to ensure adequate tacrolimus exposure and optimal efficacy of the drug.
- Pharmacogenetics-assisted tacrolimus monitoring, especially when incorporated in dosing algorithms, could be useful to determine the starting dose of tacrolimus

This box summarizes key points contained in the article.

associated with long-term immunosuppression have become increasingly evident [19]. Reducing the toxic effects of immunosuppression has become a major goal in the treatment of transplant recipients [20]. The most frequently used means of Tac monitoring is the measurement of the predose concentration (C_0) in whole blood. Some clinicians have started to question the current reliance on C_0 when performing TDM of Tac, with instances of toxicity and rejection occurring when C_0 are within 'acceptable' ranges. Amongst the transplant professionals, there is an ongoing debate as to whether the Tac C_0 sufficiently predicts kidney transplant rejection (see below) [21].

3. Controversies of TDM

The target Tac concentrations depend on the time after transplantation, immunosuppressive comedication and the presumed risk of rejection. Therapeutic ranges developed for Tac have not generally been based on statistical approaches, but rather on a mixture of empirical observations, in quite small samples of patients. In the past 20 years, there has been a substantial change in the target Tac concentration after kidney transplantation, with target concentrations as high as 20 ng/mL in the early 1990s, and with targets as low as 3–7 ng/mL after the publication of the Symphony-Elite study [3]. However, only few studies have compared different Tac concentration ranges and there is little support to promote the use of a specific therapeutic window and aim for certain target concentrations [21].

3.1. Controversies of Tac exposure C_0 with rejection

One of the reasons why the optimal target concentration is still debated is the fact that the relationship between Tac exposure (measured by C_0) and the risk of graft rejection is controversial (Table 1). A multicenter trial reported the association between low whole-blood Tac C_0 and the incidence of acute rejection in renal transplantation. The Tac regimens were designed to produce low (5–14 ng/ml), medium (15–25 ng/ml), or high (26–40 ng/ml) whole-blood C_0. A significant association was observed for decreasing rates of rejection with increasing Tac exposure. The authors suggested that the target range of whole-blood C_0 that optimizes efficacy and minimizes toxicity was 5–15 ng/ml during the first 42 days of treatment [22]. In the same year, 92 kidney transplant recipients and 721 liver transplant recipients from four clinical trials were analyzed. The Tac concentration range was also divided into low (5–14 ng/ml), medium (15–25 ng/ml), or high (26–40 ng/ml) in renal transplant recipients. Again a significant correlation between Tac C_0 and the incidence of rejection was found in renal transplant recipients [23]. In 1999, Undre *et al.* reported that the mean AUC during the early posttransplant period of Tac is significantly lower in patients who experience acute rejection than those who remain rejection free. They suggested that in order to reduce the risk of rejection, a Tac C_0 of 10 ng/mL should be achieved by day 2–3 after transplantation [24]. A decade later, Borobia *et al.* performed a receiver operating characteristic (ROC) curve analysis to determine whether particular Tac C_0 concentrations measured in the first week could discriminate between patients with an acute rejection and those who experienced no rejection.

Patients with a Tac C_0 below 9.3 ng/mL on day 5 showed a shorter graft survival in comparison with patients with Tac C_0 above this concentration [26]. A similar conclusion was also drawn by Staatz *et al.*, who estimated that a rejection rate as high as 55% would be found for patients with a Tac C_0 between 0 and 10 ng/ml, compared with no observed rejection in patients with a Tac C_0 between 10 and 15 ng/ml in the first month after kidney transplantation [25]. They suggested that in order to minimize rejection in the first month after renal transplantation, Tac C_0 had to be maintained above 10 ng/ml [25]. Gatault *et al.* recently compared the efficacy and safety of two different doses of extended-release Tac (TacER) in kidney transplant recipients between 4 and 12 months after transplantation. Stable steroid-free kidney transplant recipients were randomized (1:1) after 4 months. Group A ($n = 87$) had a 50% reduction in TacER dose with a targeted TacER C_0 of 3 ng/mL or higher; whereas group B ($n = 99$) had no change in TacER dose (TacER C_0 7–12 ng/mL). The authors observed that the eGFR was similar in both groups at 12 months, while more rejection episodes and inflammation occurred in group A than in group B. They suggested that the TacER C_0 should be kept above 7 ng/mL during the first post-operative year. However, the results of this study should be interpreted in relation to the fact that steroid-free patients were included who were receiving an average mycophenolate mofetil (MMF) between 1 and 1.5 g/day [28].

In contrast, other studies in renal transplantation did not find an association between plasma or whole-blood Tac concentrations and the risk of acute rejection [27,29]. In a recent study, data from three clinical trials were pooled ($n = 1304$) and analyzed. No correlation was found between the Tac C_0 measured at five time points (day 3, 10, and 14, and month 1 and 6 after transplantation) and the occurrence of acute rejection in the period thereafter, in the first posttransplant year [21].

