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Therapeutic drug monitoring of tacrolimus-personalized therapy: Second consensus report

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Abstract

Ten years ago, a consensus report on the optimization of tacrolimus was published in this journal. In 2017, the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicity (IATDMCT) decided to issue an updated consensus report considering the most relevant advances in tacrolimus pharmacokinetics, pharmacogenetics, pharmacodynamics and immunologic biomarkers, with the aim to provide analytical and drug-exposure recommendations to assist TDM professionals and clinicians to individualize tacrolimus TDM and treatment.

The Consensus is based on in-depth literature searches regarding each topic that is addressed in this document. Thirty-seven international experts in the field of TDM of tacrolimus as well as its pharmacogenetics and biomarkers contributed to the drafting of sections most relevant for their expertise. Whenever applicable, the quality of evidence as well as the strength of recommendations was graded according to a published grading guide. Following iterated editing, the final version of the complete document was approved by all authors.

For each category of solid organ and stem cell transplantation, the current state of pharmacokinetic monitoring is discussed and the specific targets of tacrolimus trough concentrations (pre-dose sample C_0) are presented for subgroups of patients along with the grading of these recommendations. In addition, tacrolimus AUC determination is proposed as the best TDM option early after transplantation, at the time of immunosuppression minimization, for special populations, and specific clinical situations. For indications other than transplantation, the potentially effective tacrolimus concentrations in systemic treatment are discussed without formal grading.

The importance of consistency, calibration, proficiency testing and the requirement for standardization and need for traceability and reference materials is highlighted. The status for alternative approaches for tacrolimus TDM is presented including dried blood spots (DBS), volumetric absorptive micro-sampling (VAMS) and the development of intracellular measurements of tacrolimus.

The association between *CYP3A5 genotype* and tacrolimus dose requirement is consistent (Grading[1, 2] A I). So far, pharmacodynamic and immunologic biomarkers have not entered routine monitoring, but determination of residual NFAT-regulated gene expression supports the identification of renal transplant recipients at risk of rejection, infections and malignancy (B II).

In addition, monitoring intracellular T-cell IFN-g production can help to identify kidney and liver transplant recipients at high risk of acute rejection (B II) and select good candidates for immunosuppression minimization (B II). Although cell-free DNA seems a promising biomarker of acute donor injury and to assess the minimally effective C_0 of tacrolimus, multicenter prospective interventional studies are required to better evaluate its clinical utility in solid organ transplantation.

Population pharmacokinetics (PopPK) models including *CYP3A5* and *CYP3A4* genotypes will be considered to guide initial tacrolimus dosing. Future studies should investigate the clinical benefit of time-to-event models to better evaluate biomarkers as predictive of personal response, the risk of rejection and graft outcome.

The Expert Committee concludes that considerable advances in the different fields of tacrolimus monitoring have been achieved during this last decade. Continued efforts should focus on the opportunities to implement in clinical routine the combination of new standardized pharmacokinetic approaches with pharmacogenetics, and valid biomarkers to further personalize tacrolimus therapy and to improve long-term outcomes for treated patients.

Keywords: Tacrolimus-personalized therapy, pharmacokinetics, biomarkers, pharmacogenetics, pharmacodynamics, Consensus, immunologic biomarkers, graft outcome, graft injury, tacrolimus target concentrations, methods standardization, new approaches in tacrolimus TDM, PopPK/PG modeling.

EXECUTIVE SUMMARY

This executive summary is an overview of the Consensus Report on TDM of Tacrolimus-personalized therapy that synthesizes the key points and the grading of recommendations regarding: tacrolimus exposure in different types of solid organ transplantation (SOT) and other indications; the influence of pharmacogenetic variables and pharmacodynamic biomarkers in achieving drug specific target concentrations, as well as the role of PK/PG and PK/PD models in personal drug adjustment.

The new recommendations and knowledge regarding the previous consensus report in 2009 are highlighted. During the last decade, there have been many changes regarding the clinical management and TDM of tacrolimus. The goal of these changes was to minimize the adverse effects, mainly nephrotoxicity, and improve its effectiveness. Several studies have evaluated new target concentrations for tacrolimus significantly lower than those of the previous decade. In addition, these therapeutic ranges for tacrolimus have been refined based on post-transplant time, concomitant immunosuppressive medication and according to immunological risk.

With the changes in tacrolimus monitoring, there is a requirement for highly standardized, specific, sensitive and robust methods that allow precise tacrolimus monitoring, even at low concentration ranges (2-4 ng/mL). In addition, during this last decade, we have developed a new approach from a pharmacological perspective of monitoring therapeutic drugs that includes not only the pharmacokinetics but also some pharmacogenetic and pharmacodynamic variables, since this combination can provide a more personalized treatment. Efforts have been made to evaluate the influence of genetic polymorphisms on the initial dosing of tacrolimus and the subsequent clinical effects such as the incidence of rejections in various populations in SOT. Considering that similar concentrations of tacrolimus may produce different degrees of

immunosuppression in treated patients, several groups have focused on evaluating the clinical usefulness of pharmacodynamic and immunological biomarkers predictive of the risk of graft rejection and clinical outcome.

Pharmacokinetic Monitoring

Drug exposure and within-patient variability of tacrolimus concentrations as a biomarker for therapeutic drug monitoring

Previous studies in SOT have shown a relationship between tacrolimus exposure and the risk of acute rejection and drug-related adverse events. The AUC is considered the pharmacokinetic exposure parameter best associated with clinical effects. Unfortunately, no prospective studies of clinical outcomes have been conducted in adult and pediatric transplant recipients to investigate properly the potential benefits of AUC_{0-12} monitoring compared to C_0 guided therapy. However, C_0 is used in most transplant centers for routine TDM of tacrolimus. The monitoring of tacrolimus-AUC has been proposed especially in the early period post-transplantation, from time to time to check the overall exposure, and when clinically indicated. Furthermore, the rather poor correlation between C_0 and AUC translates into very variable AUC/C_0 ratios, which means that patients with identical C_0 may have very different AUC_{0-12h}. Therefore, the authors suggest evaluating this ratio at least once in the early period and once in the stable period, for each transplant recipient.

Recent reports indicate that the within-patient variability of tacrolimus concentrations could be a useful tool for optimizing the immunosuppressive therapy in SOT. Most of the time, the within-patient variability is simply evaluated using the coefficient of variation (CV) of trough

concentrations (C_0). The hypothesis is that significant variability of tacrolimus concentrations may lead alternatively to underexposure and overexposure periods resulting in immune activations with sub-clinical rejections accumulation favoring organ lesions and drug toxicity associated with adverse events and organ damages. Furthermore, the intra-patient variability may identify patients with low adherence and patients with particular pharmacokinetic profiles. To date, none of the different tacrolimus formulations (immediate release, prolonged or extended release) has been clearly reported to decrease pharmacokinetic variability of the drug when compared to the other.

In this Consensus Report, it is explained for the first time that the intra-patient variability must be evaluated; likewise, the monitoring of the C_0 / AUC ratio is proposed, to identify those patients that are good candidates to analyze AUC-Tac instead of C_0.

Recommended tacrolimus target concentration ranges in solid organ transplantation.

Executive summary on tacrolimus exposure recommendations

In kidney Transplant recipients:

1. In immunological low-risk patients, tacrolimus may be targeted to: a C_0 of 4-12 ng/mL (and preferably to C_0>7 ng/mL) when prescribed in combination with IL-2 receptor (IL-2R) blocker induction therapy, mycophenolate and glucocorticoids (A I); or a C_0 of 4-7 (month 0-2) and 2-4 (> month 2) ng/mL when combined with everolimus and glucocorticoids and induction therapy (either Thymoblobuline or IL-2R blockers) (B II).

2. Tacrolimus C_0 targets may be higher in adult patients at higher immunological risks. (B II).

3. Although only supported by clinical experience, a C_0 target range of 10-20 ng/mL may be proposed for pediatric patients (C1 II).

4. A minimal $AUC_{0\text{-}12h}$ threshold of 150 ng.h/mL may be proposed for the twice daily formulation in adults (B II). AUC targets corresponding to different C_0 ranges were derived from AUC-C_0 correlation studies in large adult patient populations, for the twice daily and once-daily (Advagraf) formulations.

In liver transplant recipients:

Adults:

1. When prescribed in combination with mycophenolate or everolimus and corticosteroids, tacrolimus may be targeted to a C_0 of 6–10 ng/mL during the first 4 weeks post-transplantation and 5–8 ng/mL thereafter (A I).

2. Tacrolimus as a monotherapy, or when only associated with induction treatment, may require a higher C_0 target (10-15 ng/mL during the first 3 months after transplantation and 5-10 ng/mL afterwards) (C1 II).

3. A tacrolimus C_0 of 10-15 ng/mL may also be aimed for in patients on a corticosteroid-free regimen (even beyond the 4th month after surgery) (C1 II).

For pediatric patients, there is not enough clinical evidence to make recommendations.

In heart and lung transplantation:

The C_0 ranges recommended almost 20 years ago must be revised (and probably lowered), as they encompassed values between 15 and 20 ng/mL in the first weeks post-transplantation, whereas recent studies have shown that the risk of acute kidney injury within-the first two weeks post-transplantation was significantly increased for C_0 >15 ng/mL (B II).

In bone marrow transplantation:

A C_0 of 10–20 ng/mL may be targeted when tacrolimus is prescribed orally in combination with methotrexate, in adults (B II) as well as in children (B II).

For the use of tacrolimus on other indications, there is not strong enough evidence to make recommendations (C2).

MEASUREMENT OF TACROLIMUS CONCENTRATIONS

This Consensus Report encompasses the advances made in the field of the analysis of tacrolimus concentrations, with a critical discussion of pros and cons for each method, including new monitoring strategies such as dried blood spots (DBS) and intracellular tacrolimus concentrations. Stability of tacrolimus has been investigated using both patient blood samples and spiked whole-blood samples. The stability of tacrolimus concentrations was proven for samples stored up to 14 days at 22°C or 4°C, as well as at least 1 month at -20°C and 1 year at -70°C.

The use of commercial whole blood-based tacrolimus calibrators is recommended to ensure accurate measurements and to support the harmonization of results between laboratories.

Analytical methods to determine tacrolimus in whole blood

Analysis of tacrolimus in whole blood is performed either by immunoassays or by liquid chromatography-tandem mass spectrometry (LC-MS/MS), with both techniques being represented in nearly equal proportions at measurement service providers worldwide.

Chromatographic methods

The majority of the LC-MS/MS assays (~75%) were multianalyte methods that allowed for the simultaneous quantification of tacrolimus and other immunosuppressive drugs within one analytical batch using whole blood samples.

High analytical selectivity and sensitivity, and the possibility for high throughput multianalyte assays are important benefits of LC-MS/MS. Thus, this technique has met the evolving clinical requirements for fast, accurate, and precise tacrolimus assays performing well at low concentrations. Nevertheless, skilled laboratory management and trained staff are necessary to establish and operate LC-MS/MS assays for routine TDM.

***Sample preparation*:** Tacrolimus is measured in whole blood. Protein precipitation, solid-phase extraction (SPE), and liquid-liquid extraction (LLE) have been reported as sample preparation strategies prior to chromatography in LC-MS/MS assays including tacrolimus. Precipitation of whole blood samples can be performed by either a stepwise or simultaneous addition of zinc sulfate solution and organic solvent.

Chromatography: Chromatographic separation is commonly based on C18 (or C8) stationary phases combined with mobile phases of water and methanol to which an acidified ammonium buffer is added. The mobile phase constituents should be of LC-MS quality.

Mass-spectrometry: The majority of tacrolimus LC-MS/MS assays apply electrospray ionization (ESI) as the interface to get the sample compounds ionized and into gas phase. The conditions for spraying, evaporation, ionization, and acceleration of tacrolimus ions into the mass-spectrometer have to be optimized with respect to the specific instrument type. Several of the ion source parameters are global in a multianalyte assay and, consequently, optimization of these parameters may have to be prioritized for compounds yielding the lowest instrument response at the lower limit of quantification (LLOQ) (usually sirolimus and everolimus when included in a multianalyte assay for immunosuppressive drugs).

Immunoassays

First generation assays have been replaced by improved tests. Currently a choice of immunoassays is available (EMIT, ECLIA, others) and the Chemiluminescent Microparticle Immunoassay (CMIA) has rapidly become a leader of immunoassay methodology for tacrolimus due to low *bias vs.* chromatography, imprecision even better than LC-MS/MS and close agreement of results for clinical samples in proficiency tests.

Evaluation of the CMIA tacrolimus assay showed no interference with hematocrit, bilirubin or total protein, but cross-reactivity yielding 94% with 31-O-desmethyl (M-II) and 45% with 15-O-desmethyl (M-III) tacrolimus metabolites. The cross-reactivity with 13-O-desmethyl (M-I) and 12-hydroxy (M-IV) tacrolimus metabolites was negligible.

NEW MONITORING STRATEGIES

Microsample based tacrolimus concentration monitoring

The use of DBS on filter cards and other microsampling are innovative, minimal-invasive sample methods, which can replace traditional blood sampling for TDM of tacrolimus. This approach is patient-friendly and can be implemented at home by the patient themselves, collecting very small amounts of blood (typically 10-20 µL). The procedure is both cost and time saving, and it also allows for multiple sampling within a dose interval, simplifying the determination of AUC. Current challenges in microsample implementation include: extraction recovery, the hematocrit effect, correlation between venous and capillary blood measurements, the quality of the blood spot, risk of contamination, and sample stability.

New generation micro-sampling such as volumetric absorptive micro-sampling (VAMS) can possibly mitigate some of the usual bias encountered with these methods, particularly the hematocrit effect.

Intracellular and tissue tacrolimus concentration monitoring

Determining tacrolimus drug concentrations where it exerts its immunosuppressive effect might be particularly relevant to achieve personalized therapy. Several groups have contributed to the development of analytical methods to measure tacrolimus concentrations in peripheral blood mononuclear cells (PBMC) of kidney, liver and heart transplant recipients. The large variability reported in these studies highlighted the critical importance of pre-analytical and analytical steps for intracellular concentration assays. Most analytical methods employed liquid chromatography with tandem mass-spectrometric detection, but there are several analytical issues that have not

yet been fully addressed. These methods must be appropriately standardized, harmonized, and validated following international guidelines.

PHARMACOGENETICS

The association between *CYP3A5* genotype and tacrolimus dose requirement is consistent and has been observed among kidney, liver, heart and lung transplant recipients, both adult and pediatric. Although genotyping has proven effective in predicting the starting dose of tacrolimus, this has not influenced outcome of transplanted patients, provided appropriate use of TDM. As the clinical benefit of popPK models is being investigated, the value of including *CYP3A* genotypes and potentially other genetic markers in such models may be re-evaluated.

Executive summary and practical recommendations

1. Patients expressing CYP3A5 require approximately 50% higher tacrolimus dose to reach the target therapeutic range compared with non-expressors (A I).

Although there is evidence from a randomized-controlled clinical trial that basing the tacrolimus starting dose on the *CYP3A5* genotype may facilitate tacrolimus dosing, this has not been a universal finding and there is currently no convincing clinical evidence that a pharmacogenetics-based approach to tacrolimus dosing improves clinical outcomes after solid organ transplantation.

2. Of the many other candidate single nucleotide polymorphisms that have been studied, *CYP3A4*22* appears to be the most promising as it explains residual variability in tacrolimus pharmacokinetics (B II, and C2 II, for Caucasians, Asiatic and African origin populations).

PHARMACODYNAMIC BIOMARKERS FOR TACROLIMUS MONITORING

Drug specific pharmacodynamic biomarkers

Calcineurin phosphatase (CaN) activity

The activity of CaN is determined in PBMC which requires cell isolation before the assay can be performed. An inverse relationship between CaN activity in PBMC and CNI concentrations in whole blood has been observed in patients after liver and kidney transplantation. Results from *in vitro* experiments indicate that, in contrast to cyclosporine, tacrolimus had a relatively high EC_{50}, above the upper limit of the therapeutic range (20 ng/mL). This questions the relevance of CaN activity as a pharmacodynamic marker for tacrolimus' immunosuppressive effects.

NFAT regulated gene expression

The quantitative analysis of IL-2, IFN-γ and GM-CSF gene expression in whole blood is established to quantify the inhibition of the transcription of NFAT-regulated genes, based on samples collected at tacrolimus C_0 and peak concentrations (1.5 hour post-dose) after oral administration of tacrolimus.

NFAT-regulated gene expression has been performed in solid organ transplantation such as kidney (adults and children), liver, heart, and lung transplant recipients, showing that monitoring the residual NFAT-regulated gene expression could identify allograft recipients at higher risk of infections or acute rejection.

The real-time polymerase chain reaction (RT-PCR) technique provides a highly reproducible, and sensitive tool and can be set up with satisfactory analytical performance in a routine molecular biological laboratory to be used in larger patient cohorts and in multicenter clinical studies. NFAT-regulated gene expression has the potential to develop into a monitoring tool complementing pharmacokinetics, especially in long-term renal allograft recipients.

Drug non-specific pharmacodynamic biomarkers

Intracellular cytokines

Several studies have focused on the utility of intracellular expression of IL-2 and IFNγ as prognostic biomarkers for the risk of acute rejection, as diagnostic biomarkers at the time of rejection and as markers reflecting the efficacy and the safety of tacrolimus. Flow cytometry and the enzyme-linked immunosorbent spot (ELISPOT) are the two mainly used methodologies. An ongoing randomized multicenter European study (Biodrim; Health F2-2012-305147) is currently evaluating the ELISPOT assay during tacrolimus minimization therapy in order to stratify renal transplant patients into low and high responders. In stable liver transplant recipients IFN-γ expressing $CD4^+$ and $CD8^+$ T-cells has been identified as surrogate markers for the risk of rejection after withdrawal of long-term immunosuppressive treatment.

For the validation of these biomarkers as early predictive biomarkers of the risk of rejection and graft clinical outcome it is crucial to improve some methodological aspects and harmonize these functional assays.

Donor-specific anti-HLA antibodies

Today, donor-specific anti-HLA antibodies (DSA) and the subsequent development of ABMR are considered to be leading causes for graft loss. The development of standardized highly sensitive solid-phase assays for detecting anti-HLA antibodies has significantly improved the clinical utility. In kidney and heart transplantation, DSA is a biomarker of under-immunosuppression, which may be caused by non-adherence but may also occur in both CNI-free and CNI-minimization clinical protocols. Most physicians agree that tacrolimus (with levels > 5ng/ml) is the CNI of choice in case of dnDSA, although no data from prospective controlled multicenter studies are available.

Graft-derived cell-free DNA

Quantification of donor-derived cell-free DNA (dd-cfDNA) in recipient blood or urine has been evaluated as a potential surrogate biomarker of acute injury in the donor organ but lacks the specificity to distinguish between acute rejection and BK virus nephropathy injury. Plasma levels of dd-cfDNA have been correlated with allograft rejection and outcome in renal transplant recipients. In liver transplant recipients it has been demonstrated that Graft-derived cell-free DNA (GcfDNA or d-cfDNA) quantification could be used to assess the minimally effective trough concentrations of tacrolimus.

Although dcfDNA seems to be a promising biomarker for monitoring graft health after transplantation, multicenter, prospective, observational and interventional studies will be required to better define how it can be used and evaluate its clinical utility before considering it a valid biomarker in solid organ transplantation.

Executive summary and practical recommendations

1. Determination of residual NFAT-regulated gene expression supports the identification of renal transplant recipients at higher risk of acute rejection, opportunistic infections, malignancy, and cardiovascular risk (B II).

2. Monitoring residual NFAT-regulated gene expression complements CNI pharmacokinetics as an adjunct to guiding CNI therapy (B III).

3. Monitoring intracellular T-cell IFN-γ production (particularly by the enzyme–linked immune-spot, ELISPOT, assay) before and early after transplantation can help to identify kidney and liver transplant recipients at high risk of acute rejection (B II) and select good candidates for immunosuppression minimization (B II).

Pharmacodynamic monitoring of tacrolimus therapy has not entered routine monitoring yet. To advance in the process of validation of pharmacodynamic and immunologic biomarkers it is crucial to improve and standardize methods. The clinical implementation of these biomarkers as a complement to tacrolimus-TDM may have impact on patient and graft care.

PK/PGx/PD modeling

Executive summary and practical recommendations

1. The utilization of popPK model based Bayesian estimators has shown improved target achievement compared to standard TDM. While trough concentration is used in most transplant centers for TDM of tacrolimus there is some evidence that C_0 correlates poorly with $AUC_{0\text{-}12}$.

2. The use of popPK model derived Bayesian estimators based on limited sampling strategies however provides AUC predictions with bias <5% and an imprecision <20%. This seems to be an applicable way to improve future tacrolimus TDM as compared to continue with standard trough concentration based TDM, especially when considering home sampling with micro sampling devices that currently are under validation (as presented above).

3. The authors recommend the integration of *CYP3A5*3* and *CYP3A4*22* genotype information, when available, in future tacrolimus popPK models, primarily for the opportunity to optimize initial dosing.

4. More pharmacokinetic-pharmacodynamic and PB/PK modeling activities are required to enhance the understanding of factors influencing clinical outcomes in transplantation.

INTRODUCTION

Tacrolimus is among the most frequently used immunosuppressive drug in solid organ transplantation. Building on three decades of experience and a large number of clinical trials, we have arrived at the current principles for the optimal use of this drug.[3-6] These include personalization of the dosing by frequent measurements of whole blood concentrations, in order to apply target concentration ranges which have gradually been set at lower levels, based on the

results from multicenter trials that investigated various combinations of immunosuppressants.[7-9] Although these immunosuppressive protocols have reduced the first-year incidence of biopsy proven acute rejections (BPAR) in renal and liver transplant recipients to respectively 15% and 25% or lower, there is still room for improvement. Importantly, there is a range of adverse events which affect the quality of life and life expectancy of transplant patients who need lifelong immunosuppression. At the same time, we are faced with large numbers of patients who lost their grafts due to antibody-mediated rejections, most likely due to under-immunosuppression. Therefore, the search for methods that may reflect personal drug response, to further optimize and personalize tacrolimus dosing to obtain the lowest possible individual exposure, is still warranted.

