Article Type: Review

Intra-Patient Variability of tacrolimus exposure in solid organ transplantation: a novel marker for clinical outcome

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Key words: Tacrolimus –solid organ transplantation – intra-patient variability – medication non-adherence – graft survival – allograft rejection - immunosuppression

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Funding: No funding was received for this work.

Conflict of Interest: The author declared no competing interests for this work.

Abstract

The calcineurin-inhibitor tacrolimus provides an acceptable balance between prevention of allograft rejection and drug-related adverse effects, making it the standard of care in all types of solid organ transplantation for the last two decades. Recent data have demonstrated that high intra-patient variability (IPV) in tacrolimus pre-dose trough concentrations has deleterious effects on allograft survival. The underlying mechanisms by which a high tacrolimus IPV shortens allograft survival are acute and chronic rejection, donor-specific anti-HLA antibodies and progressive fibrotic damage to the graft. Modifiable causes of high tacrolimus IPV include medication non-adherence, drug interactions, nutritional interferences and concurrent diseases. Recognizing high tacrolimus IPV as an important prognostic risk factor after solid organ transplantation requires understanding of the definitions, the use of correct diagnostic metrics and methodology. Therapeutic interventions aimed at reducing tacrolimus IPV are targeted on improving medication non-adherence, avoiding or adjusting drug interactions, drug dosing assists and educational support of recipients.

Introduction

In the last two decades the calcineurin-inhibitor (CNI) tacrolimus has progressively replaced cyclosporine as primary immunosuppressive drug in solid organ transplantation (1). It is characterized by a narrow therapeutic index necessitating concentration-controlled dosing in clinical practice. Target ranges of blood tacrolimus concentrations in different organ transplantations have evolved over the years based on results from large multicenter trials and increasing clinical experience (2). After more than 20 years of use, defining target tacrolimus trough concentrations that delineate development of drug-related toxicity and effective prevention of different types of alloimmune injury to the graft, remains a challenge (3,4). Especially as knowledge about key determinants of long-term graft functional status grows because of improved diagnostics (molecular, cellular, genetic, -omics), target ranges require repeated fine-tuning to accommodate for newly acquired insights. For example chronic rejection, chronic allograft nephropathy and CNI nephrotoxicity were clinical entities that dominated kidney transplantation outcome in 2000. Currently, (surrogate) kidney allograft outcome parameters include complex histological entities (e.g. microvascular injury, interstitial fibrosis and tubular atrophy, complement activation), type and intensity of donor-specific anti-HLA antibodies (DSA), different phenotypes of cellular and humoral allograft rejection and intra-graft and blood- and urine-derived transcriptomic signatures (5).

Therapeutic drug monitoring (TDM) of tacrolimus has transformed into a clinical instrument, not only to correct for between-patient variability and intra-patient variability (IPV) in exposure, but also to react to changes in allograft (e.g. subclinical rejection) and recipient (e.g. DSA) status (6). While changes in graft and patient status would ideally benefit from small adaptations in tacrolimus exposure targets, intra-patient variability of tacrolimus exposure hampers responsive dose fine-tuning within the respective narrow target concentration windows. In addition, high IPV has emerged as an important clinical risk factor that is associated with poorer (surrogate) outcome parameters in solid organ transplantation (7). In this short review the establishment of reliable metrics for IPV, the different determinants of IPV, the clinical implications of IPV and the therapeutic options to reduce IPV is discussed in an attempt to ameliorate long-term graft and patient survival rates in solid organ transplantation.

Tacrolimus intra-patient variability: definitions and timing

Intra-patient variability is defined as *fluctuations in tacrolimus blood concentrations in an individual patient over a certain time period in which the tacrolimus dose was not changed*. The coefficient of variation (CV) is most commonly used to quantify IPV. The coefficient of variation assesses the degree of variation, represented by the ratio of the standard deviation (σ) to the mean value (μ):

$$CV\% = (\sigma^*/\mu) \times 100$$

*σ is the square root of the variance σ^2

In any given data set, CV% can be estimated:

$$CV\% = \sqrt{\sum\{(Xi - X)^2/(n-1)/X\}}\ x\ 100$$

X is the average of all tacrolimus dose-corrected C_0 concentrations measured in time period *i*; X*i* is an individual C_0 dose-corrected concentration and n is the total number of available C_0 in time period i.