Based on the available literature, we conclude that shortly after the introduction of Tac into clinical practice in the 1990s, despite high target concentrations, acute rejection rates did reach 40% or more [25]. This remarkably high incidence of rejection can be explained by the limited experience with this drug and the concomitant use of azathioprine instead of MMF and/or the lack of induction therapy. The introduction of MMF and anti-IL-2 receptor antibody and T cell depleting antibody therapy in

Table 1. The association of tacrolimus concentration and toxicity or rejection in kidney transplant recipients.

Author	Year of publication	n	Whole-blood Tac range (ng/ml)	Toxicity/rejection episode and main conclusion	Recommended range (ng/ml)	Time after transplantation
Laskow *et al.* [22]	1996	89	Low: 5–14 ng/ml Medium:15–25 ng/ml High: 26–40 ng/ml	Rejection*: decreasing rejection with increasing Tac C_0. toxicity*: increasing toxicity with increasing Tac C_0.	5–15 ng/ml	First 42 days
Kershner *et al.* [23]	1996	92	Low: 5–14 ng/ml Medium: 15–25 ng/ml High: 26–40 ng/ml	Rejection*: decreasing rejection with increasing Tac C_0. toxicity*: increasing toxicity with increasing Tac C_0.		First 42 days
Undre *et al.* [24]	1999	66	AUC>200 ng.h/mL AUC<200 ng.h/mL	Rejection*: significantly lower AUC in patients who experience acute rejection than those who remain rejection free.	>10 ng/ml to avoid rejection	Day 2–3
Staatz *et al.* [25]	2001	29	Low: 0–10 ng/ml High: 10–15 ng/ml	Rejection*: decreasing rejection with increasing Tac C_0.	>10 ng/ml to avoid rejection	First month
Borobia *et al.* [26]	2009	57	Low: >9.3 ng/ml High: <9.3 ng/ml	Rejection*: decreasing rejection with increasing Tac C_0.	>9.3 ng/ml >8.7 ng/ml to avoid rejection	Day 5 day 7
Bottiger *et al.* [27]	1999	14	A: >30 ng/ml B: 20–30 ng/ml C: 10–20 ng/ml D: <10 ng/ml	Rejection: not significant Toxicity*: increasing toxicity with increasing Tac C_0.	<20 ng/ml To avoid toxicity	First year
Ekberg *et al* [1,3]	2007 2009	1645	3–7 ng/mL (Symphony) Compared with standard CsA, low-dose CsA or low-dose sirolimus.	Low-dose Tac results in better renal function, lower acute rejection rates, and graft loss.	Low-dose tacrolimus (3–7 ng/ml) with induction and MMF	1 year and 3 year
Bouamar *et al.* [21]	2013	1304	A: 3–7 ng/ml (Symphony) B: 10–14 ng/ml (FDCC) C: 8–12 ng/ml (first month, OptiCept)	Rejection: not significant		Day 3, 10, and 14 month 1 and 6
Gatault *et al.* [28]	2017	186	A: >3 ng/ml (50% reduction dose) B: 7–12 ng/ml (normal)	Rejection*: more rejection and infections in group A	>7 ng/ml (with steroid free and MMF reduction)	4–12 months

*: significant association between Tac concentration and rejection or toxicity

Tac-treated patients led to a dramatic reduction of rejection rates to percentages below 20% [30]. The current strategy to target low-medium Tac exposure seems reasonable. The lower end of the Tac concentration range has not been clearly established but it is unlikely that a trial comparing different Tac concentration ranges will ever be performed in the era of modern immunosuppression.

3.2. Controversies of Tac exposure C_0 with toxicity

The relationship between Tac C_0 and toxicity appears to be stronger than with rejection. Several studies have demonstrated a correlation between high blood Tac concentrations and toxicity, particularly nephrotoxicity [22,23,29,31]. In the multicenter, open-label, concentration-ranging renal transplant trial, a significant trend was observed for increasing toxicity with increasing maximum Tac C_0 [22]. The relationship between Tac C_0 and toxicity was clearly established in a study which combined the data of four trials in both kidney and liver transplant recipients [23]. These toxicities included 'renal dysfunction' and 'any toxicity requiring a dose reduction.' The authors demonstrated that the incidence of toxicity requiring a dosage reduction increased significantly with increasing Tac C_0. With the high-dose Tac regimen (0.2 mg/kg, twice daily, 26–40 ng/ml), the incidence of toxicity was 62.1%, compared with 50% in the medium-dose regimen (0.15 mg/kg, twice daily, 15–25 ng/ml) and 33.3% in the low-dose regimen (0.1 mg/kg, twice daily, 5–14 ng/ml). In 1999, Bottiger *et al*. also concluded that side effects (one or more) were closely related to Tac exposure: 76% of Tac C_0 above 30 ng/ml, 41% of C_0 within the interval of 20–30 ng/ml, and 26% of the C_0 within the interval of 10–20 ng/ml and only 5.3% for Tac concentrations below 10 ng/ml. The authors recommended that Tac whole-blood C_0 should preferably be kept below 20 ng/ml to avoid side effects [27].