In September 2017, the Immunosuppressive Drugs Scientific Committee (ISDs SC) of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) decided to prepare an updated Consensus Report and to discuss the most recent advances in therapeutic drug monitoring (TDM) of tacrolimus-personalized therapy. The aim was to improve the standards of practice, and to highlight the potential of new methods and principles that may provide individualized therapy and improve patient care.

Ten years ago a consensus report on the optimization of tacrolimus was presented in this journal.[10] This report pointed to the rather poor correlation between tacrolimus trough concentrations and outcome, especially with acute rejection, and recommended area under the concentration-time curve (AUC) measurements for more precise monitoring. Furthermore, due to a high degree of pharmacokinetic and pharmacodynamic between-patient variability, the use of pharmacogenetic and immunologic biomarkers should be considered and properly evaluated in prospective, multicenter clinical trials. To support pharmacokinetic dose individualization,

limited-sampling strategies have been introduced, accompanied by population pharmacokinetic models or calculations using Bayesian forecasting.[11, 12] In the meantime, the use of mass-spectrometry in assays to measure tacrolimus has taken over in routine clinical monitoring in many centers. This has led to a modification of practice for efficient processing of large numbers of samples[13] whereas some problems related to analytical specificity have also been eliminated.[14] Furthermore, new assays have provided opportunities for measurement in other matrices, such as peripheral blood mononuclear cells and dried blood spots. It should be noted that to improve the prediction efficacy of monitoring, pharmacodynamic and specific immunological biomarkers strongly associated with the mechanism of action of tacrolimus have been assessed in several clinical trials.[15]

In this new document, the Expert Committee, consisting of thirty-seven international experts in the fields of TDM of tacrolimus and its pharmacogenetics and biomarkers, present a broad consensus on the current recommendations to achieve optimal personalization of tacrolimus therapy. The consensus is based on in-depth literature research and detailed Expert Committee discussions about pharmacology, pharmacokinetic monitoring (for once daily and twice daily formulations), analytical methods, standardization and new TDM approaches. Calcineurin (CaN) phosphatase activity measurement and a new concept for pharmacodynamic monitoring of calcineurin inhibitors (CNI), nuclear factor of activated T-cells (NFAT)-regulated gene expression as well as non-specific pharmacodynamic-biomarkers (intracellular cytokines and chemokines production) have also been introduced and documented for tacrolimus, and their potential as a supplement to blood concentration measurements is discussed. With respect to pharmacogenetics, the significance of *CYP3A5* variants has been investigated in detail in kidney, liver, heart and lung transplant recipients and several population pharmacokinetic (popPK)

models including *CYP3A5* genotype have been developed for tacrolimus. The potential contribution of other genetic factors, such as *CYP3A4*22* and the efflux transporter adenosine triphosphate-binding cassette subfamily B member 1 (*ABCB1* gene), is summarized.

These recommendations and evaluations are outlined for all discussed topics. Of note, recommended tacrolimus target concentrations are defined for each type of organ transplant. Moreover, for pharmacodynamic biomarkers and pharmacogenetics, a systematic weighing of the quality of evidence and strength of recommendations according to the Grading of Recommendations Assessment Development and Evaluation was done **(Table 1)**[1, 2]. Furthermore, this Consensus Document will incorporate the recommendations concerning the clinical utility of combining tacrolimus pharmacokinetic TDM with pharmacogenetic and pharmacodynamic biomarkers to better prevent acute rejection, subclinical rejection, drug-related adverse events and graft dysfunction.

This Consensus Report will support all professionals involved in the management of patients treated with tacrolimus in transplantation and other clinical settings and aims to improve both standards of practice in pharmacological tacrolimus TDM (pharmacokinetic/pharmacogenetic/ pharmacodynamic) and personalized patient care.

TACROLIMUS PHARMACOLOGY

Mechanism of action

Tacrolimus (known also as FK-506) binds to an immunophilin FK506 binding protein (FKBP) which constitutes the main therapeutic mechanism.[16] The tacrolimus-FKBP complex inhibits the activity of CaN, a serine threonine phosphatase, which plays an important role in interleukin 2 (IL-2) promoter induction after T-cell activation.[17, 18] This process inhibits the translocation of a

family of transcription factors of activated T-cells. It leads to reduced transcriptional activation of cytokine genes for interleukins (IL-2, IL-3, IL-4, IL-5), tumor necrosis factor α (TNF-α), interferon-gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor ((GM-CSF). Finally, proliferation of T lymphocytes is reduced.[19, 20]

Indications (registered and off-label)

The approved indications for tacrolimus vary by country and formulation. With regards to organ transplantation in adult and pediatric patients, the originator's immediate release or intravenous formulations obtained market approval in the USA for the prophylaxis of organ rejection in patients receiving allogeneic liver, kidney or heart transplants. In Europe, it is approved also for the treatment of allograft rejection resistant to treatment with other immunosuppressive medicinal products. In Japan, where the drug was developed, additional approvals were granted for lung, pancreas, allogeneic small bowel transplants, as well as for the prophylaxis of graft rejection and graft vs host disease (GVHD) in bone marrow transplantation. In Latin America, it is also approved for the treatment of severe and mild acute rejection and the prevention of GVHD.

The indications for prolonged or modified release formulations are somewhat more restrictive (except Graceptor in Japan): in the USA, Astagraf is only approved for the prophylaxis of organ rejection in kidney transplant patients in combination with other immunosuppressants. Envarsus has the additional restriction that patients should be converted from tacrolimus immediate-release formulations. In Europe, Advagraf and Envarsus are approved for kidney and liver transplantation but not for heart transplantation, as well as for the treatment of allograft rejection, but only in adults.

Tacrolimus has also been registered for a variety of other non-transplant indications across countries. In Europe, Protopic is registered for the treatment of eczema by topical administration on the skin. In Japan, oral tacrolimus is approved for the treatment of interstitial pneumonia associated with polymyositis and dermatomyositis. In Uruguay, it is indicated for refractory rheumatoid arthritis, refractory atopic dermatitis and refractory uveitis.

Interestingly, regional indications become off-label use in the other parts of the world, such as: hematopoietic stem cell, lung or small bowel transplantation, to which one can add rare composite tissue transplantations (face, hand, arm *etc*.); or refractory auto-immune diseases, not only those approved in Uruguay or Japan, but also the more common psoriasis, lupus nephritis, inflammatory bowel diseases, etc.

Pharmacokinetics of tacrolimus

Oral tacrolimus is rapidly absorbed, with a peak concentration attained within 0.5-1 hour, but it has a flat absorption profile in some liver transplant recipients with absorption that seems to be independent of bile.[21, 22] The poor dissolution of tacrolimus in gastric juices, administration with food, erratic gastrointestinal motility, extensive pre-systemic metabolism by CYP3A enzymes in the gut wall and liver and activity of the efflux-pump P-glycoprotein (P-gp, encoded by, *ABCB1* gene) are relevant factors that contribute to the variability in absorption. The mean oral bioavailability is 25%.[22]

Binding of tacrolimus to red blood cells (RBC, about 95%) is concentration-dependent.[23] In plasma, it is approximately 99% bound to plasma proteins such as α_1-acid glycoprotein, albumin and for a very small part to lipoproteins. Blood: plasma tacrolimus concentration ratios range from 13-114 (mean =15) and are dependent on hematocrit, plasma protein and tacrolimus concentration.[23]

Tacrolimus is metabolized mainly by CYP3A4 and CYP3A5 in liver and gut wall, with a minimal contribution of CYP3A7.[24] Up to 15 metabolites are formed by mono and di demethylation, hydroxylation and to some degree a combination of demethylation and hydroxylation. The major metabolites of tacrolimus are the 13-O-desmethyl tacrolimus (M-I) and the 15-O-desmethyl tacrolimus (M-III). 13-O-desmethyl tacrolimus (M-I) has 10% of the activity of tacrolimus while the 31-O-desmethyl tacrolimus (M-II) has similar activity to the parent drug but the concentrations are low-to-not-detectable in patients.[25, 26] Tacrolimus is highly lipophilic, has a low clearance and approximately 95% of its metabolites are eliminated by the biliary route. Less than 1% of unchanged tacrolimus is eliminated by urinary and biliary routes.[27] The terminal elimination half-life of tacrolimus has a range between 4-41 hours.[22]

Using PK modeling as described in a separate section later in this paper, apparent clearance and central volume of distribution as 24.0-28.5 L/h and 70.6-158.2 L, respectively, have been reported for adult kidney transplant recipients.[28] *CYP3A5* genotype, weight, hematocrit and post-operative day were identified to affect tacrolimus clearance. The between-individual variability for clearance and central volume of distribution was 54% and 110%.[28] In another study, the mean between-occasion variability in tacrolimus clearance was 17% at 6-12 months post renal transplantation.[29] Circadian variation with tacrolimus pharmacokinetics was reported, with a lower bioavailability at night[30]; but a lack of circadian variability, either with early or maintenance tacrolimus therapy, has also been reported.[31]

Clearance is two-fold higher following pediatric liver transplantation, with a shorter terminal half-life (11.5+/-3.8 hours).[32] In pediatric liver transplantation, a linear increase of tacrolimus clearance up to 21 days post-surgery was reported.[33] Thereafter, tacrolimus clearance decreased up to a period of one year after transplantation.[34] The apparent clearance decreased with time

after transplantation due to an increase in hematocrit and albumin.[35] In pregnancy, an apparent decrease in α_1-acid-glycoprotein, albumin and RBC resulted in a 39% increase in the total tacrolimus clearance and 100% increase of the unbound fraction of tacrolimus, compared to the postpartum period.[36]

Temporary elevation of tacrolimus trough concentrations (about 2-fold and more) has been observed in patients with diarrhea.[37, 38] This may be because intestinal Pgp activity is impaired in patients with persistent diarrhea, leading to increased bioavailability.[39]

Reduction in the dose of corticosteroids, from the early post-transplant months to one year after transplantation, contributes to a decrease in tacrolimus clearance, facilitating a reduction in the tacrolimus dose.[35, 40] Variability of tacrolimus exposure in African American patients compared to Caucasian and Asian patients has been reported, and is attributed to ethnic differences in CYP3A5 and P-gp.[41] In the section on Pharmacogenetics in this review, the past, present and future of pharmacogenetic testing for tacrolimus will be discussed.

Drug-drug and drug-food interactions

Compounds which affect CYP3A enzyme activity or P-gp-mediated transport may influence tacrolimus concentrations in blood. Drug-drug interactions of clinical importance are summarized in **Table 2**, based on reviews.[42, 43] Drugs that are known inhibitors of CYP3A4/5/7 include calcium antagonists, macrolide antibiotics and others as listed in **Table 2**. One group of drugs that have demonstrated profound inhibition of CYP3A enzymes is the triazole antifungals.[44, 45] In individual cases large increases in tacrolimus exposure have been reported, as exemplified by voriconazole.[46] At initiation of therapy with these drugs, an immediate tacrolimus dose reduction is recommended, as maintaining the tacrolimus dose and waiting for a tacrolimus concentration after a couple of days is likely to cause significant toxicity.

More recent reviews have focused on potent interactions between tacrolimus and the antiretroviral agents used for the treatment of HIV-infected transplant recipients. Among these drugs are classes that inhibit and induce, respectively, the CYP3A enzymes and the P-gp transporter. This knowledge is also important for the selection of the most appropriate anti-HIV regimen to combine with immunosuppressive treatment.[47]

The direct-acting antiviral agents for HCV infection can also produce drug interactions with tacrolimus.[48-51] Considerable modifications of tacrolimus dose have been recommended for combination with other regimens of the direct acting antivirals against HCV.

In contrast, as listed in Table 2, drugs that are known inducers of CYP3A include rifampin, antiepileptic drugs, some HIV antivirals and importantly also the glucocorticoids.[40, 52] These drugs enhance CYP3A activity, increase the rate of tacrolimus metabolism and lead to a decrease of tacrolimus concentration that if passing unnoticed may fall below the recommended therapeutic range, which would put a patient at risk for graft rejection. If in the first months after kidney transplantation the prednisolone dose is gradually tapered, this affect tacrolimus exposure. In this setting, a rise in serum creatinine as a result of increasing tacrolimus concentrations may be misinterpreted as a rejection following corticosteroid tapering.

The consumption of solid food when taking oral doses of tacrolimus can decrease both the rate and extent of drug absorption from the GI tract.[53] This food-effect is most pronounced after a high-fat meal.[54] Therefore, the drug label recommends taking tacrolimus on an empty stomach, or at least 1 hour before or 2 to 3 hours after a meal. Food-drug interactions with tacrolimus include grapefruit and grapefruit juice.[55] St. John's wort is a known inducer of the CYP3A.[56] and has been shown to decrease tacrolimus exposure in renal transplant recipients.[57]

Consequently, foods and drugs that are known to alter the activity of drug metabolizing enzymes should be used with caution during tacrolimus therapy, and the use of herbal medications should be avoided. The use of concomitant medications with tacrolimus may put a patient at risk of toxicity or sub-therapeutic blood concentrations.[43]

Galenic formulations & generics

Tacrolimus is now also available in immediate or modified release pharmaceutical forms for oral administration (tablet or capsule). The Advagraf prolonged release once daily formulation was developed to increase treatment adherence, ultimately leading to better prevention of graft rejection. Recently, a new prolonged-release tacrolimus formulation named Envarsus was developed utilizing so called "solid solution" delivery technology. Envarsus presents higher bioavailability and as a consequence needs to be administered in comparably lower doses based on 0.75 mg, 1 mg and 4 mg tablets (correction factor of 0.7). A potential benefit of this formulation is that less-fluctuating tacrolimus concentrations have been observed.[58]

Tacrolimus is a narrow therapeutic index drug (NTID). Several generic formulations for Prograf have been registered since patent protection expired. A list of tacrolimus brand names worldwide is currently counting 258 products, all formulations included and mostly due to the large number of immediate release generics.[59] The lack of worldwide harmonization in the registration of generic drugs, especially with respect to the criteria for bioequivalence, has resulted in regional differences in the availability of tacrolimus generics. For the once daily tacrolimus formulations patent protection was still in place at the time when the present document was written, and generic formulations based on these technologies had not entered the market yet.

Part of the transplant community has been, and still is, concerned that the pharmacokinetic properties of generic tacrolimus formulations may be too different from those of the innovator product, in all or in subgroups of special populations such as patients with cystic fibrosis or elderly patients.[60] Various national or international transplant societies have published recommendations regarding the implementation of generic immunosuppressive drugs in the transplant field.[61, 62] Typically they recommended caution with the use of generic immunosuppressive drugs until more robust clinical data are available and adequate regulatory safeguards are instituted. One of the main concerns was that bioequivalence demonstrated in healthy volunteers may not be representative of all transplant recipients. In the literature there are a large number of published studies on comparisons between one or more generic formulation and the Prograf formulation.[63] Endpoints of these studies often include pharmacokinetic parameters, including bioequivalence. Some studies also include clinical endpoints, such as acute rejection or renal function. For the latter endpoints the sample size is often too small and the confidence intervals are too wide to conclude whether the substitution to generic formulations is safe or unsafe. For studies that focused on pharmacokinetic endpoints, a major problem is the higher variability in transplanted patients compared to healthy volunteers. In registration studies using healthy volunteers, standardized conditions in a specialized research unit are the norm. However, studies in transplant patients are typically subject to a less controlled research environment with ensuing variability. One of the clear exceptions is the prospective, replicate dosing, partially blinded, randomized, 3-treatment, 6-period crossover bioequivalence study that was conducted at the University of Cincinnati in patients with kidney (n = 35) or liver transplant (n = 36). In this study it was concluded that the bioequivalence demonstrated for tacrolimus in healthy volunteers also translates to those receiving a kidney or liver transplant.[64] In contrast to

this population with a median age of 52 years for kidney and 57 years for liver transplant recipients, another study included 28 Norwegian renal transplant recipients with median age 69 years (range 60-78 years); In this study bioequivalence criteria were not met.[65] Another example is the immediate release generic Limustin which in one study including pediatric renal transplant recipients demonstrated very low bioavailability compared to the originator formulation (Prograf).[66] Further experiments by these authors showed that the pharmaceutical characteristics of this generic was dissimilar to the original and likely explained the reduced tacrolimus exposure in children.

It is unlikely that large prospective trials comparing generic and innovator tacrolimus formulations in newly transplanted patients will be conducted.[67] We consider generic tacrolimus as an attractive therapeutic option, especially in *de novo* transplant patients. As a standard of practice, the treatment should be guided by TDM, and simultaneously controlled for safety and efficacy. Conversions from innovator drug to generic or from one generic to another generic version need to be performed under careful monitoring of drug exposure and only after adequate instructions to the patient. In view of the risk of mistakes caused by patient confusion, it is important to limit the number of conversions from one formulation to another as much as possible.

PHARMACOKINETIC MONITORING

Evidence-based pharmacokinetic monitoring for tacrolimus in specific clinical situations

Relationships between tacrolimus exposure and treatment outcomes have been amply reported. The AUC can be regarded as the exposure metrics best associated with tacrolimus clinical effects, but no prospective study has been conducted in adult or pediatric transplantation to investigate the potential benefits on clinical outcomes of between-dose AUC monitoring over

therapy guided by trough concentrations, C_0 (i.e. concentration in samples drawn immediately before dose). C_0 is much easier to obtain and used in most transplant centers for routine TDM. The monitoring of tacrolimus AUC has been proposed especially in the early period post-transplantation to check the time evolution of the overall exposure and when clinically indicated (*e.g.* to investigate suspected tacrolimus adverse effects, or to guide immunosuppression minimization). Furthermore, in different conditions, the AUC/C_0 ratio is influenced by the post-transplantation time-period, the CYP3A5*3 genotype,[68] and can be drastically modified in patients with decreased intestinal motility or decreased absorption (*e.g.* diabetes mellitus, ileus, cystic fibrosis, bariatric surgery, gastrectomy, colectomy), in patients exposed to strong pharmacokinetic drug-drug interactions (*e.g.* on anti-HIV, azole antifungal drugs) or in patients with other sources of tacrolimus metabolism deficiency. The rather poor correlation between C_0 and AUC[69] translates into very variable AUC/C_0 ratios, which means that patients with identical C_0 may have very different between-dose AUC. Interestingly, tacrolimus $AUC_{0–24h}$ correlated better with C_{24h} than C_0 for both twice-daily and once-daily tacrolimus, and $AUC_{0\text{-}12h}$ correlated better with C_{12h} than C_0 for twice daily tacrolimus. The authors concluded that C_0 can only be a correct proxy of the overall exposure if blood sampling is perfectly timed.[68] However, this ratio was found to be very stable with time in individuals and the authors suggested evaluating this ratio at least once in the early period (first month) and once in the stable period (after three months) for each transplant recipient.[70]

This section summarizes the updated recommendations for tacrolimus exposure in each type of solid organ transplantation as well as in other clinical applications.

Pharmacokinetic monitoring in kidney transplantation

Tacrolimus exposure, efficacy and toxicity

Since the previous consensus paper on tacrolimus TDM,[10] only a few studies have investigated the relationships between tacrolimus exposure and the risk of acute rejection or toxicity. In the large Genomics of Deterioration of Kidney Allograft Failure (DeKAF) study in adult patients over the first 6 months after transplantation, each 1 ng/mL decrease in tacrolimus C_0 was associated with a 7.2% increased risk of acute rejection ($p = 0.03$).[71] A smaller study in low-immunological risk patients without steroids and with a moderate dose of mycophenolic acid concluded that C_0 should be maintained >7 ng/mL during the first year post-transplantation.[72] However, a pooled analysis of three other randomized, controlled clinical trials found no relationship between tacrolimus C_0 and biopsy proven acute rejection (BPAR).[73] Two rather old and small observational studies respectively showed: a significant association between tacrolimus $AUC_{0\text{-}12h}$ and acute rejection, with mean values of 157 ng.h/mL in patient with AR and 215 ng.h/mL in patients without[74]; and efficacy thresholds to avoid BPAR of 150 ng.h/mL for tacrolimus $AUC_{0\text{-}12h}$ and 45 mg.h/L for mycophenolic acid $AUC_{0\text{-}12h}$.[75]

Evidence is even more limited in pediatric patients. A retrospective study of 58 children showed that a lower frequency of chronic kidney disease and decreased graft function was observed when C_0 was maintained at ≥10 ng/mL in the first three months after transplantation.[76]

Tacrolimus exposure has been more consistently associated with the incidence of adverse events,[77, 78] but overall, the studies which investigated the relationship between tacrolimus exposure and the risk of AR and toxicity were often retrospective and/or included limited numbers of patients and/or involved co-medications different from those used nowadays.

Recommended tacrolimus target concentration ranges

Based on the current evidence, it is not possible to recommend a single target tacrolimus concentration range.[4] Rather, the tacrolimus target exposure has been defined empirically and depends on the perceived risk of acute rejection, the time after transplantation and the co-medication used. In the United States and in Europe, tacrolimus combined with mycophenolate is the backbone of immunosuppressive drug protocols. In most centers, patients also receive induction therapy (either T-cell-depleting antibody therapy or interleukin (IL)-2 receptor blockers) and glucocorticoids.[79] The best evidence for such regimens was provided by the SYMPHONY randomized, controlled clinical trial,[3] which showed that 75% of the low-immunological risk patients in the tacrolimus arm (which performed best) had a tacrolimus C_0 between 4 and 11 ng/mL in the first month after transplantation, between 4 and 10 ng/mL later on, and between 4 and 9 ng/mL between 6 and 12 months.[9] Still, as mentioned above, a more recent study advised against C_0 <7 ng/mL in a similar low-risk population.[72] Also, different targets may be aimed for when tacrolimus is combined with T lymphocyte-depleting antibody therapy or in higher risk patients.