Several other formulas are available to calculate tacrolimus IPV but no clinically relevant differences between formulas have yet been demonstrated (7). The timing after transplantation during which tacrolimus C_0 concentrations and dosing information is collected is important. Arbitrarily, the first 3 months after transplantation are not considered an optimal period to assess tacrolimus IPV by most investigators. Delayed graft function, acute rejection episodes and anti-rejection treatment (e.g.

corticosteroids), anemia, gastrointestinal motility and concomitant medications can cause variation in tacrolimus exposure. Hospitalization episodes are also often characterized by stronger fluctuations in tacrolimus C_0 due to concurrent diseases, interventions and co-medications. Ideally, IPV is determined in "a stable clinical period" which often means when the patient is routinely attending outpatient follow-up clinics. Most studies have assessed tacrolimus IPV between 6-12 months as a compromise between a stable period postoperative period with a sufficient number of tacrolimus C_0 determinations available and still sufficiently early after transplantation to allow for diagnostic and therapeutic interventions when a high IPV is observed (8-12). Tacrolimus C_0 values during hospital admissions are not included in the calculation of CV%. Importantly, several studies have shown that CV is also affected by the number of C_0 values that are available for calculation with a higher CV% with increasing numbers (8-10). One would expect a more accurate (and hence lower) estimation of true tacrolimus C_0 CV% with increasing number of measurements available but in routine practice, more frequent C_0 measurements (even outside times of hospitalization) predominantly indicate the presence of clinical problems that interfere with tacrolimus exposure (e.g. diarrhea, drug-drug interaction). Therefore, as stated in the definition of IPV, the quality of exposure data will be superior when obtained "*in a time period in which the tacrolimus dose was not changed*".

While reported tacrolimus IPV estimates range from 5% to more than 60%, most studies observe an IPV between 15 and 35% (7). The FDA reported that "highly variable" (HV) drugs are defined as drugs in which the within-subject variability (defined as the %CV) in one or more of the bioequivalence measures is 30% or greater (13). In a multicenter registry analysis in more than 6600 patients who received a deceased donor kidney between 2000-2014 and had a functioning graft for >3 years, high Tac IPV at posttransplant years 1, 2, and 3 was associated with decreased graft survival (14). Compared to patients with an IPV <30%, the risk of graft loss increased by 32% in patients with an IPV of 30% to 44% and by 66% in patients with an IPV ≥ 45% ($P = 0.002$ and $P < 0.001$)(14). Recently, a first attempt was made to establish a specific baseline reference for IPV in stable renal and liver recipients on three different formulations of twice-daily tacrolimus (innovator and 2 generics)(15). Medication adherence was assessed in stable patients by electronic monitoring (Medication Event Monitoring System: MEMS, Aardex, Palo Alto, US), pill counts, daily patient diary and daily tacrolimus C_0 measurements using a combination of Dried Blood Spot (DBS) and venous blood sampling. By these measures the population was found to be adherent with a rate of 99.9% with a mean interval between evening and morning dose of 11.86 hours (15). The median weekly CV for all patients was 15.2%; 16.8% (IQR 12.3-23.8) for kidney recipients and 14.4% (IQR 11.0-19.9) for liver

recipients. Of the 287 weekly CV values, 29 (10.1%) were greater than 30% (occurring in 16 patients)(15). These results show that baseline CV of C_0 for twice-daily formulations of tacrolimus in stable kidney and liver recipients with stringently controlled medication adherence and absence of any known drug-drug interactions, averages around a median of 15%. This study also indirectly suggests, because of the observed differences in mean CV of tacrolimus C_0 compared with other studies, that medication non-adherence seems to be the single most important driver of high IPV, given the fact that other known causes of IPV (*see Tacrolimus IPV: causes*) were not apparently different from previous IPV studies in which no controlling for adherence was performed.