The large variability in the pharmacokinetics of Tac makes it difficult to predict what drug concentration will be achieved with a particular dosage or after a change in drug dose [32]. Without TDM, the large interpatient variability in Tac pharmacokinetics would be unnoticed, and extremes in Tac exposure could occur, exposing some patients to toxic levels while others are at risk for rejection due to too low exposure. Despite the controversies surrounding TDM and the proposed targets, TDM is still considered as the standard care after transplant and widely used in clinical practice nowadays.

3.3. Monitoring TDM with AUC

The area under the concentration versus time curve (AUC) is the best marker of Tac exposure. However, in many centers, it is not feasible to perform TDM by means of a full-dosing interval AUC because of logistic and financial constraints. In addition, it poses a considerable burden on patients. Another limitation of TDM by means of Tac AUC is the absence of hard evidence to support targeting a specific AUC. Nonetheless, some centers prefer to monitor Tac by means of AUC [33]. Calculation of the AUC based on a limited number of blood samples strategy (LSS) using Bayesian estimation has been proposed as a solution [34].

It is unclear which Tac AUC should be targeted in both the early- and late-phase after kidney transplantation. Undre *et al*. and Squifflet *et al*. both suggested an AUC >200 ng/h/mL in the early phase after transplantation to be highly discriminatory for the risk of acute rejection [24,35]. The study by Scholten *et al*. performed an AUC-guided dosing study in 15 renal transplant recipients. Targets for AUC were as follows: 210 ng/h/mL for weeks 2–6 (corresponding with a C_0 of 12.5 ng/mL) and 125 ng/h/mL for weeks 6–52 (corresponding with a C_0 of 7.5 ng/mL). The authors suggest an AUC target of 150–200 ng/h/mL [36]. However, since the publication of the Symphony-Elite study [1], which demonstrated lower rates of acute rejection and improved graft function associated with low-exposure Tac (target C_0 3–7 ng/mL) in combination with MMF and glucocorticoids, the corresponding AUC may be targeted to a lower range.

Before any new monitoring strategy can be recommended, further studies are required to clarify the relationship between (abbreviated) AUC monitoring and clinical outcome. For population pharmacokinetic models and Bayesian forecasting to be useful clinically, this next step must be taken to evaluate how closely dosage predictions with these models actually achieve AUC targets and improve clinical outcomes.

3.4. Analytical issues

Immunoassays have been used for routine TDM because of their quick turnaround time, lower costs, and less complex method (from the records of the International Tac Proficiency Testing Scheme) [37]. It is well known that some Tac metabolites including M-1 (13-O-desmethyl tacrolimus, 10% of the immunosuppressive activity of Tac), M-II (31-O-desmethyl tacrolimus, immunosuppressive activity comparable to Tac), M-III (15-O-desmethyl tacrolimus, no activity), M-V (15,31-di-O-desmethyl tacrolimus, no activity) show cross-reactivity with immunoassays [38]. Up to 30% of Tac quantification may be due to nonspecific detection [37]. Liquid chromatography-tandem mass spectrometric detection (LC-MS/MS) is now increasingly being implemented as a cost-effective alternative technology for Tac TDM. LC-MS/MS is the technique of choice because of its ability to separate and simultaneously quantify Tac and its metabolites. In patients with low plasma concentrations of albumin, the results of Tac measurement as determined by immunoassay are likely to be higher compared with LC-MS/MS in the early post-surgery period. The correlation of albumin with the interassay differences may possibly be explained by the presence of unbound Tac or metabolites with a lower affinity for albumin, still able to cross-react with the antibody [39]. Some studies found that immunoassays appear unable to analyze Tac concentrations in the lower concentration range (between 3.0 and 5.0 ng/mL) and have a higher coefficient of variation (CV) [40]. Tac concentration measurement by LC-MS methods have higher sensitivity, precision, and accuracy, while the application of LC-MS in individual centers is limited. Partly, this was because of inadequate upfront payment for chromatography and difficulties in operation and maintenance. Many centers analyze too few Tac samples to justify the investment in LC-MS. Current data suggest that immunoassays will remain the backbone of assay services for Tac, so we must ensure that they are being used correctly and that the data are useful for clinical practice [36].