Tacrolimus in combination with everolimus therapy was investigated in the TRANSFORM randomized, controlled clinical trial.[80] It showed that in transplant recipients at mild to moderate immunological risk, everolimus plus low-exposure tacrolimus (target C_0 of 4-7 ng/mL (months 0-2), 2-5 ng/mL (months 3-6) and 2-4 ng/mL thereafter) is non-inferior to mycophenolate plus standard-exposure tacrolimus (target C_0 of 8-12 ng/mL, 6-10 ng/mL and 5-8 ng/mL, respectively) for a binary composite end point assessing immunosuppressive efficacy and preservation of graft function.[80]

There has been no new study in support of tacrolimus exposure targets in pediatric kidney transplant recipients. As a reminder, the recommendations are to start with an initial tacrolimus dose of 0.15 mg/kg twice a day, to achieve C_0 concentrations between 10 and 20 ng/mL during the first 2 months after transplantation and between 5 and 10 ng/mL thereafter.[10]

Concerning between-dose AUC targets, a minimal threshold of approximately 150 ng.h/mL in adult kidney transplant recipients emerges from the only two studies published.[74, 75] However, based on exposure measurement in very large numbers of patients on twice-daily[69] or once-daily[70] tacrolimus formulations, between-dose AUC target ranges were derived from the different C_0 targets proposed for low-, standard- and high-risk patients. For twice daily tacrolimus, the corresponding $AUC_{0\text{-}12h}$/ C_0-ranges proposed were: 75-140 ng.h/mL for 3-7 ng/mL; 100-190 ng.h/mL for 5-10 ng/mL; 140-210 ng.h/mL for 8-12 ng/mL; and 180-270 ng.h/mL for 10-15 ng/mL.[69] For once-daily tacrolimus (Advagraf), the corresponding $AUC_{0\text{-}24h}$ target ranges proposed were: 150-275 ng.h/mL for C_0 3-7 ng/mL; 180-350 ng.h/mL for 5-10 ng/mL; 260-400 ng.h/mL for 8-12 ng/mL; and 310-475 ng.h/mL for 10-15 ng/mL.[70] Importantly, for a given C_0 range, 3 tighter time-adjusted AUC ranges were proposed (for 0-3 months, 3-12 months and > 12 months post-transplantation), because the correlation between AUC and C_0 changes over the first 12 months post-transplantation for both formulations, owing to the natural decrease in tacrolimus apparent clearance over this time period. The AUC ranges proposed above correspond to the combination of the 3 time-adjusted ranges.

Pharmacokinetic monitoring in liver transplantation

Tacrolimus exposure, efficacy and toxicity

AUC is considered to be the pharmacokinetic exposure index best associated with clinical effects. Therefore, the monitoring of tacrolimus AUC has been proposed, when clinically

indicated, but routine monitoring of tacrolimus C_0 concentrations is usual practice for outpatients.[6] In case of combination therapy of tacrolimus with corticosteroids and either mycophenolate or everolimus, a tacrolimus C_0 of 6 to 10 ng/mL should be targeted from day-1 and over the first 4 weeks of treatment, followed by a target of 5 to 8 ng/mL.[7, 8, 81-86] Waiting 6 weeks before targeting 5 to 8 ng/mL in combination with mycophenolic acid may be considered as an alternative, as the evidence in favor of the target change at 4 weeks is weak. With such immunosuppressive regimen, C_0 >10 ng/mL seem to offer little additional advantage in terms of reduced allograft rejection, but result in a higher rate of renal dysfunction.[85] Also, C_0 <5 ng/mL should be avoided, mainly during the first month, as they may lead to worse long-term outcomes.[82] Tacrolimus given as monotherapy or only associated with induction treatment may require higher C_0 targets (10-15 ng/mL during the first 3 months after transplantation and 5-10 ng/mL afterwards), although there is some evidence in favor of minimized targets with such regimens too.[87-90] Similarly, higher targets (10-15 ng/mL even beyond the 4th month after surgery) may be aimed for in case of corticosteroid-free treatment. In general, though, C_0 value >15 ng/mL should be avoided.[91] Liver function usually becomes stable 3 weeks post-transplantation. TDM after this period may therefore be reduced in frequency beyond this point in time, if patient condition and C_0 levels are stable.

Although there are limited data regarding optimal C_0 target range(s) in pediatric liver transplant patients, a retrospective study of 72 pediatric patients aged 0.5-17.6 years indicated that adverse events such as nephrotoxicity were associated with higher tacrolimus C_0 during maintenance therapy (median tacrolimus C_0 in patients with or without adverse events: 8.2 and 4.8 ng/mL, respectively).[92]

Pharmacokinetic monitoring in thoracic transplantation

Tacrolimus exposure, efficacy and toxicity

Data on tacrolimus pharmacokinetics and TDM in thoracic transplantation are limited. All studies were conducted in <25 adult patients, except for one in 45 heart transplant [93] and another in 78 lung transplant recipients.[94]

Tacrolimus dose after thoracic transplantation is usually adjusted based on C_0 levels, mainly targeting the ranges proposed almost 20 years ago for kidney and liver transplant recipients: 15-20 ng/mL for the first 2 months, 10-15 ng/mL from months 3-6, and 8-10 ng/mL after 6 months following heart transplantation[5]; and 10-25 ng/mL for the first 2 weeks, 10-20 ng/mL for the next 6 to 10 weeks, and 10-15 ng/mL thereafter in lung transplantation.[95] These C_0 targets were never formally revised. Moreover, as these targets have not been associated with any type of assay for blood concentration measurement, they should be interpreted and implemented in routine clinical practice with caution, and further research is needed to refine them.[96] Two recent studies explored the relationship between tacrolimus C_0 and acute kidney injury, in heart[97] and in lung[98] transplantation, respectively, and showed that the risk of acute kidney injury within the first two weeks post-transplantation was significantly increased for C_0>15 ng/mL.

As in other solid organ transplantations, tacrolimus AUC rather than single concentrations may be used for TDM. A wide range of correlation coefficient values between C_0 and AUC_{0-12} have been reported.[99-102] Only one study in heart transplantation has evaluated the relationship between AUC and outcome.[101] The AUC_{0-12} after the first oral dose was significantly lower in patients who experienced acute rejection compared to those who did not (71 *vs.* 168 h.ng/mL, p = 0.012), but no information was provided on when acute rejection occurred. Unfortunately, no

prospective studies have been conducted so far in thoracic transplantation to compared different AUC_{0-12} targets, or to compare AUC monitoring with C_0 monitoring.[96]

Pharmacokinetic monitoring in bone marrow transplantation

Tacrolimus exposure, efficacy and toxicity

Graft-*versus*-host disease (GVHD) remains one of the main causes of treatment-related mortality after allo-hematopoietic stem cell transplantation (HSCT). The concomitant administration of a CNI (tacrolimus or cyclosporine) and short-term methotrexate is one of the standard regimens used to prevent GVHD.

Randomized controlled trials in both adult and pediatric patients have suggested that the combination of methotrexate and tacrolimus may be superior to methotrexate and cyclosporine to reduce acute-GVHD (aGVHD).[103-106] Tacrolimus is generally administered by continuous IV infusion beginning on the day prior to allogeneic transplantation at a dose of 0.03 mg/kg/d.[107-109] When patients are able to tolerate oral administration, the factor used for tacrolimus dose conversion from intravenous to oral administration varies between 1:3 and 1:5.[109] In most clinical trials and retrospective studies, the C_0 target concentration was between 10 and 20 ng/mL.[108, 110, 111] A retrospective cohort study of 120 consecutive adult patients undergoing first allogeneic HSCT found a lower risk of grade 2-4 aGvHD in patients with tacrolimus mean C_0 of >12 ng/mL over the first week post-grafting, while lower tacrolimus concentrations at weeks 2, 3 and 4 were not associated with a higher incidence of aGvHD. [112] Tacrolimus C_0 target ranges of 10-30 or 10-40 ng/mL have also been reported[113, 114], but the incidence of nephrotoxicity was very high[113] and significantly increased for C_0 >20 ng/mL.[114]

Few studies have evaluated tacrolimus concentration targets in children undergoing HSCT. A Japanese retrospective study of tacrolimus combined with methotrexate (97 children aged 0.4-18 years) found that mean tacrolimus concentrations <=7 ng/mL during continuous infusion over the first 4 weeks (i.e., not really representative of the steady-state concentration) were associated with an increased risk of aGVHD and poorer survival. [115] Another retrospective study, where tacrolimus was combined with mycophenolic acid (60 children aged 0.4-21 years) found that a mean tacrolimus concentration <10 ng/mL during week 3 was associated with increased incidence of aGVHD.[116]

Pharmacokinetic monitoring in other diseases where tacrolimus is prescribed.

Tacrolimus is also prescribed (off-label in most cases) for several auto-immune diseases.

Atopic dermatitis

A topical tacrolimus ointment is effective against atopic dermatitis (or atopic eczema).[117] It did not lead to relevant systemic tacrolimus exposure in adults,[118] in children aged 2-17 years old,[119] or in infants aged <2 years.[120] In adults, 94% of tacrolimus blood concentrations were <1 ng/mL and the highest value at any time point in any patient was 1.38 ng/mL.[118]

A small proof-of-concept, non-comparative study investigated the safety and efficacy of sequential therapy with short term oral tacrolimus to achieve rapid disease control followed by maintenance with topical tacrolimus 0.1% ointment in the treatment of severe atopic dermatitis.[121] Over the first 3 weeks of the study, all patients received oral tacrolimus as monotherapy. Topical tacrolimus was then added to the oral treatment in weeks 4 to 6. After week 6, patients were treated with only topical tacrolimus. Clinical improvement was noted in

about 2/3 of the patients. The average whole blood tacrolimus levels were 8.1 ng/mL at week 1, 7.7 ng/mL at week 3, and 5.5 ng/mL at week 6.

Psoriasis

In patients treated with tacrolimus following renal transplantation, remarkable improvements in incidental psoriasis have been noted.[122] Tacrolimus may be more suited than cyclosporine to a patient population with increased cardiovascular risk.[123] However, there is no evidence in favor of a tacrolimus concentration target range for this indication. Topical tacrolimus has been suggested as a suitable treatment for more localized psoriasis, such as facial, genital, and intertriginous psoriasis[124], requiring no TDM since systemic drug exposure has been shown to be low.

Lupus nephritis

The standard of care for the induction treatment of proliferative lupus nephritis remains mycophenolate mofetil and cyclophosphamide. CNIs are only recommended as a second line alternative given their side effects, especially in Asian populations.[125] However, a Bayesian network meta-analysis found that a maintenance treatment with tacrolimus in patients with biopsy-proven lupus nephritis class III, IV, or V yielded the best chance of preventing renal relapse as well as the lowest risk of withdrawals due to adverse events and leukopenia, as compared to mycophenolate mofetil, azathioprine and cyclophosphamide. [126] Tacrolimus dose was titrated to achieve a C_0 of 4-6 ng/mL in 34 Chinese patients,[127] and a C_0 of 6-10 ng/ml in the first and second month and 4-8 ng/ml thereafter in a Thai study.[128]

In pediatric patients, a single center clinical study found that once daily tacrolimus at 3 mg/day, resulting in C_0 between 1.5 and 7.5 ng/mL, improved the serological parameters, the lupus

activity index and reduced the need for steroids in patients aged 9-25 years. However, no significant relationship between treatment effect and blood concentration was noted over the 2-year study period.[129]

A meta-analysis of 23 clinical studies, all performed in Asian populations, showed that in most studies, tacrolimus dose was titrated to achieve a C_0 values of 5-10 ng/ml in the first and second months, and 4-7 ng/ml thereafter.[130] Still, the 'optimal' tacrolimus blood trough concentration has yet to be determined, and the targets used have largely been chosen to avoid toxic effects.[131] Finally, the positive results of tacrolimus in Asian patients cannot be extrapolated to other ethnicities.

Inflammatory bowel disease

In a recent review, tacrolimus and infliximab appeared to be equally safe and effective in the short-term treatment of active ulcerative colitis.[132] Tacrolimus oral dose was generally adjusted to achieve C_0 levels of 5-10 ng/mL thereafter. In a study of 65 patients with moderate to severe active ulcerative colitis two target tacrolimus concentrations (5-10 and 10-15 ng/mL) and a control group on placebo were compared double-blindly.[133] Clinical improvement was observed significantly more often in the tacrolimus high target range group. Bruns *et al.*[134] recommended frequent monitoring of whole blood tacrolimus concentrations, since efficacy and toxicity are dose-dependent.

Tacrolimus has also been investigated in pediatric patients with inflammatory bowel disease. A prospective multicenter trial including 13 pediatric patients aged 6-20 years with oral tacrolimus started at a dose of 0.1 mg/kg twice daily and subsequently adjusted to achieve C_0 concentrations between 10 and 15 ng/mL found that 69% of patients responded to oral tacrolimus within 14 days.[135] A single center, retrospective study of 18 pediatric patients aged 1-16 years, given

tacrolimus at a starting dose of 0.1 mg/kg/dose twice daily and subsequently adjusted to a target C_0 of 10-15 ng/mL for the first 2 weeks and 7-12 ng/mL thereafter, concluded that in patients with steroid-resistant colitis, tacrolimus was capable of inducing short- to medium-term remission but yielded no long-term benefit.[136]

Executive summary on tacrolimus exposure recommendations

In kidney Transplant recipients

1. In low-immunological risk patients, tacrolimus may be targeted to: a C_0 of 4-12 ng/mL (and preferably to C_0>7 ng/mL) when prescribed in combination with IL-2 receptor (IL-2R) blocker induction therapy, mycophenolate and glucocorticoids (A I); or a C_0 of 4-7 (month 0-2) and 2-4 (> month 2) ng/mL when combined with everolimus and glucocorticoids and induction therapy (either Thymoblobuline or IL-2R blockers) (B II).

2. Tacrolimus C_0 targets may be higher in adult patients at higher immunological risks. (B II)

3. Although only supported by clinical experience, a C_0 target range of 10-20 ng/mL may be proposed for pediatric patients (C1 II)

4. A minimal $AUC_{0\text{-}12h}$ threshold of 150 ng.h/mL may be proposed for the twice daily formulation in adults (B II). AUC targets corresponding to different C_0 ranges were derived from AUC-C_0 correlation studies in large adult patient populations, for the twice daily and once-daily (Advagraf) formulations.

In liver transplant recipients

For adult patients:

1. When prescribed in combination with mycophenolate or everolimus and corticosteroids, tacrolimus may be targeted to a C_0 of 6–10 ng/mL during the first 4 weeks post-transplantation and 5-8 ng/mL thereafter (A I).

2. Tacrolimus as a monotherapy, or when only associated with induction treatment may require a higher C_0 target (10-15 ng/mL during the first 3 months after transplantation and 5-10 ng/mL afterwards) (C1 II).

3. A tacrolimus C_0 of 10-15 ng/mL may also be aimed for in patients on a corticosteroid-free regimen (even beyond the 4th month after surgery) (C1 II).

For pediatric patients, there is not enough clinical evidence to make recommendations.

Heart and lung transplantation, the C_0 ranges recommended almost 20 years ago must be revised (and probably lowered), as they encompassed values between 15 and 20 ng/mL in the first weeks post-transplantation, whereas recent studies have shown that the risk of acute kidney injury within the first two weeks post-transplantation was significantly increased for C_0 >15 ng/mL (B II).

Bone marrow transplantation: C_0 of 10-20 ng/mL may be targeted when tacrolimus is prescribed orally in combination with methotrexate, in adults (B II) as well as in children (B II).

In the other conditions in which tacrolimus is sometimes employed, there is not strong enough evidence to make recommendations (C2).

Within-patient variability of tacrolimus exposure as a biomarker for therapeutic drug monitoring

In addition to the tacrolimus blood concentration itself, its within-patient (between-occasion) variability might also be a valuable tool for optimizing immunosuppressive therapy in solid organ transplantation.[137-139] Within-patient variability can be simply evaluated using the coefficient of variation (CV) of at least three consecutive C_0 measurements in a given time period. To date, none of the different tacrolimus formulations (immediate release, prolonged or extended release) clearly shows lower within-patient pharmacokinetic variability than the others.[140, 141] Significant variability of tacrolimus concentrations during immunosuppressive treatment may lead alternatively to underexposure and overexposure periods and represent a risk of treatment failure.

The first suggestions to consider the within-patient variability as a potential biomarker of treatment outcome were reported in kidney transplantation.[29] The CV of tacrolimus C_0 beyond 6 months post-transplantation was found to be a more relevant biomarker of tacrolimus toxicity and immunosuppressive efficacy than the sole C_0.[142, 143] Patients with high exposure variability were confirmed to be at higher risk of developing histologic kidney lesions,[143] graft loss,[142] and of poorer long term outcomes.[29, 144-146] Most of the studies conducted in kidney transplantation included patients with stable treatment (at least 6 months after transplantation), in a period when adherence may be regarded as a strong determinant of within-patient variability.

In liver transplantation, whether in the early post-operative period (days 8 to 30) or at mid-term (after 6 months), the within-patient variability of tacrolimus concentrations was related to patient outcome.[147, 148] In adult patients, early within-patient variability (between day 8 and day 30) was associated with long term graft and patient survival and with complications such as

nephrotoxicity, cardiotoxicity and neurological adverse events, meaning that early interventions may be undertaken to reduce variability.[148] In pediatric patients, the link between within-patient variability, calculated from month 6 of tacrolimus initiation, and biopsy-proven acute rejection was first found in retrospective studies and later confirmed prospectively, whereas there was no relationship with graft or patient survival.[147, 149]

Very recently, similar results have been reported in heart transplantation. The within-patient variability was calculated retrospectively between month 3 and month 12 and a high CV turned out to be an important risk factor for the onset of rejection.[139]

Evaluating the within-patient variability of C_0 values during tacrolimus treatment should be further considered in the clinical management of solid organ recipients. Early identification of at-risk patients, with higher within-patient variability, may allow implementing actions aimed at reducing this variability and preventing its clinical consequences.

Executive summary on within-patient variability of tacrolimus exposure

Within-patient variability of tacrolimus concentrations has emerged as a potential tool to predict adverse events during follow-up of kidney, liver and heart transplant recipients, but has not entered routine monitoring yet (B II). Evaluation of within-patient variability can easily be implemented to improve therapeutic drug monitoring (B II). The use of within-patient variability has not been validated prospectively and the timing of determination has to be defined.

MEASUREMENT OF TACROLIMUS CONCENTRATIONS

Sample stability

Short and long-term stability of tacrolimus and its major metabolites (MI, MII, MIII) have been investigated using both patient blood samples and whole-blood samples spiked with these

substances. Stability of the measured tacrolimus concentration was proven for samples stored up to 14 days at 22°C or 4°C, as well as at least 1 month at -20°C and 1 year at -70°C.[25, 150] MI, MII and MIII were found to be stable for at least 3 days at ambient temperature, 1 month at -20°C and 12 months at -80°C.[25, 151] Three freeze-thaw cycles were possible without compromising the quantitative results.[25, 151] These cycles also had no effect on the unbound tacrolimus concentration, but this fraction increased by > 30% in samples stored for 6 months at -80°C.[152] The authors concluded that samples should be ultrafiltrated when fresh to study the unbound tacrolimus concentration. For long-term storage the prepared ultrafiltrates can be frozen at -80°C.

Stability of tacrolimus concentrations was also investigated in some alternative sample matrices. For example in peripheral blood mononuclear cells ($CD4^+$ T- and $CD19^+$ B-cells), stability was demonstrated for up to 3 months at both ambient temperature and -30°C;[153] in oral fluid ambient temperature (no exact time was mentioned) and for up to 1 month at -80°C;[154] and in human bile samples over 20 hours at ambient temperature and 6 months at -80°C.[155] After 3 freeze and thaw cycles no loss of tacrolimus concentration was observed in oral fluid or bile.

Extended stability of tacrolimus in DBS samples using different grades of Whatman® paper (Whatman, Kent, UK) has been reported. For example on Whatman 31 ET CHR paper tacrolimus concentrations were consistent over 28 days when stored at either 22 °C or 37 °C [156], over 30 days on Whatman 903 Protein Saver Cards at temperatures ranging between -20 °C and 25 °C and at least 5 days at 60 °C.[157]

Analytical methods to determine tacrolimus in whole blood

More than 60% of the requests for measurement of immunosuppressive drug concentrations in clinical laboratories concerns this drug. Because TDM was recommended with the introduction of tacrolimus in the early 1990s, many analytical methods determining tacrolimus concentrations

have been developed and established for clinical services.[14] These methods offer different advantages but may also suffer a variety of disadvantages.[158] Analytical laboratories face a number of alternatives when choosing the method that is most appropriate for their local circumstances. Analysis of tacrolimus in whole blood is performed either by immunoassays or by liquid chromatography-tandem mass spectrometry (LC-MS/MS), with both techniques being represented in nearly equal proportions at measurement service providers worldwide.[159]

Chromatographic methods

In a 2013 international survey, 53 % of TDM laboratories reported that LC-MS/MS was used as their routine tacrolimus method.[159] About 60% of the LC-MS/MS procedures was laboratory developed tests (LDT), whilst the remainder was based on kits launched on the market by the diagnostic industry. The majority of the LC-MS/MS assays (~75%) was multianalyte methods that allowed for the simultaneous quantification of tacrolimus and other immunosuppressive drugs within one analytical batch, using a whole blood sample.[159]

High analytical selectivity and sensitivity, and the possibility for high throughput multianalyte assays are important benefits of LC-MS/MS. Thus, this technique has met the evolving clinical requirements for fast, accurate, and precise tacrolimus assays performing well at low concentrations. The ease of use and robustness of LC-MS/MS instruments and also the combination with automated or semi-automated sample preparation have gradually been improved and further facilitated the widespread implementation in clinical laboratories. Nevertheless, skilled laboratory management and specially trained staff are necessary to establish and operate LC-MS/MS assays for routine TDM. Furthermore, for LC-MS/MS, a rather large upfront financial investment is necessary.

The design, validation and continuous quality assurance of immunosuppressive drug LC-MS/MS assays are critical for routine performance and these aspects have recently been reviewed by the IATDMCT Immunosuppressive Drugs Scientific Committee.[14] The process of LC-MS/MS-based quantification of tacrolimus in clinical samples can be divided into sample preparation, chromatographic separation, mass-spectrometric selection as well as detection, data processing and validation, finally leading to reportable results.

***Sample preparation*:** Tacrolimus is extensively distributed into red blood cells and so it is measured in whole blood. Ethylenediaminetetraacetic acid (EDTA) is the preferred anticoagulant.[14] Protein precipitation, solid-phase extraction (SPE), and liquid-liquid extraction (LLE) have been reported as sample preparation strategies prior to chromatography in LC-MS/MS assays including tacrolimus.[160] The sample preparation and chromatography should be designed to balance each other: clean extracts may allow simple and fast chromatography, whereas crude extracts should be compensated for by more thorough chromatographic clean-up and separation to avoid impairment of the MS/MS response.