TDM of Tac is usually performed in whole blood after venous blood sampling. Dried blood spot (DBS) sampling might be an alternative. In DBS sampling, blood is obtained via a finger prick with a lancet. The drop of blood is applied to sampling paper, which is dried and posted to a laboratory [41]. An advantage of using DBS for TDM is that patients can collect the DBS at home and no phlebotomist is necessary. This technique opens up the possibility to perform extensive pharmacokinetic studies in patients at home. There is, however, a need for more standardization, quality assurance, basic research, and assay development before DBS can be widely implemented in TDM of Tac [42]. In addition, it would be extremely useful if multiple components could be assessed in the same sample. Of note, a DBS assay that measures other immunosuppressants and serum creatinine would meet a clinical need [43,44].

3.5. Intrapatient variability

In addition to being highly variable interindividually, Tac pharmacokinetics can also fluctuate within an individual patient. This so-called intrapatient variability (IPV) is defined as the fluctuation in Tac blood concentrations within an individual over a certain period of time during which the Tac dose is left unchanged (for an in-depth review of Tac IPV please see reference [45]). A high Tac IPV is considered a risk factor for poor long-term outcomes after kidney transplantation, and similar findings have been reported after liver [46] and lung transplantation [47]. The first evidence for the clinical importance of Tac IPV was obtained by Borra *et al*. who found that the within-patient variability in Tac is a significant risk factor for reaching a composite end point consisting of graft loss, biopsy-proven chronic allograft nephropathy and 'doubling in plasma creatinine concentration in the period between 12 months post-transplantation and last follow up [48]. A high Tac IPV was also associated with more acute rejection after kidney transplantation [49]. Recently, Sapir-Pichhadze *et al*. observed that a higher Tac IPV was associated with more late allograft rejection, transplant glomerulopathy, graft loss, and death with a functioning transplant [50]. In pediatric kidney transplantation, a high Tac IPV has also been associated with increased late rejection and graft loss [51,52].

Several factors can influence Tac pharmacokinetics and contribute to Tac IPV, including the type of analytical assay (see above), the concomitant intake of food, gastrointestinal disturbances, drug–drug interactions, genetic factors and importantly, non-adherence [45,53–55]. Calculation of Tac IPV is relatively easy and can be done automatically in the electronic patient record or by use of apps, and may help to identify high-risk patients during routine follow-up visits to the outpatient clinic. Once a patient is recognized as having a high IPV, physicians need to find out what is the underlying cause and try to resolve the problem [56]. Education with regard to the effects of food and over-the-counter medication on Tac exposure should be attempted. When non-adherence is suspected, interventions aimed at improving drug compliance and possibly, switching to a Tac once-daily formulation should be considered [57].

3.6. Drug–drug interactions

Drug interactions occur when the efficacy or toxicity of a medication is changed by coadministration of another drug. As Tac is a substrate for cytochrome P450 (CYP) 3A enzymes and P-gp, drugs that inhibit or induce these mechanisms may increase or decrease blood Tac concentrations, such as glucocorticoids and antifungal agents. Detailed knowledge of potential drug–drug interactions with immunosuppressive drugs to avoid significant clinical effects is of great importance in the clinical management of transplant patients [53].

Tac drug interactions have been extensively studied [58–61]. Drug interaction with P-gp may change Tac tissue distribution and modify its toxicity and immunosuppressive activity [62]. There is also evidence that ethnic and gender differences exist for Tac drug interactions [63].

3.7. Once-daily Tac

Tac was originally formulated as Prograf®. Newer once-daily (QD) prolonged-release formulations of Tac (Advagraf® and Envarsus® XR) and various generic versions of Prograf® are becoming available now. The Envarsus® XR formulation using MeltDose® technology was introduced as an innovation in the field of the immunosuppressive drugs [64]. Envarsus® XR is associated with consistent Tac exposure (AUC) at an approximately 30% lower dose compared to twice-daily Tac. On the basis of the stricter criteria for narrow therapeutic-index drugs, Prograf®, Advagraf® and Envarsus® XR are not bioequivalent. Patients may require a daily dosage increase if converted from Prograf® to Advagraf®, while a daily dosage reduction appears necessary for conversion from Prograf® to Envarsus® XR [65].

Studies found similar results for both once-daily formulations in terms of patient survival, graft survival and renal function [66,67]. One recently published review concluded that once-daily Tac is non-inferior to twice-daily Tac, with a concentration-dependent risk of rejection risk [68]. For the toxicity, some studies suggested that once-daily Tac may have favorable effects on blood pressure, the lipid profile and glucose tolerance [68–70]. Larger randomized, controlled trials are needed in different transplant populations to determine whether there are differences in efficacy and toxicity across the formulations and whether formulation conversion is worthwhile in the long term.

There is also a trend for improved adherence with QD formulation [71] and in one study, patients stated a preference for once-daily Tac dosing [72]. In addition to improved adherence, studies showed that after conversion, intraindividual variability appears to be lower with Tac QD [73,74]. A close monitoring of C_0 levels, or Bayesian estimation of the AUC when needed, is mandatory because of the high interindividual variability in Tac pharmacokinetics [75,76].