Precipitation of whole blood samples can be performed by either a stepwise or simultaneous addition of zinc sulfate solution and organic solvent. Annesley and Clayton developed a protocol in which five volumes deionized water was added to whole blood before step-wise addition of zinc sulfate 0.1 mol/L and methanol. Apparently, the water hemolysis step improved the extraction efficiency and lowered the imprecision of the tacrolimus measurements in clinical samples.[161] This extraction protocol was further validated by Seger *et al.* as part of a standardized multianalyte LC-MS/MS assay: 50 µL EDTA whole blood was added to 250 µL water and 750 µL methanol:zinc sulfate 0.1 mol/L (2:1), then vortex mixed at room temperature and centrifuged at 4 °C.[162] The water hemolysis step and subsequent precipitation with methanol:

zinc sulfate may be regarded as state-of-the art for this type of sample preparation. However, visual inspection of the precipitate is recommended.

Chromatography: The hydrophobic nature of tacrolimus makes it suitable for reversed-phase chromatography. Conditions for chromatographic separation are commonly based on C18 (or C8) stationary phases combined with mobile phases of water and methanol to which an acidified ammonium buffer (*e.g.* formic acid plus ammonium acetate) is added.[160] The mobile phase constituents should be of LC-MS quality. Tacrolimus is retained on a C8/C18 analytical column with methanol content up to approximately 50 % in the mobile phase, thereby allowing the separation of less hydrophobic compounds. Further on, tacrolimus can be eluted with adequate peak symmetry by increasing the methanol content to ≥ 90 %.[13, 162] Some chromatographic compromises may be necessary when cyclosporine is included in the assay, because the latter demands highly optimized conditions to obtain acceptable peak shapes. Column temperatures, in the range 35 °C to 75 °C, may be used to facilitate peak narrowing, also allowing a higher flow rate due to reduced back-pressure.

Online extraction with two-dimensional chromatography is a widely used strategy for clean-up before directing the extracts into the mass-spectrometer. The prepared matrix is then injected on an extraction column with large particles and a low-organic mobile phase is pumped at a high rate (turbulent flow). Subsequently, the system switches to a high-organic mobile phase which back-flushes tacrolimus from the extraction column and through an analytical column.[160] Such online clean-up techniques may be applied to avoid potential ion suppression effects on the signal intensity and also to minimize contamination of the mass spectrometer.

The chromatography of tacrolimus should be designed to minimize co-elution with compounds causing ion suppression/ ion enhancement. Tracking of the time intervals with potential signal

suppression/ enhancement (post-column infusion) and monitoring the retention times for glycerophosphocholines will guide such achievements during assay development.[163, 164]

Mass-spectrometry: The majority of tacrolimus LC-MS/MS assays apply electrospray ionization (ESI) as the interface to get the mobile phase solvent and sample compounds into gas phase.[165] The conditions for spraying, evaporation, ionization, and acceleration of tacrolimus ions into the mass-spectrometer have to be optimized with respect to the specific instrument. Several of the ion source parameters are global in a multianalyte assay and, consequently, optimization of these parameters may have to be prioritized for compounds yielding the lowest instrument response at the lower limit of quantification (LLOQ) (usually sirolimus and everolimus when included in an multianalyte immunosuppressive drugs assay).

Sample matrix components will potentially influence the evaporation and ionization efficiency of tacrolimus (ion suppression or enhancement). Proper sample preparation and chromatographic separation is mandatory to minimize such matrix effects on the analyte response. Highly sensitive mass-spectrometers enable less impact of matrix effects since they allow less biological matrix per sample to be loaded into the system. It is generally recommended to use a stable isotope-labeled internal standard (SIL-IS) to correct variations throughout the assay procedure, including compensation of matrix effects. There are commercially available SIL-IS for tacrolimus (*e.g.* ^{13}C, D_2-TAC).

Selective reaction monitoring (SRM) is applied for tacrolimus quantification, usually with the positively charged ammonium adduct as precursor ion (m/z 821.5) and product ion set at m/z 768.5.[160] The corresponding mass transitions should be used for the IS.

Assuring adequate quality of clinical tacrolimus LC-MS/MS assays: The use of commercial whole blood-based tacrolimus calibrators is recommended to ensure accurate measurements and

to support the harmonization of results between laboratories. Indeed, 78% of TDM laboratories have reported the usage of commercial tacrolimus calibrators.[159] Preferably the quality control samples should be sourced independently from the calibrators. Tacrolimus measurements should be based on a multilevel calibration curve and a proper calibration model with adequate weighting should be applied. Natural tacrolimus isotopes may contribute to the IS response when a SIL-IS is used and a nonlinear calibration curve should be considered in such cases. Also, there may be tacrolimus impurities in the IS solution generating an analyte response in tacrolimus-free samples. Impurity interferences like this should be balanced against requirements for the LLOQ.

Reproducibility of the peak integration can be ensured with automated data processing using custom-adjusted algorithms in the instrument software. Manual integration should generally be avoided but, if necessary, it should be justified and documented in each case. Since the MS/MS signal intensity may fluctuate over time on an instrument, it is useful to include daily testing of the absolute assay response to verify that requirements for the LLOQ are fulfilled.

Hospital TDM laboratories may receive tacrolimus samples from both in-house and outpatient clinics and the requirements for the turnaround time will often be different depending on the clinical setting. LC-MS/MS assays should be designed to meet a turnaround time of approximately 3 hours for in-house tacrolimus samples. Alternating injections into two LC-channels coupled to a single MS/MS allow overlapping chromatography and can be used as a strategy to obtain a reduced batch run-time.[13] Laboratories performing therapeutic monitoring of tacrolimus should perform adequate assay validation or verification before implementation of the assay as a routine service. They should adhere to pre-defined acceptance criteria for analytical performance of their routine runs and participate in external proficiency testing (PT).[14]

Immunoassays

Tacrolimus TDM has benefited extensively from immunochemical methods since the drug was introduced into clinical practice. First generation assays including the PRO-Trac II ELISA (DiaSorin), the Microparticle Enzyme Immunoassay (MEIA) produced by Abbott and the Cloned Enzyme Donor Immunoassay (CEDIA) originally produced by Microgenics, subsequently Thermo Fisher, were replaced by improved tests. Currently a choice of immunoassay is available.

EMIT: The Enzyme Multiplied Immunoassay Technique (EMIT) is offered by Siemens (formerly Dade-Behring) from the early 2000s. The assay may be performed on several analyzers (*e.g.* Vital Viva - different models, Roche Cobas Mira - different models and Integra, Beckman Synchron LX20, Bayer Advia 1650, Abbott Architect c8000, Hitachi 902 *etc*). The EMIT reagents suffer from cross-reactivity with tacrolimus metabolites, resulting in significant overestimation of tacrolimus concentrations in patient samples as compared to LC-MS/MS, reaching up to 30-36%.[25, 166] It has also been noted that another factor contributing to the bias could be nonspecific cross-reactivity.[25] Poor LLOQ ranging between 2.8 ng/mL and 4.6 ng/mL has been reported,[167-168] suggesting that EMIT is not reliable for monitoring tacrolimus concentrations below about 3.0-5.0 ng/mL. LeGatt *et al.* reported total imprecision of 13.7% and 6.0% for tacrolimus concentrations of 3.4 and 19.1 ng/mL, respectively.[167] Commonly observed poor repeatability of determinations between analytical runs, as well as a wide dispersion of the results by EMIT seen in proficiency testing [14, 168] were partially related to reagent instability after opening and method calibration at a particular laboratory. Currently, use of EMIT for tacrolimus monitoring is steadily diminishing.

***ACMIA*:** Another assay from Siemens is the Antibody-Conjugated Magnetic Immunoassay (ACMIA) developed for the Dimension analyzer family. This application, which has been available since 2007 for general biochemistry analyzers, had the advantage of no manual pretreatment step.

The LLOQ for ACMIA was reported as 2.5-5.36 ng/mL[168-171]

A mean positive bias of 1.78 ± 1.51 ng/mL *vs.* LC-MS/MS was found in patient samples by Tempestilli *et al.*[170] and practically no bias (+1.7%) by Cangemi *et al.*[172] Total imprecision of ≤ 12.9% [173, 174] was reported. Tempestilli *et al.* observed the influence of albumin on tacrolimus concentrations and suggested the risk of inappropriately low tacrolimus dosage in low albumin patients.[170] However, ACMIA results were not affected by hematocrit values.[175, 176]

Interestingly, the method's main asset became its primary disadvantage. No pretreatment could lead to lower accuracy and to an overestimation of the results in as many as 1% of patient samples.[170, 177] In some patients the assay was affected by endogenous blood constituents (incl. heterophilic or anti β-galactosidase antibodies) giving falsely elevated results.[172, 177]

Recently, the manufacturer has made modifications,[178] which are expected to resolve the problem. The “new” ACMIA for tacrolimus is characterized by an improved LLOQ of 0.86 ng/mL, fitting well with recent TDM requirements[14], negligible bias of 1.7% to LC-MS/MS and total imprecision of ≤5.5%.[178] If the modified ACMIA proves resistant to erroneous results, it could be an attractive tool for tacrolimus TDM.[179]

***CMIA*:** The Chemiluminescent Microparticle Immunoassay (CMIA) was developed by Abbott for a family of Architect analyzers. After receiving FDA approval, the CMIA can be used for routine tacrolimus TDM in US clinical laboratories.[180] The first analytical step is a manual

pretreatment of a whole blood sample in order to precipitate proteins and extract tacrolimus into a supernatant, followed by immunoassay. Evaluation of the CMIA tacrolimus assay showed no interference with hematocrit, bilirubin or total protein[169, 171, 181] but cross-reactivity yielding 94% with 31-O-desmethyl tacrolimus (M-II), and 45% with 15-O-desmethyl tacrolimus (M-III) tacrolimus metabolites. The cross-reactivity with 13-O-desmethyl tacrolimus (M-I) and 12-hydroxy tacrolimus (M-IV) metabolites was negligible.[171]

Functional sensitivity (CV = 20% of the fitted curve) of 0.5 ng/mL,[169, 181, 182] even lower than the manufacturer's claim (0.8 ng/mL) was reported. However, CV <10% was noted only beyond 1 ng/mL tacrolimus concentration.[169] In Wallemacq's multicenter study, the total CMIA imprecision was ≤8.2% using QC materials[171] and it was ≤8.8% using samples of Chinese renal transplant patients as reported by Li et al.[183] Saint-Marcoux *et al.* noted 4.4 ± 0.2% *bias vs.* LC-MS/MS in pooled clinical samples, whereas in 2 analytical sites involved in Wallemacq's comparison, the bias was reported as +0.51 and +1.63 ng/mL.[171, 184] The assay rapidly became a leader of immunoassay methodology for tacrolimus due to a comparatively low *bias vs.* chromatography, imprecision even better than LC-MS/MS and close agreement of results for clinical samples in proficiency testing data.

ECLIA: The Electrochemiluminescence Immunoassay (ECLIA, Elecsys) is a semi-automated diagnostic assay developed by Roche for use in a family of Cobas e analyzers to monitor tacrolimus in whole blood samples. A comprehensive study has been conducted in five centers in Europe to evaluate the performance of ECLIA for tacrolimus determination. Three different models of Cobas analyzers (e 411, e 601, e 602) were used.[185] The manufacturer declares that the assay shows no interference from bilirubin, hematocrit, or total protein, and that cross-reactivity is 70% for metabolite M-II but no detectable cross-reactivity with tacrolimus metabolites: M-I,

M-III and M-IV. A study by Shipkova showed method linearity between 0.5 and 40 ng/mL, functional sensitivity (CV ≤20%) was 0.3 ng/mL, and CV ≤10% was at 0.8 ng/mL;[185] Within-run imprecision was ≤8.9%; laboratory-to-laboratory imprecision ≤12.1%. This performance was confirmed in more recently published studies.[180, 186] Fung *et al.* reported total imprecision of 3.9-9.4%.[187] Whilst the ECLIA tacrolimus assay has a shorter analysis time (18 *vs.* 30 min) than the CMIA, it uses a higher sample volume (300 *vs.* 200 µL), a factor to be considered when choosing the assay best suited for a particular laboratory.[185, 187, 188]

***Immunoassays under evaluation*:** A new test for tacrolimus monitoring, the Quantitative Microsphere System (QMS) Tacrolimus Immunoassay, has been introduced recently by Thermo Scientific (Fremont, CA, USA). Method description as well as its clinical evaluation is available in a publication by Leung *et al.*[189] They found that the assay is free from interference from bilirubin, hemolysis and lipemia. The method was reported as linear up to 30 ng/mL with a LLOQ at 0.7 ng/mL (CV = 14.4%). Imprecision was stated as 3.9-8.1% and 4.7-10.0% (within-run and between-run, respectively). A comparison made between QMS and LC-MS/MS measures in 145 patient samples showed a bias of +1.6 ng/mL.[189]

A similar type of assay, latex agglutination turbidimetric immunoassay (LTIA, Nanopia TDM Tacrolimus assay kit) from Sekisui Medical has been tested in Japan. LTIA was compared to three other IAs (CMIA, ECLIA and ACMIA) by Akamine *et al.*[190] In this evaluation, LTIA had the best profile for cross-reactivity with three major tacrolimus metabolites (M-I, M-II and M-III), and yet LTIA presented the highest bias (+1.88 ng/mL) which was additionally influenced by *CYP3A5* genotype and hematocrit value.[190] Further investigations are necessary to judge whether the LTIA tacrolimus assay is suitable for tacrolimus TDM.

The evolution observed over more than 20 years for tacrolimus immunoassays shows improved specificity, precision, limit of quantification, as well as time of analysis and automation. Thus, the new generation of immunoassays is increasingly an alternative to LC-MS/MS methods in tacrolimus monitoring.

Consistency of tacrolimus results generated by different analytical methods

Method inconsistency may have an impact on patient care for several reasons including but not limited to its effect on clinical decisions and drug dosing. It also has an impact on long-term outcomes for patients and on the correctness of retrospective analysis of clinical data or the interpretation of pooled data from clinical trials, since these analyses may be used for regulatory purposes or to establish clinical decision points. The range of immunoassays available for tacrolimus each shows a different spectrum of cross-reactivity to tacrolimus metabolites and different susceptibilities to interactions with heterophilic antibodies and endogenous factors such as hematocrit or albumin.[14] Therefore, dealing with the issue of consistency of results generated with different methods is not easy. A recent study of the performance of current tacrolimus immunoassays among Japanese hospitals demonstrated large between-laboratory variability even when using samples spiked with the drug.[168] The very low level of method harmonization with laboratory developed tests (LDTs, *e.g.* the majority of LC-MS/MS methods) presents further obstacles. According to data from the Analytical Services International (ASI) Proficiency Testing (PT) program collected between 2014 and 2017 **(Figures 1A and 2A)** the ratio between the maximum and minimum averages of the concentrations determined by different methods when analyzing the same samples was about 1.3-fold. This was true both for spiked and pooled patient samples. However, considering the within-method CV (%) found for each of the different peer groups **(Figures 1B and 2B)** a much greater difference between minimum and maximum

reported results can be anticipated. Recently Agrawal *et al.*[191] evaluated, under "real-world" clinical conditions, the effect of a change in analytical methodology from CMIA (run on an Abbott Architect system) to a kit based LC-MS/MS method (Waters TQD Acquity mass-spectrometer with Mass Trak) on patient classification according to the tacrolimus concentration. Although a good analytical performance has been demonstrated for both of these methods in the past,[192, 193] the results of this study demonstrated that 40% of patient samples were discordantly classified by the two methods when the applied therapeutic ranges were 2 ng/mL wide. The discordance rate improved to 3% when the width of the target range was increased to 4 ng/mL. A change in patient classification due to lack of comparability between methods might lead to the patient receiving either an insufficient dose and rejecting the organ, or receiving a high, potentially toxic dose. Although introducing laboratory- (or method-)specific target ranges may be a helpful approach to attenuate the impact of between-method differences on patient classification, this approach may pose a hidden danger, particularly when laboratories need to change the methodology at short notice (*e.g.* due to problems with reagent supply) or when transplant physicians have to simultaneously interpret results provided by different laboratories.

The narrow therapeutic index of tacrolimus sets stringent requirements for the performance of analytical methods. For instance, estimation by different approaches that try to take into account the within-individual biological variability of pre-dose tacrolimus concentrations shows that an adequate application of a 4-5 ng/mL wide target concentration range would require analytical imprecision and bias of ≤ 6% each.[14, 194] As discussed in recently published IATDMCT recommendations for proper analytical performance[14] and demonstrated in **Figures 1 and 2**, such goals are hardly achievable in current routine TDM services. Targeting of even more narrow therapeutic ranges for clinical purposes seems, with reference to current analytical

practices, unrealistic.[159] The recommended achievement of between run imprecision of at least ≤ 10% with a total error of ≤ 15% (among other performance characteristics) should be considered obligatory for tacrolimus analytical methods to rate them as acceptable.[14]

Furthermore, to properly deal with patient non-adherence issues that are particularly critical in transplantation not only method precision but also the ability to measure very low concentrations is important. The recommended LLOQ for tacrolimus is ≤1 ng/mL, and can be achieved currently by LC-MS/MS methods, the ECLIA, and CMIA techniques,[14] as well as the newest generation of ACMIA. The measurement range of the QMS method as reported by the manufacturer can theoretically also cover such low concentrations, but the imprecision observed in PT programs is broader than those of the latter methods (**Figure 1B**). Hence, between-method consistency in analytical sensitivity is lacking too.

Last but not least, the fact that tacrolimus is prescribed as a long-term therapy clearly highlights the importance of consistent analytical performance of methods and laboratories over long periods of time. However, as can been seen in **Figure 1**, long-term inconsistency with analytical methods over time is still an issue, and the within-method variability of the bias to the same spiked concentrations frequently reached 15% and even more when looking at the interquartile ranges.

In summary, when looking back to the early years of tacrolimus TDM, a continuous improvement of method performance can be seen. However, the analytical performance of currently available methods still varies broadly which needs critical consideration when implementing or changing TDM services for tacrolimus.

Method calibration and proficiency testing

In the case of tacrolimus analytics, there are multiple PT programs available (both national and international) with the College of American Pathologists (CAP) scheme and the programs formerly run by ASI (now provided by LGC) being most popular. Results from these programs have been used in the past to reveal analytical problems and their causes.[195] When choosing a PT program it is important to consider whether specimens based on human whole-blood spiked with tacrolimus metabolites, real patient material from transplant recipients and blank samples without tacrolimus and metabolites are part of the distributions by the program. Such samples are important to check for matrix effects on the accuracy of the measurement.[14]

In general, users can check on accuracy using third party prepared calibrators or controls or compare their results with those given by PT data. Fortunately, since 2013, a higher order whole blood reference material for tacrolimus has been available commercially (ERM-DA110a) and listed in the database of the Joint Committee for Traceability in Laboratory Medicine (JCTLM). Because commercially available calibrators and controls are in general traceable to this material their use in routine services is preferable to reduce the risk of calibration bias. Participation in PT is particularly supportive if own results can be compared not only to the peers but also to results traceable to a higher order reference method, however unfortunately this possibility is still not available for tacrolimus. In this situation, comparison to results generated with the LC-MS/MS technique (the LC-MS/MS participant group) can provide some helpful information when evaluating immunoassays. However, the drawback here is that the LC-MS/MS group is not consistent.[193] and the level of validation as well as the overall quality performance management at the participating analytical sites are unknown. Therefore, for the evaluation and verification of immunoassays accuracy, confirmation of their results using a fully validated LC-MS/MS as a reference and real (not pooled) patient samples is advised before application to routine services.

Acceptance criteria for such between-method comparisons have been recommended by the IATDMCT Immunosuppressive Drugs Scientific Committee:[14]

a. A linear regression slope within ±10% of the theoretical value of 1.0.

b. A linear regression intercept not significantly different from zero.

c. A standard error for the estimate, Syx ≤10% of the average of the therapeutic concentrations.

Figures 1 and 2 summarize data regarding the performance of currently used tacrolimus methods as collected between 2014 and 2017 (after the introduction of ERM-DA110a) from the ASI PT program. They show that, although, the bias of method means when compared to spiked target concentrations (4, 8 and 12 ng/mL**, Figure 1A**) lies with few exceptions (EMIT at 4 ng/mL) within a range of ±10%, it varies broadly between distributions. When comparing the performance of immunoassays with pooled patient samples **(Figure 2A)** versus the LC-MS/MS group, all of them showed some overestimation, in agreement with published data generated with non-pooled patient samples.[169, 185] In contrast to the EMIT and QMS assays, the median overestimation with the CMIA, ECLIA, and ACMIA was <10%.

Looking at the long-term method-specific percentage CVs (both for spiked and pooled patient samples), they clearly favor LC-MS/MS, CMIA, and ECLIA methods compared to ACMIA, QMS and EMIT **(Figures 1B and 2B)**. Moreover, the percentage CVs of the first group of methods seems not to be concentration-dependent, in contrast to the methods of the second group, for which much broader within-group variability at the lowest concentration (4 ng/mL) compared to 8 and 12 ng/mL was identified. During the observation period the CMIA and ECLIA methods demonstrated better long-term precision than the very heterogeneous LC-MS/MS group.

All these results underline the strengths of PT and demonstrate that there is without doubt a high potential for improvement of tacrolimus analytics to further standardize method and laboratory performance.

Standardization of tacrolimus TDM

The applicability and reliability of analytical figures generated by laboratories is strongly linked with data quality, particularly their accuracy. This general remark holds true for any kind of measurement service and is not limited to clinically relevant entities such as tacrolimus. Laboratory medicine adopted relatively early the general metrological concept of traceability,[196, 197] and established a close relationship with national metrological institutes. By founding the Joint Committee for Traceability in Laboratory Medicine (JCTLM) located at the International Bureau of Weights and Measures,[198] chemical and biological entities in laboratory medicine have been raised to the same level of international consistency and used classical SI units for measuring time, weight, and length.

Measurement procedure accuracy is achieved through ensuring specificity of the applied methods. These methods are the responsibility of individual laboratory units offering defined measurement services. Total error or measurement uncertainty calculations[199-201] can be used to investigate the error components associated with the processes of measuring, namely bias and precision which do combine up to the accuracy of a measurement system.[202] Error budgets may be used to estimate the expected total error (TE) which combines systematic error components (associated with bias) and stochastic (random) error components (associated with precision). They can be either calculated by “top-down” or “bottom-up” approaches. Whereas bottom-up approaches are used in the assessment of reference measurement systems,[201] top-down approaches allow the monitoring of systems in routine use; *e.g.* either individual users or global

measurement services. For tacrolimus, a recent top-down investigation showed that this approach, either using single laboratory validation data or proficiency testing data, gave identical results.[203] Different top-down approaches to estimate measurement uncertainty of whole blood tacrolimus mass concentration values meet the assay quality criteria set up by a recent IATDMCT guidance paper.[14]

If individual laboratories use the same test principle, *e.g.* an automated immunoassay, traceability of locally applied calibrators to an "industrial master calibration" is usually assured and guaranteed by the assay vendor. The vendor (*e.g.* operating under FDA clearance or within the framework of an obtained IVD-CE certification) has responsibility for the trueness, *i.e.* lack of *bias* of the local calibration to this master calibration. The expected assay precision is also stated by the assay producer, local deviations to the given numbers must be carefully monitored, since they increase the total error of an assay. If such deviations exceed (definable) thresholds, the local laboratory should ensure the assay vendor takes corrective actions (*e.g.* running an additional service, changing a pipetting unit *etc.*) to prevent the occurrence of irregular analytical errors.[204] If a laboratory has decided to develop an "in house assay" (LDT), the responsibility for maintaining the trueness and precision of a measurement service is entirely the responsibility of the individual laboratory.[205] Failure can be minimized if the trueness of the assay is kept under control by using commercial calibrator materials. In the past it has been impressively shown that this approach minimizes the risk of between-laboratory imprecision for both 25-OH vitamin D[206] and tacrolimus,[192] which improves the comparability of patient results obtained by individual laboratories.

In the current global situation, with several FDA approved / IVD-CE certified tacrolimus measurement systems on the market and at least two additional calibrator material vendors

serving the LTD community, one has to assume that the trueness between this tacrolimus TDM measurement platform realizations is limited. Proficiency data **(Figures 1A and 2A)** supports this assumption,[14] emphasizing the need for traceability of individual tacrolimus measurement services beyond the industrial or commercial calibrator systems.

LGC, a National Measurement Institutes of the United Kingdom, took up this challenge some years ago and presented two certified reference materials to the public: ERM-DA110a, a whole blood matrix containing tacrolimus in 2014 (secondary higher order reference material) and, in 2017, ERM-AC022a, which is pure tacrolimus (neat substance, primary higher order reference material). ERM-AC022a was characterized by quantitative NMR but it is not clear which reference method was applied for value assignment, since the primary reference method has not been disclosed, as has been done in other fields, e.g. for steroid hormone measurements.[207, 208] However, ERM-AC022a and ERM-DA110a are both listed by the JCTLM, implying that the responsible JCTLM working group has reviewed the reports associated with both, and concluded that they are in accordance with the JCTLM regulations.[209] Nevertheless, the availability of peer reviewed reference methods for tacrolimus is long overdue. Unfortunately, in the whole field of ISD-TDM, only one such method has been presented and it was for cyclosporine.[210] Within the Scientific Division (SD) of the IFCC (International Federation of Clinical Chemistry), a work group (WG-ID) was founded in 2018 to focus on activities in this field, including the generation of reference materials and the placement of appropriate reference procedures for immunosuppressive drugs including tacrolimus.[211]

New TDM approaches

It should be noted that all of these new TDM approaches were made possible by important analytical improvements developed during the last decade (*i.e.* sensitive and precise liquid chromatography with tandem mass-spectrometry).

Microsample based tacrolimus concentration monitoring (DBS and others)

DBSs on filter cards and volumetric absorptive micro-sampling are innovative, minimal-invasive sample methods, which can replace traditional blood sampling for the TDM of immunosuppressive drugs but can also be used for a number of other applications. This approach is patient-friendly and can be implemented at home by the patient themselves, collecting very small amounts of blood (typically 10-20 µL). The procedure is both cost and time saving, and it also allows for multiple sampling within a dose interval, enabling the determination of a patient's AUC. The AUC reflects patient's systemic drug exposure. Micro sampled AUC determinations have advantages in special populations such as neonates, pediatric and older patients, in whom venipunctures are difficult and the blood volume that can safely be collected is limited. Furthermore, in a busy out-patient clinic it is logistically difficult to draw multiple blood samples throughout a 12-24 hours dosing-interval. Thus, for tacrolimus TDM, a microsample approach is particularly appealing.

Preanalytical and analytical requirements and pitfalls: Microsample methods require extensive validation with some criteria beyond the usual recommendations for analytical method validation.[212, 213] This approach also requires sufficient analytical sensitivity because of the low volume of blood collected. The use of microsamples with dried blood is a destructive method meaning that the complete sample is used for the analysis and no confirmation of drug measurement can be conducted using the same sample, only using parallel samples. Current challenges in microsample implementation include but are not limited to: extraction recovery,

matrix effect on blood volume (hematocrit effect), correlation between venous and capillary blood measurements, the quality of the blood spot, risk of contamination, and sample stability. These issues require additional validation steps and have to be properly addressed during method validation.[214] New generation microsampling such as volumetric absorptive microsampling (VAMS) can possibly mitigate some of the usual bias encountered with these methods, particularly hematocrit effect.[215] Several DBS methods for tacrolimus TDM have been published. These methods are often multiplexed with other immunosuppressive drugs and may include creatinine determination, which is of particular relevance in kidney transplantation.[216-218] To date, microsample validation is only addressed in a few guidelines.[219, 220]

***Future developments and clinical perspective*:** The use of micro-sampling for tacrolimus TDM needs to be cross-validated with established TDM strategies based on venous EDTA whole blood, to determine whether this approach is suitable for clinical purposes. One of the main advantages over established sampling procedures for tacrolimus TDM is the potential to improve the patient's quality of life. One study has been conducted recently in transplanted children. In parents and children completing the satisfaction survey, all but one reported preference for DBS sampling over venous blood sampling.[221] Clinical performance of microsampling in routine practice should also be evaluated in various transplant settings. Analytical performance together with patient training to ensure appropriate sample quality and motivation will be key factors for the implementation of micro-sampling in clinical practice.

Intracellular and tissue tacrolimus concentration monitoring

TDM of immunosuppressive drugs results in a decrease in acute cellular rejection (ACR) rates as well as a decrease in treatment-related toxicity, particularly the nephrotoxic effects of the CNI.[91] However, despite intensive use of TDM, the global outcomes of transplantation appear to be only

marginally improved.[10] This has led pharmacologists to develop newer ways to optimize drug treatment, including measuring the concentration of immunosuppressive drugs directly at their site of action. Currently, standard TDM of tacrolimus is based on the measurement of drug concentrations in whole-blood. Determining tacrolimus drug concentrations where it exerts its immunosuppressive effect, in the T-cell or, for practical reasons, in peripheral blood mononuclear cells (PBMC), might be particularly relevant. Moreover, measuring tacrolimus directly in graft tissue may be of interest as it is reasonable to expect that local concentrations better reflect drug effect. Interest in such approaches has been strengthened by observations of the weak relationship between intracellular and whole blood tacrolimus concentrations in patients following various types of transplantation, suggesting potential added value.[222-225] A study confirming the link between intracellular tacrolimus concentrations and patient outcomes following liver transplantation suggested that this new TDM approach was a valuable option,[226] but definitive clinical verification and validation remain to be generated. The work conducted on this topic has been reviewed recently.[227, 228]

Preanalytical and analytical requirements and pitfalls: The first investigations evaluating intracellular concentrations of tacrolimus were conducted in the 1990s. Several groups have contributed to the development of analytical methods to measure tacrolimus concentrations in PBMC of kidney, liver and heart transplant recipients.[153, 222, 224, 225, 229, 230] The large variability reported in these studies highlighted the critical importance of pre-analytical and analytical steps for intracellular concentration assays. These methods must be appropriately validated following international guidelines and be sufficiently sensitive to quantify low intracellular concentrations, especially in the setting of immunosuppressant minimization protocols. Most analytical methods have involved liquid chromatography with tandem mass-spectrometry detection. Normalization

of the concentrations measured is also needed and can be achieved based on the number of cells, on the mean corpuscular volume of cells for PBMC, or based on the sample weight for tissues. There are several potential issues that have not yet been fully addressed, such as tacrolimus passive or active transport across the cell membrane before cell separation, equilibrium modifications during cell separation or washing steps and the risk of sample contamination with red blood cells. Proper evaluation of the impact of each of these potential issues has to be conducted as these may cause measurement bias. There is a crucial need for practice harmonization for the TDM of intracellular tacrolimus concentrations. Moreover, to date, no quality control programs exist.

***Relationship with whole blood concentrations*:** Early studies to evaluate the relationship between whole blood and cellular concentrations of tacrolimus have reported a lack of correlation.[224-226] However, as the approach is further refined, a trend to better correlations has emerged. Notably, a recent study conducted in a large population of stable kidney transplant recipients (n = 213) showed a linear relationship between whole blood and intracellular tacrolimus concentrations, although agreement between the two concentrations remained modest (r = 0.67).[231] Among factors influencing tacrolimus compartmentalization in cells, the role of P-gp (or ABCB1) has already been identified as a determinant of tacrolimus efflux and *ABCB1* genotype relates to intracellular tacrolimus exposure.[224] Other factors may include the circulating free-fraction (influenced by protein binding and hematocrit), and the intracellular expression of CYP3A5/4.[224] More research must be carried out to further explore the role of these factors in the high variability of immunosuppressive efficacy of tacrolimus. A more detailed knowledge of such factors may also allow the identification of sub-populations of patients who may benefit from alternative TDM strategies, such as intracellular measurement of tacrolimus.

***Clinical evidences and future perspectives*:** To date, the most convincing proof of concept study was reported by Wallemacq's group in Belgium.[226] In a study conducted in 90 liver transplant recipients treated with tacrolimus monotherapy, they reported a relationship between tacrolimus concentrations in PBMC as well as in liver biopsy samples and histological rejection grade determined at day-7 post-transplantation, whereas whole blood concentrations were not associated with the severity of rejection.[226] The results of this study suggested that intracellular tacrolimus concentrations early after liver transplantation seem to be related to rejection risk and severity. The lack of a relationship between whole blood tacrolimus concentrations and clinical outcome during this early period may be a good argument for using intracellular tacrolimus concentrations as a biomarker of immunosuppressive drug effect. Another observational study highlighted the case of a liver transplant recipient experiencing an ACR while having the lowest intracellular tacrolimus exposure among study participants.[232] However, the definitive evidence for the relevance of intracellular tacrolimus concentrations as a longitudinal biomarker usable for clinical practice with standard immunosuppressive regimens is still lacking. The time-consuming pre-analytical sample work up makes this approach complex and still requires extensive validation. Another perspective could be obtained by multi-variate mathematical modeling predicting intracellular concentrations, based on increasing experience in genetic polymorphism, together with a better understanding and identification of covariates influencing intracellular tacrolimus concentrations. Besides intracellular measurement, determining the tacrolimus free-fraction also appears to be an appealing approach. This unbound (*i.e.* the pharmacologically active moiety) fraction of the drug might be of interest in reflecting immunosuppressive drug effect. This newer way of TDM should also be investigated but is still at an early stage of development.[152, 233] Finally, it should be mentioned that the analysis of tacrolimus in oral fluid

samples has been investigated. One study concluded that the correlation with whole blood concentrations was poor and due to several problematic methodological problems could not be recommended.[234] A more recent report indicated that some of these problems could be overcome, however, blood contamination may still pose a problem[235] and this kind of sampling seems to not have gained any further interest so far.

PHARMACOGENETICS

After oral administration, tacrolimus is metabolized by gastrointestinal and hepatic cytochrome P450 (CYP) 3A isoenzymes, predominantly CYP3A4 and CYP3A5 (other members of the CYP3A family are CYP3A7 and CYP3A43). The main enzyme involved in tacrolimus biotransformation is CYP3A5, with CYP3A4 having a lower efficiency for catalysis.[236]

The expression of CYP3A5 is largely determined by genetic polymorphisms, with only 15-25% of Caucasians expressing CYP3A5 at a detectable level. The main genetic factor responsible for this is an intron 3 single-nucleotide polymorphism (SNP; *CYP3A5*3*, rs776746, g.6986A>G) which causes aberrant splicing, resulting in the absence of protein and, thus, CYP3A5 activity.[237, 238] Individuals with at least one *CYP3A5*1* allele (defined as the "wild-type" allele) are classified as CYP3A5 expressors (*CYP3A5*1/*1* or *CYP3A5*1/*3*). The minor allele frequency (MAF) of *CYP3A5*3* varies widely across different ethnicities, resulting in significant differences in CYP3A5 expression **(Table 3)**. Another rare SNP located in exon 7 (*CYP3A5*6*, rs10264272, g.14690G>A) has also been shown to result in loss of functional CYP3A5 activity.[237]

Until recently, and in contrast to CYP3A5, no common SNP in the *CYP3A4* gene could be related to CYP3A4 activity and to explain the significant between-individual variability in CYP3A4 activity. However, in 2011, Wang et al., demonstrated that an intron 6 SNP

(*CYP3A4*22*, rs35599367, C>T) significantly influenced CYP3A4 hepatic expression, suggesting that this SNP may be a biomarker for the prediction of the response to drugs metabolized by CYP3A4.[239]

In addition to CYP3A4 and CYP3A5, the efflux transporter ABCB1 also plays a role in tacrolimus pharmacokinetics.[240, 241] Over the last decade, more than 50 SNPs have been identified in *ABCB1*.[242, 243] The three most common SNPs in the protein-encoding region are rs1128503 (1236C>T, Gly412Gly), rs2032582 (2677G>T/A, Ala893Ser/Thr), and rs1045642 (3435C>T, Ile1145Ile).[244, 245] These three SNPs (with a MAF of around 50% in Caucasians) are in strong linkage disequilibrium (LD) and have been investigated extensively. Other less frequent SNPs have been described and, potentially, can explain part of the between-individual variability observed in the expression and/or function of ABCB1. Of particular interest, the *ABCB1* 1199G>A coding SNP located in exon 11 (rs2229109) is relatively frequent, with a reported allelic frequency of about 6% in the Caucasian population. This SNP is associated with a serine to asparagine substitution at position 400 in a cytoplasmic loop of ABCB1 which is involved in substrate recognition and with intracellular accumulation of tacrolimus in HEK293 and K562 recombinant T-cell lines.[246]

A SNP in the gene encoding P450 oxidoreductase (*POR*28*; rs1057868, C>T, Ala503Val) has been associated with an increased *in vivo* CYP3A activity using midazolam as a drug probe.[247] Because tacrolimus is metabolized by CYP3A isoenzymes, this SNP might affect tacrolimus pharmacokinetics.

Finally, two different SNPs in peroxisome proliferator-activated receptor alpha (PPARα) (rs4253728 G>A and rs4823613 A>G) have been shown to influence CYP3A4 activity both *in vitro* and *in vivo*.[248]

Pharmacokinetic-Pharmacogenetic relationships in kidney transplantation

In relation to pharmacokinetic parameters and TDM, it has been clearly demonstrated that the *CYP3A5*3* variant is the main genetic factor influencing tacrolimus dose requirement (defined as the dose-adjusted, whole blood pre-dose concentration or C_0) in stable renal transplant recipients, both in the early and late phase after transplantation (*i.e.* several weeks to months after transplantation).[249-251] Since the original publication, this genetic association has been confirmed in a large number of studies[252] and meta-analysis.[253, 254] Based on these observations, dose adjustments have been proposed for the first tacrolimus dose after kidney transplantation, according to the *CYP3A5* genotype of the recipient, with a doubling of the tacrolimus starting dose in patients who are CYP3A5 expressors (carrying at least one wild-type *CYP3A5*1* allele).[255] Indeed, genotype-based adjustment of the initial tacrolimus dose has already proven useful, with a greater proportion of patients reaching the therapeutic concentration range shortly after transplantation.[256] Such a pre-emptive strategy, without additional dose adaptation (only based on *CYP3A5* genotype) during the first four days after transplantation, has been proven to be safe and easy to implement.[257] The level of evidence of this pharmacogenetic-pharmacokinetic association justified a specific guideline from the Clinical Pharmacogenetics Implementation Consortium (CPIC).[258] However, the benefit in terms of reaching the targeted tacrolimus exposure earlier with the use of *CYP3A5* genotype-based dosing has not been a universal finding[259] and no trials have so far demonstrated improved clinical outcomes.

Besides *CYP3A5*3*, CYP3A4 activity has also been shown to be an important factor influencing tacrolimus dose requirement and clearance in renal transplant patients.[260] To a lesser extent than *CYP3A5*3*, *CYP3A4*22* has been shown to influence tacrolimus dose-adjusted pre-dose concentrations and dose requirements in stable renal transplant patients.[261, 262] A similar approach

to genotype-based dose adjustment has been proposed with the advice that both *CYP3A5*3* and *CYP3A4*22* should be taken into consideration.[263] Updated guidelines for the tacrolimus starting dose based on the recipient's *CYP3A5* and *CYP3A4* combined genotype **(Table 4)** have been validated through popPK modelling.[264, 265] However, the clinical utility of this approach still remains to be proven in prospective studies before it can be included in a future revised version of the CPIC guidelines.

Although numerous studies have shown no influence of *ABCB1* genotype on tacrolimus pharmacokinetic parameters [249, 251, 266, 267], some studies and/or meta-analyses have reported weak but significant associations,[268, 269] mainly during the first month after transplantation.[270] In those, latter publications, *ABCB1* 3435 CC patients were reported to have a lower tacrolimus concentration-to-dose ratio and a higher dose requirement for tacrolimus compared with patients having the CT and TT genotype, although no genotype-based dose adjustments have been proposed according to *ABCB1* genotype of the recipient. An explanation for the lack of the anticipated correlation between *ABCB1* variants and tacrolimus pharmacokinetics could be that the expression and the function of ABCB1 is highly variable and is influenced by several factors, including non-genetic factors.[271]

It is interesting to note that *ABCB1* genotype has been shown to influence intracellular tacrolimus concentrations, particularly in PBMCs. Indeed, 3435T and 1199A carriers had a significantly higher intracellular tacrolimus concentration compared with homozygous wild-type patients, suggesting a reduced ABCB1 activity towards tacrolimus in PBMCs of patients with these *ABCB1* variants.[272] Theoretically, such effects of *ABCB1* genotype on PBMC concentrations could influence pharmacodynamics without any significant impact on whole-blood pharmacokinetic parameters.[273]

In addition, in relation to pharmacokinetic parameters and TDM, it has been demonstrated that patients carrying at least one *POR*28* allele have a higher tacrolimus dose requirement than those not carrying this allele (*POR*1/*1*), but this association was only found in CYP3A5 expressors.[274-277] Only one study reported no association between *POR* genotype and tacrolimus pharmacokinetic parameters.[278] Going one step further, another study, which confirmed that the *POR*28* allele was associated with increased *in vivo* CYP3A5 activity towards tacrolimus in CYP3A5 expressors, also showed that *POR*28* homozygosity (*POR*28/*28*) was associated with a significantly higher CYP3A4 activity towards tacrolimus in CYP3A5 non-expressors.[279] Up until now, no specific genotype-based dose adjustments have been proposed according to recipient's *POR* genotype but, based on the consistency of the available data, future recommendations could include *CYP3A5*3*, *CYP3A4*22* and *POR*28* genetic information.

Finally, tacrolimus dose-adjusted pre-dose concentrations were not different depending on peroxisome proliferator-activated receptor alpha *PPARA* genotype in a cohort of 241 Caucasian kidney transplant patients.[278] In another study (n = 177), a limited effect (15% higher tacrolimus concentration) was observed in the *PPARA* variant allele carriers.[274]

Pharmacogenetic-Pharmacodynamic relationships in kidney transplantation

FK-binding protein-12 (FKBP-12), the principal binding protein for tacrolimus, is polymorphically expressed. The same holds true for CaN and the nuclear factor of activated T-cells (NFAT), which is the main downstream target of CaN (reviewed in Pouche *et al.*[280]). In an *in vitro* study, it was demonstrated that genetic polymorphisms in the genes encoding proteins of the CaN pathway were associated with the inhibitory effects of tacrolimus.[281] However, in a study including 160 kidney transplant recipients, no statistically significant associations between

these genetic variants and tacrolimus pharmacodynamics was demonstrated.[282] Taken together, the limited available data do not suggest a clinically relevant effect of genetic polymorphisms in FKBP-12, CaN and NFAT and tacrolimus pharmacodynamics.

Pharmacogenetics in liver transplantation

CYP3A isoenzymes are expressed in both the liver and the intestine. Both organs contribute to the pre-systemic metabolism of tacrolimus but, unlike other forms of solid organ transplantation, the hepatic enzyme content is determined by the donor genome in liver transplantation whereas the intestinal content is determined by the genome of the recipient.