4. Novel matrices

4.1. Intracellular Tac

As the site of action of Tac is within the lymphocyte, it seems logical to assume that the Tac concentration at its target site is more relevant than the concentration in whole blood to predict the efficacy of treatment [77]. Over the last few years several assays have been published that were able to measure Tac in peripheral blood mononuclear cells (PBMC), obtained following gradient density centrifugation. In 2007, the first published assay

was an immunoassay [78], but since then several (UP)LC-MS/MS assays have been published [79–81]. Capron *et al*. studied the intracellular Tac concentration in 96 renal transplant recipients. They concluded that the intracellular concentrations seemed to be strongly dependent on *ABCB1* polymorphisms [82]. Based on histological findings, there tended to be an association between acute rejection episodes and significantly lower Tac intracellular concentrations [82]. The same research group conducted a study in liver transplant recipients and observed that patients experiencing clinical rejection one week after transplantation had significantly lower Tac PBMC concentrations on day 7 after transplantation than patients who did not suffer from a rejection episode. In contrast to the intracellular concentration, the whole-blood Tac concentration was not associated with clinical rejection. The authors concluded that the Tac concentration in PBMCs could be a better matrix for the measurement and TDM of Tac [83]. Lemaitre *et al*. failed to demonstrate a relationship between Tac whole-blood concentrations, Tac PBMC concentrations, and intracellular CN activity. This was probably caused by the small cohort of patients ($n = 10$) [84]. That same year Pensi *et al*. were able to characterize the PBMC compartment as a significant Tac reservoir in 37 pediatric liver transplant recipients, with intracellular concentrations being approximately 12.7 times higher than whole-blood concentrations. For the first time, a correlation between intracellular and whole-blood concentrations was demonstrated [81]. Fairly recently, the relationship between Tac concentrations in PBMCs, the whole-blood Tac concentration, the factors affecting this relationship, and the risk of rejection was studied in 237 renal transplant recipients [85]. The correlation between whole-blood and intracellular Tac concentration was linear. This relationship was affected by sex, hematocrit, and time after transplantation. The Tac ratio (intracellular concentration divided by whole-blood concentration) was not significantly associated with acute rejection [85].

The intracellular Tac concentration could be a better matrix to ensure adequate Tac exposure in addition to whole-blood Tac levels. The major drawback of implementing intracellular Tac concentration measurement in clinical practice is the complex analytical technique. Furthermore, there is only limited evidence that the intracellular concentration correlates better with clinical outcomes than whole blood exposure. Given the fact that one of the determinants of the ratio between intracellular and whole-blood Tac concentration is the activity of efflux pumps in the cell membrane of PBMCs, and as polymorphisms in the genes encoding for these pumps will result in different ratios between individuals, it is expected that intracellular concentration will offer a better reflection of biological action than whole blood Tac concentrations.

4.2. Unbound Tac concentration

The disposition of Tac is affected by protein binding. The unbound concentration of a drug is considered the pharmacologically active part. This may be attributed to the fact that only unbound drug in plasma can migrate to tissue compartments and interact with its receptor. It thus seems reasonable to assume that the unbound or free, Tac concentration may predict the efficacy or toxicity of treatment better than whole-blood concentrations. The unbound concentration of Tac is low (<3% of the total plasma concentration and <0.5% of the whole-blood concentration) [86]. In 2004, the distribution and plasma protein binding of Tac was studied in 40 liver transplant recipients. The unbound fraction was significantly lower in patients experiencing mild rejection compared to patients who did not experience a rejection episode (0.32 and 0.53, respectively). Interestingly, no difference was observed for total whole-blood concentrations between rejecting and non-rejecting patients [87]. A second study in 10 liver transplant recipients also showed significantly lower unbound Tac concentrations during rejection episodes. In patients experiencing Tac-related side effects, the unbound Tac concentration was significantly higher (0.84 ± 0.19 *vs*. 0.53 ± 0.19 ng/L). The whole-blood concentrations were not different for both rejection and toxicity [88]. These studies support the lack of correlation between Tac whole-blood concentration and the incidence of rejection and side effects, and suggest that the unbound Tac concentration could be a better predictor of Tac efficacy.

Due to the complex analytical technique, not much research was published on unbound Tac concentrations the last decade. Fairly recently, a novel, and less complex LC-MS/MS method was published [86]. This will hopefully facilitate more research on the relationship between unbound Tac and clinical outcomes.