The association between tacrolimus pharmacokinetics and the *CYP3A5* genotype of both the intestine (recipient) and the liver (donor) has been assessed in living-donor liver transplantation (LDLT) and in deceased-donor liver transplantation. Some studies have demonstrated that donor and recipient *CYP3A5*3* genotypes are of major influence for tacrolimus blood concentration to dose ratio (C/D) which serves as an index of clearance and tacrolimus dose requirement[283-285], whereas other authors have suggested that donor *CYP3A5*3* seems to contribute more than the recipient.[286, 287] Of note, the lowest (C/D) ratio values were seen when both donor and recipient were CYP3A5 expressors.[285, 288, 289] A meta-analysis combining data from living- and deceased-donor liver transplantation (694 donor & recipient genotypes) confirmed that both the genotype of the donor and recipient are important in determining the C/D ratio. The *CYP3A5* genotype of the recipient, the determinant of intestinal genotype, has the greatest influence on tacrolimus dose requirement in the immediate post-transplant period, whereas the donor genotype, affecting hepatic expression, becomes more important after the first few weeks.[290]

Another study, which included more than 400 LDLT cases from Japan suggested that the *CYP3A5* genotype of the recipient was more important than that of the grafted liver as an

indicator of systemic tacrolimus exposure for at least 5 weeks after transplantation.[291] The increasing role of the donor *CYP3A5* genotype with time is compatible with the progressive liver graft recovery and growth. Of note, in this study there was a higher frequency of acute cellular rejection among patients receiving a liver with at least one *CYP3A5*1* allele compared to those receiving a liver with the *CYP3A5*3/*3* genotype, suggesting an association between local (intrahepatic) concentration of unmetabolized tacrolimus and rejection. This effect of donor and recipient *CYP3A5* genotype is apparently independent of the tacrolimus formulation. This association was reported in two studies in stable liver transplant recipients treated with the once-daily tacrolimus formulation.[283, 292]

Opinion is divided on the association of the *CYP3A5* genotype with the nephrotoxicity of tacrolimus. In both liver and heart transplantation, it was reported that CYP3A5 expression in the kidneys could play a role in the individual susceptibility to the nephrotoxic effects of tacrolimus.[293-295] Another report suggested that expression of CYP3A5 (in both the graft liver and the intestine of the recipient) may result in a higher incidence of infectious complications in LDLT recipients. The exact mechanism for these complications related to excessive immunosuppression is unclear but it was postulated that these may relate to differences in tacrolimus metabolism.[296, 297]

Only a few studies have been conducted on *CYP3A4*22* in liver transplantation. In stable liver transplant recipients, Moes *et al.* found no association between this SNP and the pharmacokinetics of the once-daily formulation of tacrolimus (considering both the donor and the recipient genotypes).[292] The *CYP3A4*22* SNP was also investigated among Asian patients but was not identified in this population.[298] Results from adult liver transplant recipients receiving a twice-daily formulation of tacrolimus have shown that donor *CYP3A4*22* genotype

influences tacrolimus pharmacokinetics; a substantial reduction in tacrolimus dose was required in the first weeks after transplantation to achieve target concentrations.[299, 300] In addition, donor and recipient *CYP3A4*22* genotypes seem to have opposite effects, with absence of effect of the combined recipient and donor statuses on tacrolimus C/D ratio value.[289] In pediatric liver transplantation, a popPK study suggested that the apparent clearance of tacrolimus decreased by 29% when a graft carried a *CYP3A4*22* allele (with no effect of the recipient genotype). This effect was quantitatively similar to that observed in the case a donor liver expressing CYP3A5 (*i.e.* an increase of 30% for apparent clearance).[301] However, comparison between pediatric and adult liver transplant patients is difficult since young transplant recipients have a higher liver graft to body weight ratio, leading to a higher tacrolimus apparent clearance and dose requirement.

Results from these studies revealed controversial data, so further studies are needed to better evaluate the apparently limited influence of *CYP3A4*22* that might be masked by the more dominant effect of *CYP3A5*3* on tacrolimus pharmacokinetics.

Regarding CYP2C19 status and its influence on tacrolimus exposure, liver transplant patients with a *CYP2C19* defect genotype have a higher risk of clinically significant drug-drug interactions between tacrolimus and drugs whose major metabolic pathway involves *CYP2C19*, such as omeprazole and lanzoprazole[302] and voriconazole.[45]

As for other types of transplantation, the association between various *ABCB1* SNPs and tacrolimus pharmacokinetics and pharmacodynamics in liver transplantation is debated. The mean tacrolimus C_0 correlated significantly with ABCB1 messenger ribonucleic acid (mRNA) expression in PBMC and the ABCB1 messenger ribonucleic acid (mRNA) expression level in the small intestine was associated with acute rejection risk and one-year graft survival.[303]

Pharmacogenetics in heart transplantation

As in other solid organ transplant populations the effect of the defective *CYP3A5*3* allele on tacrolimus dose-requirement is also well established in heart transplant recipients. In fact, 7 different candidate-gene association studies related this SNP to differential tacrolimus exposure regardless of the period after transplantation or the design of the study (*i.e.* longitudinal or cross-sectional).[304-310] It has also been consistently reported that the tacrolimus dose required by *CYP3A5* expressors to achieve the target therapeutic range is approximately 2-fold higher when compared to *CYP3A5* non-expressors. However, it is estimated that only about 25% of the variability in tacrolimus dose requirement is explained by a patient's *CYP3A5* genotype, which is slightly lower than the value observed among the renal transplant population. This observation suggests that other pharmacogenetic markers may explain part of this residual variability.

So far, only two studies have assessed the effect of the *CYP3A4*22* allele on tacrolimus dose requirement in adult heart transplant recipients. The first of these studies was characterized by a cross-sectional design with a relatively small number of patients (n = 76).[308] In contrast to observations made in kidney and liver transplantation, no significant association was found between the *CYP3A4*22* SNP and tacrolimus dose requirement, even if the combined *CYP3A4* and *CYP3A5* genotype was studied. In the second study no association between *CYP3A4* genotype and tacrolimus dose requirements was observed. However, only one *CYP3A4*22* carrier was detected among the 52 tacrolimus-treated heart transplant recipients.[310] In contrast, in a cohort of 60 pediatric heart transplant recipients, it was observed that *CYP3A4*22* carriers needed a 30% lower tacrolimus dose to reach similar exposure compared with *CYP3A4*1/*1* patients.[307] This study was characterized by a longitudinal design in which 13 time-points were analyzed during the first 2 weeks after transplantation. This more rigorous design might explain

why the difference was significant despite the low number of patients included in the analysis. The differences were even more significant when patients were categorized into the different *CYP3A* genotype clusters. It was demonstrated that CYP3A poor metabolizers required 17% less tacrolimus compared with intermediate metabolizers and 48% less than extensive metabolizers. These findings suggest that the combined *CYP3A4* and *CYP3A5* genotype can provide useful information to guide tacrolimus immunosuppressive therapy after heart transplantation.

As for other solid organ transplantation populations the majority of studies have not reported a clear association between *ABCB1* genotype and tacrolimus pharmacokinetics or dose requirement among heart transplant recipients.[304, 306, 309, 310] A minor association between the *ABCB1* 1236C>T SNP and tacrolimus dose requirement was observed in a mixed cohort of 60 heart transplant recipients[311] in which 36 SNPs were investigated in relation to the efficacy, safety and pharmacokinetics of immunosuppressive drugs, including tacrolimus (n = 23). In this small study, it was shown that patients homozygous for the 1236C allele had a lower tacrolimus C_0/D than carriers of the 1236T variant allele, although this difference was not statistically significant.

Only one study in heart transplantation has evaluated the association between *POR*28* and tacrolimus pharmacokinetics.[310] In this study, associations between 7 SNPs and dose-adjusted tacrolimus C_0 at 1, 3, 6, and 12 months after heart transplantation were evaluated in 52 patients. Carriers of the *POR*28* variant had a higher dose-adjusted tacrolimus C_0 at all-time points but the differences were only significant at months 3 ($p = 0.025$) and 6 ($p = 0.047$) after transplantation. Interestingly, this *POR*28* effect was observed without consideration of the *CYP3A5*3* allelic status, whereas in other forms of solid organ transplant the defect caused by the *POR*28* allele was only apparent among CYP3A5 expressors.

Pharmacogenetics in lung transplantation

As expected, among lung transplant recipients, *CYP3A5*1* carriers require higher doses of tacrolimus to reach the target therapeutic range throughout the first post-transplant year, suggesting that doubling the dose in CYP3A5 expressors might also be an option in lung transplantation.[312] This was confirmed in a popPK study in which the tacrolimus clearance was found to be 40% higher compared with non-expressors.[94] With regard to the influence of *ABCB1* genotype, available data are limited but 2 studies suggested that *ABCB1* haplotypes associated with high ABCB1 pump function are characterized by a lower exposure to tacrolimus in adult lung transplant recipients. The magnitude and the variation over time post-transplant of the influence of *ABCB1* haplotypes on tacrolimus dose requirements were not definitely characterized in these two studies since the cohorts were relatively small and a combined analysis of all CYP3A5 and ABCB1 variants was not performed.[312, 313] No study on the association between *CYP3A4*22* and tacrolimus pharmacokinetics / dose requirement after lung transplantation has been reported.

Executive summary and practical recommendations

The association between *CYP3A5* genotype and tacrolimus dose requirements is consistent and has been observed among kidney, liver, heart and lung transplant recipients, both adult and pediatric. Patients expressing CYP3A5 require an at least 50% higher tacrolimus dose to reach the target therapeutic range compared with non-expressors (A I). Although there is evidence from a randomized-controlled clinical trial that basing the tacrolimus starting dose on an individual's *CYP3A5* genotype may facilitate tacrolimus dosing, this has not been a universal finding and there is currently no convincing clinical evidence that a pharmacogenetics-based approach to tacrolimus dosing improves clinical outcomes after solid organ transplantation. Of

the many other candidate SNPs that have been studied, *CYP3A4*22* appears to be the most promising as it explains residual variability in tacrolimus pharmacokinetics (B II, and C2 II, for Caucasians, Asiatic and African origin populations, respectively). We believe that future studies should investigate the clinical benefit of popPK models including *CYP3A5* and *CYP3A4* genotype (and possibly other genetic markers), to guide tacrolimus dosing.

PHARMACODYNAMIC BIOMARKERS FOR TACROLIMUS MONITORING

Pharmacodynamic biomarkers for the action of tacrolimus

Pharmacodynamic biomarkers can be either drug-specific or non-drug specific.[314] In the case of tacrolimus, drug-specific biomarkers are related to the signal transduction pathways and enzyme activities inhibited by the drug, whereas non-specific biomarkers reflect the inhibition of T-cell activation and proliferation in general, including cytokine production. Pharmacodynamic biomarkers can be determined either directly in whole blood, in whole blood stimulated with mitogens, antibodies, in donor leucocytes or third-party cells and in isolated lymphocytes, either quiescent or stimulated. Pharmacodynamic biomarkers used to monitor tacrolimus pharmacodynamic effects and activities are listed in **Table 5** and illustrated in Figure 3.

Drug specific pharmacodynamic biomarkers

Calcineurin phosphatase activity

Attempts to measure CaN activity to monitor the effect of tacrolimus have been pursued since very early after the release of the drug. One of the earliest approaches was to follow the dephosphorylation of a radioactively labeled peptide substrate by measuring the ^{32}P released.[315] To avoid radio isotopes, a phosphorylated peptide substrate R II has been synthesized which can be measured in its dephosphorylated state by HPLC.[316] However, it turned out that this approach

is less sensitive compared to the radiometric method and recently an LC-MS/MS-based method was published by Carr *et al.*[317] All methods to measure CaN activity using a synthetic substrate suffer from non-specificity because other intracellular phosphatases besides CaN also dephosphorylate the peptide. Therefore, this non-specific activity must either be measured separately or subtracted from the assay readout, or specific inhibitors and CaN activators must be added to the incubation mixtures to achieve a CaN specific result.[318] CaN activity is determined in peripheral blood mononuclear cells (PBMC) which requires cell isolation before the assay can be performed. Unfortunately, results from different assays are reported in method specific units, which make the comparison of data difficult. Several groups have measured CaN activity in patients after liver and kidney transplantation and, in general, observed an inverse relationship between CNI concentrations in whole blood and CaN activity in PBMC, but with a high between-individual variability.[319-321] A common observation with *in vitro* experiments is that, in contrast to the CNI cyclosporine, tacrolimus does not attain 100% inhibition of the enzyme even at very high concentrations.[317, 320, 321] In one study, the EC_{50} of tacrolimus was 26.4 ng/mL above the accepted upper limit of the therapeutic range (20 ng/mL).[320] In line with this observation, tacrolimus C_0 have been shown to be without effect on the CaN activity.[317] This questions the measurement of CaN activity as a pharmacodynamic marker of tacrolimus effect, particularly in blood samples which are drawn as trough samples immediately before the next dose. However, several groups observed an association between CaN activity and clinical events such as nephrotoxicity in liver transplant patients [320] or rejection in kidney graft recipients.[322] Surprisingly, CaN measured by HPLC did not correlate at all with intracellular tacrolimus concentrations in PBMCs.[232]

There are contradicting results about the appropriate time point for PBMC isolation after tacrolimus administration. Some authors observed better effects 2 hours after dosing, which reflects tacrolimus peak concentrations, whereas others noted a greater inhibition of enzyme activity after 4 hours.[316] An intrinsic drawback of all pharmacodynamic assays to measure enzyme activities in isolated PBMC is the fact that the drug is lost during the isolation and washing steps and that the cells are incubated in a non-physiological environment. Furthermore, CaN assays are not standardized and are, except for the LC-MS/MS method, barely validated in terms of their analytical performance.[317]

Nuclear translocation of NFAT by flow cytometry

A downstream event of CaN is the translocation of the dephosphorylated NFAT to the nucleus. NFAT is a family of transcription factors and NFAT has been reported to be particularly sensitive to inhibition by tacrolimus.[323] Intra-nuclear translocation of NFAT1 can be followed by flow cytometry image stream (AMNIS).[324, 325] For this purpose, whole blood is stimulated *ex vivo* with phorbol myristate acetate (PMA)/ionomycin for 30 minutes and the nuclear localization of NFAT1 is visualized in stimulated and non-stimulated $CD4^+$ and $CD8^+$ T cells using an anti-NFAT1 antibody and 4',6-Diamidino-2-Phenylindole (DAPI) staining.[325] Maguire *et al.* observed in a small study with 3 renal transplant patients an inverse correlation between tacrolimus concentrations and nuclear translocation of NFAT1.[325] The assay is time consuming, requires special equipment and fresh blood samples. Furthermore, it has yet to be thoroughly validated.

Using multi-parametric flow cytometry, Noceti *et al.* investigated the effect of tacrolimus along the CaN pathway in lymphocytes in healthy human participants (n=35)[326] and in patients on the waiting list for liver transplantation (n = 19).[281] As described by I/I_{max} models, increasing doses

of tacrolimus proportionally inhibit NFAT1 translocation as well as IL-2 and CD25 expression. Moreover, IL-2 and CD25 response to the inhibition of NFAT1 expression in PBMC nuclei follow allosteric sigmoidal models, suggesting tight signal translation along the CaN pathway.[326]

Between-individual variability was higher under non-stimulated than under stimulated conditions, as well as in the presence of tacrolimus.[327] IL-$2^{+}CD8^{+}$ cells at tacrolimus I_{max} showed the highest tacrolimus between-individual variability, suggesting its usefulness as a biomarker of individual effects integrating many different sources of regulation and variability. Multivariate statistical analysis showed the influence of pharmacogenetic polymorphisms on tacrolimus pharmacodynamics.[281, 326]

The aforementioned study also demonstrated the feasibility of using non-stimulated PBMCs to explore the CaN pathway under more physiologic conditions while integrating more variability than in the equivalent stimulated status.[281] The largest variability was observed at tacrolimus IC_{50}, which is in line with the large between-individual differences observed in clinical drug effects among patients. NFAT1 translocation might reflect the degree of individual immunological capacity, as Sommerer et al. and Zhan *et al*., reported a correlation between lower NFAT-regulated gene expression and increased frequency of infection episodes in transplant patients.[328, 329]

NFAT1 translocation to PBMC nuclei promises to be a suitable candidate biomarker to monitor tacrolimus pharmacodynamics after transplantation. However, further evidence in terms of potential associations of the extent of NFAT1 translocation with clinical outcomes is still needed.

NFAT regulated gene expression

As CNI trough or peak concentrations are only marginally associated with clinical outcome, if at all, several approaches to measure the biologic effects of CNI-based immunosuppression have been developed, including the assessment of CaN phosphatase activity, cytokine release and gene expression.[315, 330-336]

Quantitative analysis of gene expression has been established to calculate the functional effects of CaN inhibition, specifically the inhibition of the transcription of NFAT-regulated genes in peripheral blood.[315, 328, 337, 338] This assay is based on the quantitative analysis of IL-2, IFN-γ and GM-CSF gene expression in whole blood samples collected at cyclosporine / tacrolimus C_0, and peak concentrations (2 hours for cyclosporine and 1.5 hours for tacrolimus) after oral administration.

The real-time polymerase chain reaction (RT-PCR) technique provides a rapid, highly reproducible, and sensitive tool for the quantitative analysis of gene expression.[337] This method can be semi-automated and standardized and performed in specialized laboratories. Whole blood samples are stable for 24 hours at 20 °C. Although overall gene expression is reduced upon storage, the relative degree of NFAT inhibition remains stable during this period. NFAT-regulated gene expression has shown low analytical variability (less than 10%) in repeated measurements. Whereas between-patient variability is high, within-individual variability is low in patients on stable CNI doses and stable immunosuppressive co-medication.[336, 337]

This assay can be set up with satisfactory analytical performance in a routine molecular biological laboratory.[328, 339-341] Linearity, imprecision, and limit of quantification, as well as sample stability were investigated. A between-laboratory comparison showed comparable results.[339] The reproducibility of the NFAT-regulated gene expression assay across laboratories could facilitate the implementation of this assay for the pharmacodynamic routine monitoring of

CNI in different centers. Therefore, this monitoring technique could be used in larger patient cohorts and in multicenter clinical studies.

NFAT-regulated gene expression has been performed in solid organ transplantation such as kidney (adults and children), liver, heart, and lung transplant recipients.[318, 333, 339] Beneficial effects in the early post-transplant period were confirmed, as well as in the long-term follow-up after transplantation.[318, 340, 342] However, most of the evaluations included maintenance allograft recipients.[318, 328, 343-350] These results summarized data on acute rejection, opportunistic infections, malignancy (*e.g.* non-melanoma skin cancer), and cardiovascular risk or outcome. In observational, cross-sectional and prospective clinical trials, including one prospective case-control study, monitoring of residual NFAT-regulated gene expression has been proven as a tool to reduce cyclosporin A therapy in stable renal allograft recipients.[318, 333] In one prospective randomized controlled clinical study, the novel approach of monitoring residual NFAT-regulated gene expression led to a significantly reduced cardiovascular risk and improved allograft function in stable renal transplant recipients compared to the standard monitoring by cyclosporine trough concentrations.[351]

In a considerable proportion of patients treated with tacrolimus, the inhibition of NFAT-regulated gene expression is lower compared to cyclosporine treatment, possibly partially due to a low relative increase of tacrolimus concentrations from C_0 to C_{max}.[348] The lack of CaN inhibition in patients treated with tacrolimus has been described previously by direct quantification of the enzyme activity,[317, 320, 321] suggesting additional, as yet unidentified targets of the drug. Nevertheless, several studies on tacrolimus treatment showed that monitoring residual NFAT-regulated gene expression could identify allograft recipients at higher risk of infections or acute rejection.[340, 345, 348, 349]

NFAT-regulated gene expression is a promising biomarker in CNI therapy in regard to infectious complications, malignancies, acute rejection and cardiovascular risk. Within the last years, an assay which detects NFAT-regulated gene expression was introduced in clinical studies in several transplant centers in Europe and USA with encouraging results. The assessment of residual expression of NFAT-regulated genes is a minimally-invasive, rapid, robust, and reliable assay system, which has proven its validity and practicality in clinical and research settings. In patients treated with cyclosporine or tacrolimus, NFAT-regulated gene expression has the potential to develop into a monitoring tool complementing pharmacokinetics, especially in long-term renal allograft recipients.

Dephosphorylated proteins

The inhibitory effect of tacrolimus on the phosphorylation of intracellular signaling molecules can be measured by phospho-specific flow cytometry in T-cells and their subsets. There are scarce examples in which this approach has been used. One recent study by Kannegieter *et al.* followed the phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and Protein kinase B (AKT) in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells.[352] In non-stimulated whole blood samples, p38 MAPK and AKT were inhibited after kidney transplantation in $CD4^+$ and ERK in $CD8^+$ cells in patients treated with a tacrolimus-based immunosuppressive therapy. Stimulation of whole blood samples with PMA/ionomycin revealed lower phosphorylation of p38 MAPK and AKT in $CD4^+$ and $CD8^+$ cells compared to samples obtained before initiation of immunosuppressive therapy. Patients experiencing acute rejection episodes, but on therapy with belatacept, showed higher phosphorylation of ERK in both $CD4^+$ and $CD8^+$ cells compared to patients without rejection. Authors from the same group expanded monitoring of p38 MAPK, ERK, and AKT phosphorylation to $CD14^+$ monocytes in 20 kidney

transplant recipients receiving a tacrolimus-based immunosuppressive regimen. However, no association with clinical outcome or tacrolimus dose adjustments was reported.[353] A small conversion study of a twice-daily to a once-daily formulation of tacrolimus with 12 stable kidney graft recipients was accompanied by the monitoring of p38 MAPK phosphorylation. After conversion to the once daily formulation, a higher phosphorylation of 11.4% in $CD4^+$ T-cells and 15.6% in $CD8^+$ T-cells was observed despite comparable tacrolimus C_0. The authors considered phosphoflow as a sensitive approach to assess the pharmacodynamic effects of tacrolimus.[354] An advantage of phosphoflow cytometry is that no cell isolation is required. However, samples should be freshly analyzed and experience with the technique as well appropriate instruments are required, which currently precludes a wider application.

IL-2 mRNA expression and production in leukocytes

The terminal downstream effector of the CaN pathway is the cytokine IL-2, which can be followed as a pharmacodynamic read-out of tacrolimus action by studying its mRNA expression. Alternatively, the intracellular formation can be followed in T-cells by flow cytometry or the release from leukocytes into cell culture media.[355] In a study with 8 renal transplant patients treated with tacrolimus, mycophenolic acid and steroids, PBMC were isolated and stimulated with an anti-CD3 mouse monoclonal antibody.[355] The authors observed an inverse association between tacrolimus whole blood concentrations and IL-2 mRNA expression, the number of IL-2 producing $CD4^+$ T-cells and the extracellular IL-2 concentration.[355] Vadafari *et al.* showed an effect of the *ABCB1* genotype on intracellular IL-2 expression as a pharmacodynamic read-out of the tacrolimus effect on T-cells from kidney transplant recipients.[273] The *ABCB1 3435CT* SNP has been shown to affect transporter activity, whereby the *CC genotype* has been associated with

a higher activity compared to the *TT genotype*. In turn, this leads to lower tacrolimus concentrations inside the cells, which are associated with less IL-2 expression.

Drug non-specific pharmacodynamic biomarkers

ATP release from CD4$^+$ T-cells

T-cell activation can be assessed by a commercial assay (ImmuKnow, Viracor-IBT Laboratories, Lee's Summit, MO, USA) to follow ATP release from stimulated CD4$^+$ T-cells. The assay has been used to assess the pharmacodynamic effect of switching from a twice daily formulation of tacrolimus (Prograf) to a once daily formulation (Advagraf) in simultaneous pancreas-kidney graft recipients.[356] Unlike the effects observed in kidney transplant patients on p38 MAPK phosphorylation,[352] no significant effect was seen on the ATP production, suggesting an equal pharmacodynamic effect of both formulations.[356] Similar results have been reported before in living donor liver transplant recipients.[357] In a recent study involving liver transplant patients, the tacrolimus dose was individualized in 102 of 202 patients on the basis of the ImmuKnow assay results. Tacrolimus doses were either reduced or increased by 25% when ATP concentrations were <130 ng/mL (strong immune response) or >450 ng/ml (low immune response), respectively. Patient survival was higher, and the incidence of infections lower in the interventional arm compared to the 200 controls who were dosed according to trough blood level results only. Patients without adverse events had, in general, a lower tacrolimus dose and trough concentrations.[358]

T-cell proliferation and surface activation markers

The impact of tacrolimus on inhibition of T-cell proliferation was measured *ex vivo* by flow cytometric quantification of the proliferation cell nuclear antigen (PCNA) in peripheral blood of

heart transplant recipients.[359] Furthermore, in patients with chronic kidney disease, *ex vivo* peripheral blood analysis by flow cytometry showed inhibitory effects of tacrolimus on T-cell proliferation after labelling with carboxylfluorescein diacetate succinimidyl ester (CFSE).[360]

Regarding early T-cell activation markers, investigations involving renal transplant patients have demonstrated that CD40L and CD69 are suppressed during tacrolimus immunosuppression [330], which was even more evident after conversion from cyclosporine to tacrolimus.[361] In general, tacrolimus treatment suppresses T-cell markers (CD25, CD95, CD154), co-stimulatory molecules (CD28, ICOS) and adhesion molecules (CD54) in patients after solid organ transplantation (**Supplementary Table 1, http://links.lww.com/TDM/A321**).[330, 359, 361-364]

Tacrolimus immunosuppression also affects T-cell differentiation into distinct effector cell types like $CD4^+$, $CD8^+$ and Th17 cells. While the percentage of $CD4^+$ and $CD8^+$ T-cells has been reported to be reduced [361, 365, 366] without changing the $CD4^+/CD8^+$ T-cell ratio [367], the CD4 suppressor activity was enhanced.[361] The percentage of natural killer (NK) cells was not affected by tacrolimus treatment; however the NK cell proliferation, NK cytotoxicity and cytokine secretion in response to IL-2 were inhibited following tacrolimus treatment.[368] In addition, tacrolimus impaired IL-2 receptor and signal transducer and activator of transcription 3 (STAT3) signaling and induced a downregulation of NK receptors, which in turn induced proliferative and functional defects of NK-cells [368] resulting in an impaired innate immunity.[365]

Investigation of the effects of T-cell activation has its limitations. For example, the immunosuppressive effects of tacrolimus are rapidly lost once dosing is briefly interrupted,[366] and it is unknown to what extent tacrolimus-impaired T-cell function may be altered by infection or rejection of a transplanted organ.[359] Furthermore, the effects of tacrolimus, which has a 10- to 100-fold greater immunosuppressive potency than cyclosporine,[330] could be influenced by

genetic variations. For example, a strong association between tacrolimus pharmacodynamics and polymorphisms in the genes encoding cyclophilin A, CaN catalytic subunit and CD25 has been reported.[326] Moreover, the pharmaceutical formulation of tacrolimus may result in different concentration-time curves, which may impact T-cell activation. A study based on liver transplant patients suggested that the originator formulation of tacrolimus may affect regulatory T-cell (Tregs) differently than generic tacrolimus formulations.[368] Furthermore, switching between once and twice daily tacrolimus formulations may influence drug efficacy.[354, 369] The fact that T-cell proliferation and activation measured by CD25 and CD71 expression trended higher after conversion from mycophenolate mofetil to enteric coated mycophenolate sodium,[370] suggests that investigating the effects of tacrolimus on immune function also needs to consider immunosuppressive co-medication.

Intracellular cytokines

Based on its mechanism of action, the clinical utility of analyzing the expression of different cytokines in lymphocytes, with a notable role on T-cell response ($CD4^+$ and $CD8^+$ T-cells), has been evaluated to assess the individual effects of tacrolimus on the immune response after solid organ transplantation.[15, 371-373] Most studies published so far focused on the intracellular expression of IL-2 and IFN-γ, two cytokines that play a key role in the activation of the alloresponse.[15, 372, 374]

From an analytical point of view, two methodologies are mainly used: flow cytometry and the enzyme-linked immunosorbent spot (ELISPOT).

Several studies have focused on the utility of intracellular expression of IL-2 and IFNγ as prognostic biomarkers for the risk of acute rejection, as diagnostic biomarkers at the time of rejection and as markers reflecting the efficacy and the safety of tacrolimus. Until now, only a

few multicentre prospective trials have been reported. Millán *et al.*[375] evaluated the ability of these biomarkers to predict the risk of acute rejection in 142 transplant recipients (63 liver/79 kidney) recruited from four Spanish centers. Changes in the percentage expression of IL-2 in $CD8^+CD69^+$, IFN-γ in $CD4^+CD69^+$ and IFN-γ in $CD8^+CD69^+$ were evaluated using flow cytometry before transplantation and during one year after transplantation. The results demonstrated that, in those patients who suffered acute rejection, a significant increase of the intracellular expression of these cytokines was observed.

An ongoing randomized multicenter European study (Biodrim; Health F2-2012-305147) is currently evaluating the utility of IFN-γ and IL-2 ELISPOT assay during tacrolimus minimization therapy in order to stratify renal transplant patients into low and high responders. The CTOT-01, a prospective, multicenter, observational study designed to determine the diagnostic and prognostic utility of a panel of non-invasive biomarkers for transplant outcomes in renal recipients included IFN-γ ELISPOT analysis before and after transplantation. The same group recently evaluated the adverse outcomes of tacrolimus withdrawal in a cohort of non-sensitized primary renal transplant recipients. Analysis of donor-reactive IFN-γ ELISPOT correlated with the development of donor-specific antibodies (DSA) and/or acute humoral rejection in the tacrolimus withdrawal group.[376]

Results from Boleslawski *et al.*[377] showed that percentage of $CD3^+CD8^+IL\text{-}2^+$ expression (measured pre and post-transplantation) could be a surrogate marker to identify patients treated with tacrolimus or cyclosporine at high risk for acute rejection. In line with these results, Akoglu *et al.*[378] found that IL-2 production in $CD8^+$ T-cells correlated with Banff score (Spearman's rho = 0.81; p = 0.027) in adult liver transplant recipients treated with CNI. Ahmed *et al.*[379] demonstrated that the frequency of $CD8^+$ and $CD8^-$ cells that synthesized IL-2 and IFN-γ

correlated with the biologic effect of tacrolimus in kidney and liver transplant patients. In stable liver transplant recipients undergoing weaning from immunosuppressive therapy, Millan *et al.*[380, 381] identified the percentage of IFN-γ expressing $CD4^+$ and $CD8^+$ T-cells as surrogate markers for the risk of rejection in stable liver transplant recipients after withdrawal of long-term immunosuppressive treatment, using flow cytometry [380] and also in *de novo* adult liver transplant recipients receiving tacrolimus, mycophenolic acid and prednisone.[381] Furthermore, the latter study [381] demonstrated that the analysis of the degree of inhibition of IFN-γ and IL-2 expression in $CD8^+$ T-cells could be useful in identifying those patients with a high susceptibility to tacrolimus, avoiding unnecessarily high levels of immunosuppression in this group. Patients with an inhibition of these biomarkers lower than 40% during the 1st week post-transplantation developed acute rejection. Moreover the capacity to inhibit IFN-γ during the 1st week post-transplantation was related to the severity of acute rejection, in agreement with the results reported by Akoglu *et al.*[378] All together, these results suggest that measuring expression of IL-2 and IFN-γ in lymphocytes may reflect the individual response to tacrolimus and may be useful for dose adjustment. Recently, Noceti *et al.*[326] investigated the CaN/NFAT pathway in a new model of non-stimulated PBMC and its response to increasing tacrolimus concentrations. This group reported that intracellular IL-2 expression in $CD4^+$ T-cells correlated with tacrolimus concentrations and, in agreement with other groups, a large between-individual variability was found, especially for IL-2 producing $CD8^+$ cells.

Another intracellular cytokine that has been explored to evaluate tacrolimus effect on T-cell activation is IL-17. This proinflammatory cytokine is produced by the third subset of effector T-cells named Th17.[382] To date, only a few studies have assessed the effect of tacrolimus on Th17 responses. First, Abadja *et al.*[383] evaluated by ELISPOT how tacrolimus and mycophenolic acid

interfere with IL-17 production. The main finding of this study was that both drugs are capable of inhibiting Th17-related response. Mycophenolic acid seems to lead to an even stronger IL-17 suppression than tacrolimus. The combination of mycophenolic acid with low doses of tacrolimus tends to produce lower circulating IL-17 levels than after treatment with a conventional dose tacrolimus given alone. Thus, tacrolimus minimization strategies might help to better control Th17 immunity when mycophenolic acid is part of the immunosuppressive therapy. Second, in a prospective sequential study including renal allograft recipients receiving tacrolimus-based therapy, Chung *et al.*[384] showed that Th1 effector T-cells subsets were decreased after transplantation due to the effect of the immunosuppressive therapy, but not Th17 and IL-17-producing effector memory T-cells. One possible explanation for these discrepancies between both studies was the difference in PBMC sampling: in one study PBMC were sampled from patients exposed to tacrolimus for a long time and who showed a stable clinical course, while the second PBMC sampling was performed within 3 months after transplantation. More recently Merino *et al.*[385]demonstrated in an *in vitro* study that tacrolimus reduced the production of IL-2 in memory T-cells, whereas it completely inhibited naïve T-cells, but the production of IL-17 did not change significantly. Therefore, tacrolimus seems to be more effective in controlling alloreactive memory T-cells responsible for potential rejection episodes.

In summary, the measurement of intracellular cytokines, such as IFN-γ and IL-2, may reflect the status of T-cell response and the immunomodulatory effect of tacrolimus in each transplant patient, whereas the role of IL-17 requires further investigation. To advance in the process of validation of these biomarkers as early predictive biomarkers of the risk of rejection and graft clinical outcome it is crucial to improve some methodological aspects. Thus, commercial and common cellular reagent standards and panel of reactive T-cells (*e.g.* pool of donor antigens that

reflect the potential of organ donors) should be available to further standardize and harmonize these functional assays.

Donor-specific anti-HLA antibodies

With recent advances in the diagnostic armamentarium the understanding of the importance of anti-human leukocyte antigen (HLA) antibodies has increased.[386-388] The development of standardized highly sensitive solid-phase assays for detecting anti-HLA antibodies has significantly improved the clinical utility of this biomarker to predict antibody-mediated rejection (ABMR), and allograft injury in kidney transplant recipients. However, current methods have their limitations, and are only semiquantitative.[389] Today, donor-specific anti-HLA antibodies (DSA) and the subsequent development of ABMR are considered to be leading causes for graft loss. The negative impact of de novo DSA (dnDSA) on long-term outcome after kidney transplantation has been demonstrated in many studies[386-392] and it is estimated that 20-30% of kidney allograft recipients will develop dnDSA after transplantation. The detection of anti-HLA DSA is widely implemented in clinical practice as an important biomarker for the assessment of pre-and post-transplant risks of ABMR and allograft loss.[386] However, predictive capabilities of this biomarker alone are limited in the individual.[389, 390, 392] Outcome in patients with dnDSA is variable, with some patients rapidly developing acute ABMR, while others having an indolent clinical course for years.[386-392] It is thought that among other factors, differences between DSA with regard to the complement-fixing capability, IgG subclass, concentration, affinity, and avidity are r'esponsible for the observed differences in outcome.[386-389, 393] In addition, the presence of complement-fixing DSA may guide treatment[394] and scoring systems with additional clinical data improving its utility.[395] Although many questions on the predictive capabilities of

DSA are under investigation[396], there is consensus that dnDSA constitute an important non-invasive biomarker after renal transplantation and regular testing is recommended.[386, 388]

Despite the progress in diagnostics, there is an unmet medical need with respect to therapeutic approaches[396] and data on immunosuppression and in particular data on effects of tacrolimus exposure on DSA are scarce. For two decades, tacrolimus is an integral part in immunosuppressive protocols for immunologically high-risk patients and in desensitization protocols.[397] Despite lack of firm evidence, higher tacrolimus exposure is usually employed in high-risk patients despite potential overimmunosuppression and toxicity concerns. Several studies demonstrate, that poor adherence is an important risk factor for the development of dnDSA.[386-388, 398, 399] Poor adherence may lead to "drug holidays", to low and variable tacrolimus trough levels, which might result in high within-patient tacrolimus variability. Several retrospective studies demonstrate a negative impact on long-term outcome in patients with high within-patient tacrolimus variability[137, 400, 401] (see Pharmacokinetics Section). Similar to non-adherence, physician-guided CNIs withdrawal may result in the development of dnDSA.[376, 402] In this regard, it is interesting to note, that CNIs have no direct effect on B-cell proliferation and function, thus effective suppression of the T-cell interaction with B-cells seems important for the prevention of dnDSA.[403, 404]

So far only very few studies have investigated tacrolimus levels with regard to the development of DSA.[405, 406] Still, retrospective studies have to be interpreted with caution, as bias by indication may limit the conclusion: e.g. patients with low CNI levels may have been put on purpose on low exposure (e.g. due to CNIs toxicity, elderly marginal kidney); or low exposure is a consequence of non-adherence. Only one prospective study[406] of a steroid-free cohort treated with 1.2-1.4g/d mycophenolic acid reported that patients with a 50% lower tacrolimus dose

starting at month 4 after transplantation developed more dnDSA (tacrolimus levels at month 6: mean 4.1 vs. 6.7ng/ml). Obviously, more data are needed to address the important question.

Donor-specific antibodies (DSA) after heart transplantation are integral to the development of ABMR[407], which is, accompanied with graft failure, a major cause of mortality up to 40%[408] or even higher in terms of a late onset after heart transplantation.[409] Furthermore, DSA also evidently promote cardiac allograft vasculopathy.[410] ABMR rates of approximately 25-40% occur in desensitized patients after heart transplantation. Moreover, approximately 20-30% of non-sensitized heart transplant recipients develop dnDSA with associated ABMR in most cases. Although an ISHLT consensus report on the management of antibodies pre- and postoperatively in heart transplantation[411], well-designed studies in heart transplantation are scarce, and published results are mainly about retrospective single-center experience as published recently.[412] Thus, strategies of monitoring and treatment of DSA vary widely among heart transplant centers.

In general, DSAs develop as a result from inadequate immunosuppression in the long-term after heart transplantation or because of activation of established memory responses to allo-antigens in sensitized heart transplant recipients. Consequently, the first step after detection of DSA is to initiate or to intensify tacrolimus exposure as the CNI of choice,[413] because tacrolimus significantly decreases rejection compared to cyclosporine.[414]

In summary, in kidney and heart transplantation DSAs are a biomarker of under-immunosuppression, which may happen if patients are non-adherent but may also occur in both CNI-free and CNI-minimization clinical protocols. Most physicians agree that tacrolimus (with trough blood levels > 5ng/ml) is the CNI of choice in case of dnDSA, although no data from prospective controlled multicentre studies are available.

Graft-derived cell-free DNA

Quantification of donor-derived cell-free DNA (dd-cfDNA) in recipient blood or urine has been evaluated as a potential diagnostic biomarker for graft injury[415-417] and acute rejection.[415, 418] Results from Sigel *et al.* demonstrate that serial monitoring of urinary dd-cfDNA can be a sensitive surrogate biomarker of acute injury in the donor organ but lacks the specificity to distinguish between acute rejection and BK virus nephropathy injury.[419] More recently, plasma levels of dd-cfDNA, from 102 kidney transplant recipients, were correlated with allograft rejection and outcome. The results suggest that dd-cfDNA may be used to assess allograft rejection (T-cell mediated as well as antibody-mediated) and injury.[420]

In a study including 10 adult liver graft recipients Oellerich *et al.* investigated whether graft-derived cell-free DNA (GcfDNA or d-cfDNA) quantification could be used to assess the minimally effective trough concentrations of tacrolimus, they observed an increase in GcfDNA of more than 10% at a tacrolimus cut-off of 6.8 ng/mL.[421] The authors concluded that measuring GcfDNA could be useful to establish effective tacrolimus concentrations in liver transplant patients and to individualize immunosuppressive drug regimens. Later a prospective, observational multicentric study including 107 adult liver transplant recipients[422] showed that monitoring GcfDNA in plasma by droplet digital PCR (ddPCR) based on a limited number of predefined single nucleotide polymorphisms allowed for earlier and more sensitive discrimination of acute rejection as compared with conventional liver function tests.

Although dcfDNA seems to be a promising biomarker for monitoring graft health after transplantation,[423] multicenter, prospective, observational and interventional studies will be required to better define how it can be used and evaluate its clinical utility before considering it a valid biomarker in solid organ transplantation.

Executive summary and practical recommendations

- Determination of residual NFAT-regulated gene expression supports the identification of renal transplant recipients at higher risk of acute rejection, opportunistic infections, malignancy, and cardiovascular risk (B II).
- Monitoring residual NFAT-regulated gene expression complements CNI pharmacokinetics as an adjunct to guiding CNI therapy (B III).
- Monitoring intracellular T-cell IFN-γ production (particularly by the enzyme–linked immune-spot, ELISPOT, assay) before and early after transplantation can help to identify kidney and liver transplant recipients at high risk of acute rejection (B II) and select good candidates for immunosuppression minimization (B II).

Pharmacodynamic monitoring of tacrolimus therapy has not entered routine monitoring yet. To advance the validation of pharmacodynamic and immunologic biomarkers, it is crucial to improve and standardize some methodological aspects. The clinical implementation of these biomarkers as a complement of tacrolimus-TDM may have a tremendous impact in patient and graft care.

PHARMACOKINETIC/PHARMACOGENETIC MODELING AND PHARMACOKINETIC/ PHARMACODYNAMIC MODELING FOR TACROLIMUS

Population pharmacokinetic modeling of tacrolimus

The pharmacokinetics of tacrolimus have been described extensively in the literature using one- and two-compartmental disposition models with first order elimination in 61% and 39% of PopPK studies, respectively.[12] About a fifth of models incorporated a lag time to describe delayed drug absorption, while a few studies attempted to describe a more complex absorption

process using a gamma distribution to describe the absorption rate and then to convolute it with a single or multi-exponential impulse response.[424] The integral of this function represents an asymmetrical, S-shape absorption phase, the asymmetry of which increases when the absorption rate decreases, followed by drug distribution in one or two compartments and elimination from the central compartment. An Erlang model with a range of transit compartments has also been proposed, which represent a special case of the gamma distribution, in which the exponent is an integer that represents the number of transit compartments that the drug has to cross to reach the central compartment.[425] Most models have been based on oral pharmacokinetic curves of whole blood tacrolimus concentrations and most commonly tacrolimus whole blood apparent clearance (CL/F) was characterized.

Although the absolute bioavailability of tacrolimus has been reported to be on average 25-30%,[426] in four studies in which both intravenous and oral pharmacokinetic data for bid tacrolimus were available, typical bioavailability (F) was estimated and reported to range from 7.3 to 19.7%.[12]

Variability in tacrolimus whole blood apparent clearance amongst transplant recipients in these models was most commonly related to *CYP3A5* genotype (rs776746), patient hematocrit, patient weight, corticosteroid dose, postoperative day and a significant reduction in hepatic function (aspartate aminotransferase). Although co-medication is also an important determinant of tacrolimus disposition, most datasets did not contain information about the use of strong CYP3A inhibitors or inducers.[12]

Trough concentrations are used in most transplant centers for the TDM of tacrolimus. Although much easier to obtain and convenient for the patient, C_0 monitoring seems far from the ideal biomarker. Indeed, it has been shown that rejection and toxicity could occur even if the C_0 was

within the proposed therapeutic window.[10, 91] As explained previously in the Pharmacokinetic Section, there is some evidence that C_0 correlates poorly with AUC_{0-12}.[427, 428]

An alternative to single time point measurements or full concentration-time profiling to calculate AUC is the use of a population PK model in a Maximum A Posteriori (MAP) Bayesian forecasting technique to estimate AUC based on a limited number of measurements, generally taken in the first few hours of the dosing interval. MAP Bayesian forecasting with use of this population PK models can be a beneficial tool for accurate TDM.[11]

Bias in the prediction of tacrolimus AUC using PopPK models has ranged from 15 to 10% (assessed by mean prediction errors), imprecision ranging from 0.81 to 40% (measured as root mean squared error (RMSE) or mean absolute prediction error (MAPE)) and R^2 values ranged from 0.27 to 0.99. About two thirds of MAP Bayesian forecasting models that used two or more tacrolimus concentrations showed *bias* of 10% or less, but only 39% showed imprecision ≤ 10%.[12]

Currently five tacrolimus formulations have been described in the transplant population (intravenous, twice daily oral suspension, twice daily oral capsule, once daily oral capsule and once daily melting dose) all with slightly different models and optimal limited sampling models. The combination of sampling times at 0, 1 and 3 hours post dose consistently showed bias and imprecision values of less than 15% with one exception; the new prolonged tacrolimus melting dose formulation requires sampling times of 0, 8 and 12 hours after dose intake.[12, 429] This difference suggests that an alternative blood sampling procedure is required, such as dried blood spot monitoring, in order to make AUC monitoring feasible for this formulation.

Pharmacokinetic/Pharmacogenetic modeling

It is now well accepted that *CYP3A5*3* (rs776746) genetic profiling is informative to guide initial tacrolimus dosing.[258, 430] and several popPK models including *CYP3A5* genotype have been developed for tacrolimus, as summarized above. As outlined in the different previous sections, pharmacogenetics may influence a large part of the between-individual difference in the clearance of tacrolimus or its intracellular distribution. This influence might be quantified through popPK modeling strategies. There are several advantages of popPK modeling over other pharmacogenetic analyses: (i) the ability to quantify the effect of covariables of interest and, thereby, make quantitative assumptions about the magnitude of SNP effects on all pharmacokinetics; (ii) it facilitates routine dose adjustments using MAP-BE and (iii) it enables simulation studies, allowing dose recommendations.[11] This approach has given rise to numerous interesting studies but, except for *CYP3A* polymorphisms, no clear conclusions can be drawn at this stage, especially due to the ethnic diversity in allelic frequencies and the lack of large studies and/or unbiased data.