5. Pharmacogenetic monitoring

Genetic polymorphisms in genes encoding Tac-metabolizing enzymes partly explain the interpatient variability in Tac pharmacokinetics. The key enzymes involved in the metabolism of Tac are CYP3A4 and CYP3A5 [89]. Individuals are considered expressers of CYP3A5 if they carry at least one *CYP3A5*1* allele, whereas individuals homozygous for the *CYP3A5*3* allele are classified as CYP3A5 non-expressers. In addition to *CYP3A5*3*, the *CYP3A5*6* and *CYP3A5*7* variant alleles can also lead to nonfunctional CYP3A5 protein [90]. There are also ethnic distribution differences of *CYP3A5* variant alleles with expressers (carriers of the *CYP3A5*1* variant allele) being more frequently found among non-Caucasian populations. Approximately 10–40% of Caucasians are CYP3A5 expressers, 33% of Asians and 55% of African Americans [91–93]. CYP3A5 expressers require a Tac dose that is approximately 1.5–2-fold higher than non-expressers to reach the same exposure [94–96]. This implies that following a standard, bodyweight-based Tac dose, CYP3A5 expressers are prone to have subtherapeutic Tac concentrations whereas non-expressers are expected to have supratherapeutic Tac concentrations [32]. Genetic testing prior to the initiation of Tac treatment would allow to more quickly reach the target concentration. The CPIC guideline recommends that if *CYP3A5* genotype of a transplant patient is known, expressers should receive a 1.5–2 times higher starting dose, while CYP3A5 non-expressers should get the standard starting dose. The guideline, however, does not advise nor discourage pharmacogenetic testing prior to the start of Tac therapy [97].

Two large randomized-controlled trials have attempted to determine the clinical relevance of basing the Tac starting dose on an individual patient's *CYP3A5* genotype. In both trials, kidney

transplant recipients were randomized to either receive the standard, bodyweight-based Tac dose (0.2 mg/kg/day) or to receive a dose customized to the *CYP3A5* status of the patient (expressers 0.3 mg/kg/day and non-expressers 0.15 mg/kg/day) [98,99]. In the first study, genotype-based dosing resulted in significantly more patients being within the target Tac concentration range, 3 days after starting Tac (43.2%), compared with patients receiving the standard, bodyweight-based dose (29.1%). The genotype-based group also required significantly less time and fewer dose adjustments to reach the target Tac concentration [99]. The second trial, however, found no such advantage of *CYP3A5*-based dosing with 37.4% of patients receiving the standard bodyweight-based dose being within the Tac concentration range compared with 35.6% of the genotype-based group at first steady state. There was also no difference in the time to reach the target Tac concentration or the number of dose adjustments [98]. The explanation for this finding was that in the genotype-based arm, CYP3A5 non-expressers tended to have subtherapeutic concentrations more often after receiving the reduced starting dose. The reverse was seen among CYP3A5 expressers in the genotype-based arm, who tended to be above the target Tac concentration [98].

Both trials, performed in largely Caucasian populations, failed to demonstrate a decreased risk of acute rejection or any other clinical benefit, concluding that optimizing the initial Tac dose based on *CYP3A5* genotype alone does not improve clinical outcomes when extensive TDM is performed [100]. It appears that TDM rapidly corrects any Tac concentrations outside the therapeutic concentration range and therefore the under- or over-exposure does not last long enough to cause a clinically relevant increase in the incidence of acute rejection or side effects. The current outcome studies do not support routinely genotyping kidney transplant recipients for *CYP3A5*. However, a genotype-based strategy may hold promise for patients having a high immunological risk and for ethnic populations with higher prevalence of CYP3A5 expresser status, such as Asians and African Americans [101,102].

In both trials, the percentage of patients within the desired Tac concentration range 3 days after initiating Tac was rather low, implying that there is a considerable variability in Tac pharmacokinetics that cannot be explained by *CYP3A5* genotype alone. In Caucasians, polymorphisms in the *CYP3A5* gene explain 40–50% of the variability in Tac dose requirement [98,99]. The *CYP3A4* genotype has also been associated with altered Tac clearance [89]. Fairly recently, research has shown that *CYP3A4*22* is associated with lower Tac dose requirements, whereas *CYP3A4*26* is associated with extremely low-dose requirements [103–105].

Perhaps a more precise and novel strategy is to use a pharmacokinetic dosing algorithm for the starting dose of Tac. Recently developed algorithms usually incorporate the *CYP3A5* genotype, and occasionally also the *CYP3A4* genotype. Moreover, these algorithms use a combination of clinical, demographic, and genetic information to determine the Tac dose [106]. So far, a few dosing algorithms suitable to determine the starting dose have been developed [107]. The most extensively researched dosing algorithm was developed by Passey *et al*. [108]. It incorporated *CYP3A5* genotype, age, days posttransplant, steroid and calcium channel blocker use. It was later successfully validated externally in an independent cohort [109]. A few years later, this dosing algorithm was prospectively tested by an independent research group and unfortunately it was not able to predict the estimated Tac clearance accurately [110]. Fairly recently, a dosing algorithm for the starting dose and subsequent dosages was developed using data from 304 renal transplant recipients [111]. The pharmacokinetic model included *CYP3A5*3, CYP3A4*22*, age and hematocrit. External validation confirmed the prediction ability of the model. This algorithm has not been prospectively tested. Recently, a dosing algorithm for the starting dose and subsequent dosages of Tac following pediatric renal transplantation was published [112]. Recipients with a higher bodyweight, lower eGFR, higher hematocrit levels, CYP3A5 non-expressers and who received a kidney from a living donor, had a lower Tac clearance. The pharmacokinetic model was successfully externally validated. The dosing algorithm is currently being tested in a prospective study. To our knowledge other dosing algorithms, developed in children or adults, have not been validated externally nor tested prospectively. We feel that this is essential before these dosing algorithms can be implemented in routine clinical practice.