Multiple popPK models describing the pharmacokinetic effect of *CYP3A5*3* have been developed so far in different types of transplantation populations and, on average, they showed that introducing the *CYP3A5*3* genetic status of the patient in the model explains approximately 30% of the variability in the tacrolimus CL when using a parametric model approach.[28, 33, 94, 292, 301, 431-447] Statistical considerations demonstrated that it also decreased the Akaike information criteria (AIC) and improved the population and individual predictions in non- parametric models.[265, 447]

In addition, confirming results observed in simple candidate gene association studies, it seems that popPK models including *CYP3A4*22* (rs35599367) are more accurate than those not taking this SNP into consideration. However, this SNP seems relevant in Caucasians not expressing

CYP3A5 only as it has been demonstrated that *CYP3A4*22* alone does not significantly improve the performance of tacrolimus popPK models.[292, 441] In contrast, two recent studies have shown that including *CYP3A4*22* genotype information beyond *CYP3A5*3* for clustering the patients into poor (PM), intermediate (IM) and extensive (EM) metabolizers improved the predictability performance of such models.[265, 441] Indeed, almost 40% of the residual variability was explained by CYP3A clustering in a popPK study using a parametric approach [441] whereas this clustering strategy was associated with the highest reduction in -2 log-likelihood in a popPK study using a non-parametric approach.[265]

By performing dosage simulations with their popPK model, Andreu *et al.* defined that the highest percentage of patients with a C_0 within the target therapeutic range (5-10 ng/mL) occurred after 4, 3, and 2 mg every 12 hours for EMs, IMs, and PMs, respectively (for hematocrit fixed at 34%).[441] Consistently, Woillard *et al*. developed a double gamma absorption model using a non-parametric approach (Pmetrics) including *CYP3A4*22/CYP3A5*3* to refine the initial dose requirement of tacrolimus. Monte Carlo simulations were performed leading to the recommended starting doses of 0.07 mg/kg bid for poor metabolizers, 0.13 mg/kg bid for intermediate metabolizers and 0.2 mg/kg bid for extensive metabolizers. These recommendations have been approved recently and discussed and refined in a discriminant analysis of principal component. These proposed revisions are summarized in **Figure 4** and are in line with the CPIC or Pharmacogenetics National French Network recommendations.[430] The principal difference between the developed strategy based on popPK studies and CPIC recommendations consists of decreasing the dose for *CYP3A4*22* carriers-*CYP3A5* defectives, *i.e.* the so-called PM clusters, and to allow up to 0.4 mg/kg/day prescribed to the EM cluster. For these EM it is specifically proposed to start therapy at 0.35 mg/kg/day and to further fine-tune these doses using TDM.

Other SNPs of interest such as *POR*28* (rs1057868) or *CYP3A4*1G* (rs2242480), have also been investigated and some researchers have even tried to model their impact on tacrolimus pharmacokinetics variability using popPK models. However, in comparison to *CYP3A5*3* or *CYP3A4*22*, their effect on tacrolimus systemic exposure appears to be clinically non-significant, at least in the Caucasian population. In contrast, in the African population in which *CYP3A4*22* has not yet been described and *CYP3A5*1* carriers are the majority, other SNPs might still be important for explaining the residual pharmacokinetic variability. In this particular case, *POR*28* status might be of interest to individualize the tacrolimus dose among CYP3A5 expressors as the *POR*28* allele has been noted to influence tacrolimus pharmacokinetics but only in CYP3A5 expressors. To our knowledge, none of the popPK studies reported to date has explored that possibility in the African population. In addition, in Asian patients, in whom the number of CYP3A5 expressors is between those of Caucasians and Africans, it has been demonstrated that the combined genotype of CYP3A5-POR was the only covariant significantly related to the apparent clearance of tacrolimus. The situation is less clear for *CYP3A4*1G*, found exclusively in the Asian population. While this SNP has been associated with increased CYP3A4 activity and linked with tacrolimus pharmacokinetics in simple association studies in renal.[448] and hepatic transplantation,[449] this effect has not yet been observed in popPK studies,[450] limiting the relevance of these associations. Many other SNPs (*e.g. ABCB1, NR1I2, IL10, PPARa*) have also been investigated in popPK models, but mostly their inclusion failed to demonstrate any significant improvement of the model predictability and/or applicability. Concerning *ABCB1* SNPs, the popPK studies investigating the influence of *ABCB1* SNPs, such as the coding but synonymous 3435C>T or even the nonsynonymous 1199G>A, suggest a limited impact of these SNPs on tacrolimus blood exposure or bioavailability. Thus, it is generally accepted that *ABCB1*

genotype is not likely to have a clinical value when considering systemic pharmacokinetic. However, it seems that *ABCB1* variants (associated with a decrease in its transport) may explain differences in tacrolimus tissue distribution and might influence the effective fraction of the drug that is available to exert its immunosuppressive activity in lymphocytes[246, 272] or its toxic effect in the kidneys.[451-453] Unfortunately, none of the reported studies has used a popPK modeling approach to analyze those intracellular pharmacokinetic data. Hopefully, with the improvement of analytical techniques, this domain will be more extensively explored in the near future.

Pharmacokinetic/Pharmacodynamic modeling

The number of studies addressing tacrolimus pharmacokinetic-pharmacodynamic relationships using modeling approaches is still very few compared to those related with pure PK modeling. Similar to other therapeutic areas,[454, 455] modeling and simulation methods for immunosuppressive drugs in transplantation are crucial for the quantitation and prediction for new clinical scenarios. This has already been outlined in the last biomarker consensus document[15] but very few advances have occurred since then. Improved analytical techniques have enhanced our ability to measure various biomarkers that could be related to CNI use. The increasing knowledge of the underlying physiological mechanisms involved in CNI pharmacodynamics could allow the transition from an empirical to a quantitative framework. Development of pharmacokinetic-pharmacodynamic models could help to confirm the underlying physiological mechanisms and to facilitate the expansion and improvement of immunosuppressive treatments.

The **Supplementary Table 2, http://links.lww.com/TDM/A321** gives an overview of some of the most relevant studies that have attempted to use mathematical or statistical modeling approaches in transplantation. Some of the so-called pharmacokinetic-pharmacodynamic studies

found in the literature are based on correlations between pharmacodynamic-effects and drug exposure [456-458] (and additional references of interest that are shown in **Supplementary Table 2, http://links.lww.com/TDM/A321)**,[321, 340, 348, 355, 359, 375, 380, 381, 459-461] while others have considered the use of pharmacodynamic models, the most widely applied being the direct inhibitory Emax models.[319, 320, 322] None of these cases applied the indirect response models proposed by Jusko *et al.*[462] or disease progression models[463] to account for changes in disease unrelated to drug action. Most of the models were developed for descriptive purposes, without the evaluation of their predictive capability being reported. Lately, some new pharmacokinetic-pharmacodynamic models based on relationships between longitudinal measurements of drug exposure and clinical outcomes have appeared, but still efforts should be made to move forward from the current scenario to modeling discrete data.[464-466]

In conclusion, more pharmacokinetic-pharmacodynamic and PB/PK modeling activities are still required to enhance the understanding of factors influencing clinical outcomes in transplantation. In spite of possible inconveniences for routine clinical practice, optimal sampling study designs are essential to allow robust conclusions.

Executive summary and practical recommendations

-The utilization of popPK model based Bayesian estimators has shown improved target achievement compared to standard TDM.[467] While trough concentration is used in most transplant centers for TDM of tacrolimus there is some evidence that C_0 correlates poorly with AUC_{0-12}. The use of popPK model-derived Bayesian estimators based on limited sampling strategies (LSS), however, provides AUC predictions with bias <5% and an imprecision <20%. This seems to be an applicable way to improve future tacrolimus TDM as compared to continue

with standard trough concentration based TDM, especially when considering home sampling using microsampling devices that are currently under validation (as presented above).

-We strongly recommend the integration of *CYP3A5*3* and *CYP3A4*22* genotype information, when available, in future tacrolimus popPK models, primarily for the opportunity to optimize initial dosing.

-More pharmacokinetic-pharmacodynamic and PB/PK modeling activities are required to enhance the understanding of factors influencing clinical outcomes in transplantation.

CONCLUSIONS

In this Consensus Report we have assessed the evolution of best practice for the TDM of tacrolimus to allow for personalized treatment. We have taken into consideration the influence of standardized and harmonized analytical methods, as well as pharmacogenetic, pharmacodynamic and immunologic biomarkers, and their ability to act as early predictors of clinical events, such as rejection or drug-related adverse events.

The first Consensus Report on optimization of tacrolimus therapy[10] confirmed that the TDM of tacrolimus has a significant impact on patient management. However, it was concluded that there was the need to concentrate efforts on developing and establishing new standardized and harmonized analytical strategies to fine-tune the target concentrations to be achieved in adult and pediatric populations, considering risk factors, comedication drug-interactions, and validated biomarkers.

One decade later, the evolution of graft and patient clinical outcomes in transplantation remains insufficiently studied in the context of personalized immunosuppressive treatment early and long-term after transplantation. Tacrolimus, in its different formulations, remains and is

considered the cornerstone of immunosuppressive therapy. Tacrolimus, mycophenolic acid and steroids are the most commonly used treatment combinations in solid organ transplantation. However, our understanding of immunosuppression to achieve personalized therapies is still evolving.

For some time now, tacrolimus-related nephrotoxicity has been considered to be a major risk factor, and dose minimization was readily applied in an attempt to prevent kidney damage. Currently, the histologic graft lesions are also known to be attributed to other factors, one of the main causes being an actively maintained allo-immunity. Several studies have demonstrated that it is essential to ensure consistent tacrolimus exposure over-time and instead of aiming for very low tacrolimus trough concentrations clinicians have to put more emphasis on the prevention of under-immunosuppression and a lower limit of 4 ng/mL or greater is suggested on most indications. In the present Consensus Report, whenever possible, the recommendations for achieving specific target concentrations of tacrolimus are based on the results obtained from multicenter prospective clinical trials for different types of solid organ transplantation in specific cohorts of low or high immunological risk patients, also taking the combination with other immunosuppressive drugs into consideration. With the recent developments of novel sampling techniques and improved dosing methods (*e.g.* popPK model-based Bayesian estimators), future focus on finding optimal AUC target should be emphasized. Few studies have evaluated the optimal tacrolimus target concentrations in children undergoing different types of clinical transplantation or in adult and pediatric populations when tacrolimus has been administered for other clinical indications. So, there is still room to better determine and adjust the optimal tacrolimus target concentrations for each patient group.

When looking back on the last ten years of tacrolimus TDM, there has been a continuous and notable improvement of analytical assay performance. Several FDA approved and IVD-CE certified tacrolimus measurement systems have been introduced, but only two standardized calibrator materials are available. Tacrolimus assay inconsistencies may have a negative impact on clinical decisions, drug adjustment, and patient outcomes, demonstrating the need for traceability, the generation of standardized reference materials as well as the placement of appropriate reference procedures for tacrolimus monitoring.

When interpreting whole blood drug exposure (C_0 target concentrations, AUC ranges) and clinical trial outcomes, clinicians should keep in mind the analytical aspects (*i.e.* the assay used and the between-method bias) as well as pharmacogenetic characteristics associated with tacrolimus disposition and effects. Furthermore, it is well-known that immunosuppressive drugs modulate the immune system of each patient differently. As tacrolimus is a narrow therapeutic index drug, small variations in systemic exposure can lead to substantial differences in the pharmacodynamic response influencing graft and patient clinical outcomes. The combined knowledge of pharmacokinetics, with pharmacogenetics together with pharmacodynamic biomarkers could provide further prognostic and diagnostic information regarding the risk of rejection and condition of the allograft at earlier time points and allow anti-rejection therapy to be adjusted at an early stage before severe graft injury ensues. Accordingly, the recommendations of the Consensus Report regarding pharmacogenetics, pharmacodynamics and immunological biomarkers are summarized as follows:

- The association between *CYP3A5* genotype and tacrolimus dose requirement is robust and has been observed among both adult and pediatric kidney, liver, heart and lung transplant recipients (grading of recommendation A I), but currently there is no convincing clinical evidence that a

pharmacogenetics-based approach to tacrolimus dose selection improves graft and patient clinical outcomes after solid organ transplantation. *CYP3A4 *22* genotype is associated with residual variability in tacrolimus pharmacokinetics, but further studies should investigate the clinical benefit of some pharmacogenetic clusters in tacrolimus disposition and effect.

- Pharmacodynamic monitoring of tacrolimus therapy has not entered routine monitoring yet, but NFAT-regulated gene expression is a candidate biomarker for personal response to tacrolimus and the identification of renal and liver transplant recipients at high risk of rejection and infection (B II).

- Monitoring intracellular T-cell IFN-γ production, (particularly by the ELISPOT-assay before and early after transplantation, can help to identify kidney and liver transplant recipients at high risk of acute rejection (B II) and select good candidates for immunosuppression minimization (B II).

The authors believe that future studies should continue to investigate the clinical benefit of PopPK models including pharmacogenetic phenotypes, as well as pharmacokinetic /pharmacodynamic modeling and the potential application of such models into clinical routine, in order to facilitate personalized tacrolimus dosing.

In conclusion, the Expert Committee emphasized that it is reasonable to expect that routine monitoring of tacrolimus pharmacokinetics, combined with pharmacogenetics and predictive pharmacodynamic and immunologic biomarkers will modify and control risk factors, improve long-term outcomes post-transplant as well as graft and patient survival. There is a need to harness the information we have generated, re-evaluate how we monitor tacrolimus exposure/effect and graft outcomes by incorporating early predictive biomarkers into

multidisciplinary designed prospective robust clinical trials to support evidence for patient stratification and immunosuppression guidance, and achieve timely regulatory approval.

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Figure Legends

Figure 1. Data from the Analytical Services International (ASI) proficiency testing (PT) program showing:

(A): Between-laboratory and between-method variability of the bias of reported to target tacrolimus concentrations (4 ng/mL, 8 ng/mL and 12 ng/mL).

(B): Between-laboratory and between-method imprecision observed with whole blood samples spiked with tacrolimus to concentrations of 4 ng/mL, 8 ng/mL or 12 ng/mL.

Included are 5-6 separate distributions of whole blood samples spiked with tacrolimus and sent to the PT participants between 2014 and 2017. The methods compared include liquid chromatography-tandem mass spectrometry (LC-MS/MS, 171-200 participants), chemiluminescent microparticle immunoassay (CMIA, 131-160 participants), electrochemiluminescence immunoassay (ECLIA, 16-49 participants), antibody conjugated magnetic immunoassay (ACMIA, 28-34 participants), enzyme multiplied immunoassay

technique (EMIT2000, 10-27 participants), and quantitative microsphere system (QMS, 8-13 participants).

Figure 2. Data from the Analytical Services International (ASI) proficiency testing (PT) program collected between 2014 and 2017 and demonstrating the method-dependent between-laboratory bias (A) and imprecision (B) using pooled samples from transplant patients on therapy with tacrolimus. Included are 31 separate PT distributions with a median tacrolimus concentration (based on the results reported by the LC-MS/MS group) of 8.7 ng/mL (range: 7.5 – 16.5 ng/mL). The methods compared include liquid chromatography-tandem mass spectrometry (LC-MS/MS, 171-200 participants), chemiluminescent microparticle immunoassay (CMIA, 131-160 participants), electrochemiluminescence immunoassay (ECLIA, 14-49 participants), antibody conjugated magnetic immunoassay (ACMIA, 28-36 participants), enzyme multiplied immunoassay technique (EMIT2000, 10-27 participants), and quantitative microsphere system (QMS, 7-13 participants).

Figure 3. Pharmacodynamic targets of tacrolimus

PI3K=Phosphoinositide 3-kinase; PIP3=phosphatidylinositol (3,4) triphosphate; AKT=protein kinase B; IKK=IkB kinase; NFkB=nuclear factor kappa-loght-chain enhancer of activated B cells; ZAP70=zeta chain associated protein kinase 70); DAG=diacyl glycerol; RAS=rat sarcoma protein; RAC=GTPase; MEK=mitogen activated protein kinase kinase; MKK=dual specificity mitogen-activated protein kinase kinase; p38MAPK=p38 mitogen activated protein kinase 3; IP3=inositol-1,4,5-triphophate; NFAT=nuclear factor of activated T cells; C-RAF=RAF proto-

oncogene serine/threonine-ptotein kinase; ERK=extracellular signal-regulated kinase; FKBP12=FK binding protein 12; IFNy=inteferon gamma; GM-CSF= granulocyte macrophage colony-stimulating factor.

Figure 4. Recommendations for initial tacrolimus dose according to CYP-genotype.

Table 1. Grading system for recommendations and evidence level used in the consensus document

Category, grade	Definition
Strength of recommendation	
A	Good evidence to support a recommendation for: -Specific target concentrations -Biomarker (BM) monitoring
B	Moderate evidence to support a recommendation for: -Specific target concentrations -BM monitoring
C1	Regardless of poor evidence, recommendation for: -Specific target concentrations -BM monitoring
C2	Poor evidence to support a recommendation for: -Specific target concentrations -BM monitoring
Quality of evidence	
I	Evidence from ⩾1 properly randomized, controlled multicenter clinical trial using validated methodology
II	Evidence from ⩾1 well-designed cohort or case-controlled non-randomized clinical trial, multiple time series, standardized methodologies.

III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports from expert committees

- Grading Guide. UpToDate® - Wolters Kluwer Health [web site] 2015. Available at: http://www.uptodate.com/home/grading-guide. Accessed July 2015.
- Guyatt GH, Oxman AD, Vist GE, et al. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations.*BMJ*.2008;336:924

Table 2: Drug interactions with tacrolimus[41, 42]

Drug	Clinical Effect (Risk)
Aminogycosides Amphotericin B Cisplatin Cyclosporine Ibuprofen Kanamycin	Additive or synergistic nephrotoxicity
Antacids Carbamazepine Dexamethasone HIV antivirals Modafinil Phenobarbital Phenytoin Pioglitazone Rifabutin Rifampin Troglitazone	Reduce tacrolimus concentrations (Transplant rejection)
Bromocriptine Chloramphenicol Cimetidine Cisapride Clarithromycin Clotrimazole Cyclosporine Danazol Ditiazem Elbasvir Erythromycin	Increase tacrolimus concentrations (Toxicity

Esomeprazole Ethinylestradiol Fluconazole Grazeoprevir Itraconazole Ketoconazole Omeprazole Methylprednisolone Metoclopramide Miberfradil Nafazodone Nicardipine HIV Protease Inhibitors Theophylline Troleandomycin Verapamil Voriconazole	

Table 3: Minor allele frequencies (by ethnic group) for relevant tacrolimus biotransformation enzymes and transporters

	EUR	AFR	AMR	EAS	SAS
*CYP3A5*3, rs776746*	0.94	0.18	0.80	0.71	0.68
*CYP3A5*6, rs10264272*	<0.01	0.15	0.02	<0.01	<0.01
*CYP3A4*22, rs35599367*	0.05	<0.01	0.03	<0.01	<0.01
ABCB1 3435T, rs1045642	0.52	0.15	0.43	0.40	0.57
ABCB1 1199A, rs2229109	0.03	<0.01	0.02	<0.01	0.01
*POR*28, rs1057868*	0.30	0.17	0.28	0.37	0.35
PPAR, rs4253728	0.28	0.03	0.16	<0.01	0.10
PPAR, rs4823613	0.29	0.40	0.28	0.20	0.16

From 1000 Genomes Project data (http://www.internationalgenome.org/), all populations have been divided into 5 super populations according **EUR**, European; **AFR**, African; **AMR**, Ad Mixed American (Mexican, Puerto Ricans, Colombians and Peruvians); **EAS**, East Asian; **SAS**, South Asian

Table 4: CYP3A combined genotype classification according to [258]

	*CYP3A4*22* carriers (*CYP3A4*1/*22* or **22/*22*)	*CYP3A4*22* non carriers
*CYP3A5*1* non carriers or CYP3A5 non expressers (e.g. *CYP3A5*3/*3*)	CYP3A poor metabolizers **PM**	CYP3A intermediate metabolizers **IM**
*CYP3A5*1* carriers or CYP3A5 expressers (e.g. *CYP3A5*1/*3* or **1/*1*)	CYP3A intermediate metabolizers **IM***	CYP3A normal metabolizers **NM**

*This category of CYP3A is very rare according to the relative MAF of *CYP3A5*3* and *CYP3A4*22* in different ethnicities (see table 3)

Table 5. Pharmacodynamic biomarkers and assay platforms to assess the effect of tacrolimus

Biomarker	Assay
CaN activity	^{32}P release from a synthetic phosphorylated peptide
	Dephosphorylation of a synthetic peptide by HPLC
	Dephosphorylation of synthetic peptide by LC-MS/MS
Dephosphorylated proteins in signal transduction pathways	Phosphoflow cytometry
Nuclear translocation of NFAT	Flow cytometry
NFAT regulated gene expression	Real-time PCR
Intracellular cytokines and chemokines	Flow cytometry
Cytokine production by T-cells	ELISPOT
T-cell subsets (regulatory T-cells)	Flow cytometry, qPCR.
T-cell surface marker expression	Flow cytometry
T-cell proliferation	PCNA expression by qPCR, CSFE staining by flow cytometry
Graft derived cell-free DNA	Digital droplet PCR
ATP release from $CD4^+$ T-cells	Luminescence

ATP, adenosine triphosphate; CaN, calcineurin; CFSE, carboxylfluorescein diacetate succinimidyl ester; DNA, desoxyribonucleic acid; ELISPOT, enzyme-linked immunospot; HPLC, High-Performance Liquid Chromatography; NFAT, nuclear factor of activated T-cells; LC-MS/MS, Liquid Chromatography-Mass Spectrometry and Tandem Mass Spectrometry; PCNA, proliferating cell nuclear antigen; Real-time PCR, real-time quantitative polymerase chain reaction; qPCR, quantitative polymerase chain reaction.