6. Pharmacodynamic monitoring

6.1. Calcineurin activity

CN activity assays directly measure the effect of CNIs on their target enzyme CN. This enzyme is selectively targeted by CNIs and can therefore be used to monitor the pharmacodynamics of Tac [113]. Van Rossum *et al*. published a comprehensive review on this topic in which the pros and cons of this assay were extensively discussed [114]. Quantifying the degree of inhibition of CN determines the biological effect/pharmacodynamics of Tac and may better reflect the biological effect of Tac, compared to pharmacokinetic monitoring [115]. Data by Sellar *et al*. showed that the activity of CN in patients treated with Tac correlated with CN activity in whole blood, leukocytes, and PBMCs [116–118]. The first hours after Tac intake, a clear inhibition of CN activity was observed; however, after six hours CN activity could no longer be distinguished from pre-intake CN activity, whereas whole-blood concentrations were still elevated [117,118]. Mortensen *et al*. demonstrated that on day 14 posttransplantation, the CN activity before, as well as 1, 2, 3, and 4 h after oral intake of Tac, was significantly inhibited compared with healthy subjects not treated with a CNI. In contrast, in the same patients 5 years posttransplantation, the CN activity measured at the same time points was not significantly different from CN activity in healthy subjects, despite relevant Tac concentrations [119]. This could be explained by the lower doses and Tac target concentrations 5 years posttransplant, but also raises the question whether monitoring the CN activity is the correct pathway.

In both liver and kidney transplant recipients, the CN activity just before intake of the next Tac dose was increased in patients suffering from acute rejection [120,121]. Fukudo *et al*. demonstrated that the CN activity rapidly increased a few days before onset of acute rejection [122]. However, all the CN activity studies were conducted in small groups of patients. The correlation between CN activity and clinical events, *e.g*. acute rejection or Tac toxicity in renal transplantation therefore remains unclear [114]. In our opinion, CN activity measurement is currently not a

clinically useful marker for TDM of Tac [123]. Furthermore, the analytical techniques to measure CN are complex, time consuming, and expensive.

6.2. Expression of nuclear factor of activated T-cell-regulated genes

Another biomarker that may reflect the individual's sensitivity to CNI therapy is the assessment of NFAT-regulated gene expression. CNIs inhibit the transcription of the NFAT-regulated genes IL-2, IFN-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF) in lymphocytes [124,125]. The expression of NFAT-regulated genes in patients treated with Tac, shows an inverse correlation between Tac whole-blood concentrations and the expression of these genes [126]. At the time of peak Tac concentrations, the highest inhibition of gene expression occurred. Data on NFAT-regulated gene expression are more comprehensive in CsA therapy, compared with Tac therapy [127].

The first study on NFAT-regulated gene expression in renal transplant recipients treated with Tac, demonstrated that the residual expression of NFAT-regulated genes was significantly higher in patients with acute rejection, whereas the Tac predose concentrations were comparable. Of the patients with gene expression above 30%, a quarter developed BPAR [126]. A small study in liver transplant recipients showed similar results [128]. Two prospective observational studies concluded that low NFAT-residual gene expression was associated with signs of over-immunosuppression, including CMV and BKV viremia [127,129]. A small study in liver transplant recipients demonstrated a lower NFAT-regulated gene expression in patients with CMV-viremia [130]. NFAT-regulated gene expression might be used as a biomarker for detecting patients with an increased risk of rejection or virus-associated complications.

An RCT is currently being performed which evaluates the improvement of cardiovascular risk in CsA-treated stable renal transplant recipients by monitoring the CsA C_0 and residual NFAT-regulated gene expression [127]. Secondary objectives include the incidence of BPAR, adverse events, and renal function [127]. To our knowledge, no such studies in Tac-treated patients are currently being executed. NFAT-regulated gene expression is less specific for Tac exposure than measuring CN activity, but it seems easier and more reproducible. At this moment in time, NFAT-regulated gene expression is not a useful clinical marker for adequate Tac exposure but in the future it could possibly be used in addition to TDM.

6.3. Phosphospecific flow cytometry

Cytokines binding to the IL-2 receptor family act via activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. JAK enzymes are key elements in cytokine signaling. JAKs phosphorylate the γ_c receptor of the IL-2R that subsequently serves as docking molecule for the STAT signaling molecules. Vafadari *et al.* published a comprehensive review on this topic [131]. For other cytokines *e.g.* IL-6, IL-10, IL-17 and interferons, JAK-STAT activation is also critical for signaling. Phosphospecific flow cytometry measures activation-induced changes of signaling molecules and can be used to monitor the effect of immunosuppressive drugs on intracellular signaling pathways and how molecules are activated in response to stimuli [131].

NF-κB plays a key functional role in T cell activation and is considered a mediator of rejection processes following organ transplantation [132,133]. The effect of Tac on the NF-κB activation pathway was studied by quantitative analysis of NF-κB phosphorylation in primary T cell subsets. This study concluded that Tac has a suppressive effect on NF-κB signaling in peripheral T cell subsets [134].

It was recently discovered that Tac also suppresses the phosphorylation of the mitogen-activated protein kinase (MAPK) pathway [135]. The amount of phosphorylation of this signaling molecule seems to be inversely correlated with Tac C_0 in kidney transplant patients. Increased p38MAPK phosphorylation was associated with higher T cell activation status. Recent research has shown that conversion to once-daily Tac results in increased p38MAPK phosphorylation in T cells of kidney transplant patients [136]. Three months post conversion, p38MAPK phosphorylation increased significantly in $CD4^+$ (11.4%) and in $CD8^+$ (15.6%) T cells, whereas the Tac C_0 did not decrease significantly [136]. Another study demonstrated that Tac inhibits p38MAPK phosphorylation by 30% in $CD14^+$ monocytes [137]. Tac also partially inhibited p-AKT (14%) and p-ERK (extracellular signal-regulated kinases, 15%) [137]. Activation of these pathways plays an important role in monocyte/macrophage responses. The authors concluded that Tac does not strongly affect monocyte function [137].

The observations in the above-mentioned pharmacodynamic studies suggest that measuring NF-κB or p38MAPK phosphorylation may better reflect the biological effects of Tac therapy compared with classic pharmacokinetic monitoring. More precise information on T cell activation status is obtained, but the relationship between NF-κB or p38MAPK phosphorylation and acute rejection is yet to be established.

7. Expert opinion

Immunosuppressive therapy is necessary to prevent both acute and chronic rejection after kidney transplantation. Tac is the preferred drug and it is to be expected that in the next 10 years, patients will continue to receive Tac as part of standard immunosuppressive regimens.

Due to a narrow therapeutic index and large interpatient pharmacokinetic variability, TDM is routinely performed for individualization of the Tac dose to maintain drug efficacy and minimize the consequences of under- and overexposure. Unfortunately, the evidence for the optimal Tac C_0 is more limited than one would expect of a drug so extensively prescribed and studied. Based on the current literature, there is little support to promote a specific therapeutic window. Besides this, the relationship between Tac concentration and either acute rejection or toxicity remains controversial. Acute cellular rejection episodes occur when the Tac concentration is within the target concentration range and patients having supra-therapeutic exposure sometimes do not suffer from side effects. This suggests that Tac whole-blood predose concentrations do not always correlate with its pharmacological effect and indicate that novel matrices or monitoring strategies are needed to better predict and monitor the effect of Tac treatment.

Novel options include the measurement of Tac concentrations within the lymphocyte and the unbound concentration. Both options are technically demanding but seem feasible with the recent availability of sophisticated analytical methods. The intracellular Tac concentration is the most extensively studied option of the two. An association between the Tac PBMC concentration and acute rejection has been demonstrated and at this point in time is the most promising matrix to optimize the monitoring of Tac. Nonetheless, results need to be consistent before we can abandon classic TDM.

As Tac is mainly metabolized by CYP3A5, and as it is known that CYP3A5 expressers require a twofold higher dose to reach the same exposure compared with non-expressers, it seems reasonable to implement preemptive pharmacogenetic testing. However, two RCTs failed to demonstrate a decreased risk of acute rejection or any other clinical benefit of basing the Tac starting dose on an individual's *CYP3A5* genotype. More sophisticated dosing strategies are needed. A more precise strategy would be to develop and validate a population pharmacokinetic dosing algorithm for the initial Tac dose. Implementation of a dosing algorithm may allow recipients to reach the target Tac concentration more quickly and may lead to less patients being exposed to extremely high or low Tac concentrations.

A relatively unknown but promising technique is the pharmacodynamic monitoring of Tac. Different strategies are currently under investigation, of which measuring the NFAT regulated gene expression or phosphospecific flowcytometry show the most encouraging results. It is possible that after two decades of Tac predose concentration measurements and TDM, in a few years pharmacodynamic monitoring will be conducted in combination with classic TDM to adequately describe the effect of Tac. With no new immunosuppressive drugs in the pipeline, an improved monitoring strategy of Tac seems the next best thing to optimize patient outcomes.

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