Tacrolimus intra-patient variability: causes

In a clinical setting solid organ transplant recipients are routinely informed and educated about potential causes of variability in tacrolimus exposure and how to prevent them. The clinical relevance of these potential sources of Tac IPV varies as well as the extent to which they are modifiable (Figure 1) For example, the timing of tacrolimus dosing in relation to food ingestion is part of most patients education schemes. Simultaneous or delayed dosing of tacrolimus with food reduces tacrolimus bioavailability (16,17) . Patients are also instructed to avoid combining tacrolimus dosing with certain types of food (e.g. high-fat meal, grapefruit)(18,19), what to do in case of vomiting after tacrolimus intake, to be careful with certain herbal (over-the-counter) preparations that interfere with tacrolimus disposition [e.g. St John's wort (*Hypericum perforatum*), *Schisandra sphenanthera* extract] and the potential risks of unsupervised generic substitution (20-22).

Clinicians treating transplant recipients should be aware of drug-drug interactions (DDI) that can interfere with tacrolimus absorption and elimination, predominantly by inhibiting or inducing CYP3A4 and CYP3A5 activity, and that can lead to increased IPV (23,24). Severe diarrhea, anemia and switching analytical quantification assays are other examples of potential causes of tacrolimus IPV in clinical practice that should be identified and reacted upon promptly (23,24).

Less well understood or documented causes of IPV are the circadian rhythms that alters tacrolimus disposition and the potential effects of drug formulations (see Tacolimus IPV: interventions) and pharmacogenetics on intra-patient variability (25). Studies proving a direct effect of the *CYP3A5* genotype on Tac IPV have not been reported. Considering that CYP3A5*1 expressers have about doubled Tac dose requirements compared to non-expressers (23), they should theoretically be more susceptible to higher IPV. Pashaee N *et al*. did not find any differences in the distribution of the

different *CYP3A5* genotypes among renal recipients with a low versus high IPV (≤15.6% *vs.* >15.6%) in Tac apparent oral clearance (26). This was confirmed by Spierings N *et al*. in a different patient cohort (27). Ro H *et al*. observed that a high Tac IPV (>17.9% median) was associated with acute kidney allograft rejection but only in patients expressing CYP3A5 while Tac IPV itself was not determined by the *CYP3A5*1* allele (28).

Finally, medication non-adherence (MNA) is probably the single most important cause of intra-patient variability and early diagnosis of MNA is paramount for remediating this clinical problem effectively from the start. Immunosuppressive medication non-adherence has a reported prevalence between 8% and 55% in kidney and 12% and 73% in liver allograft recipients respectively, depending on the applied diagnostic methodology (29). The use of combined diagnostic tools [e.g. a questionnaire, drug levels (including IPV)] to detect and quantify MNA is advised, preferably including electronic monitoring devices (29). The latter are still relatively costly but are the gold standard for both detection and quantification of MNA. In addition, interventional studies examining the effect of drug regimen simplification or educational/supportive measures on MNA rely on electronic monitoring devices as primary outcome readout systems (29). Recent studies have demonstrated that MNA and high IPV are often coinciding and associated with identical poor clinical outcome. Indeed, high Tac IPV and MNA have both been linked with (late) acute rejection, poor graft function, donor-specific antibodies, antibody-mediated rejection, transplant glomerulopathy, kidney fibrosis and graft loss (*see Tacrolimus IPV: consequences*). The recent findings of Leino A *et al*. also indirectly point toward a significant effect of strictly controlled drug adherence on tacrolimus IPV compared to other studies where drug intake was not supervised (15). Because real life data are lacking it is difficult to estimate the impact of a single missed Tac dose in a stable patient. Saint-Marcoux F *et al*. developed a validated model using a total of 145 full dose-interval (12-hour) PK profiles of immediate-release Tac (IR-Tac) obtained from 32 renal recipients to simulate steady-state Tac PK-profiles in different situations (30). Simulations were performed for estimating the quantitative effect of a single missed IR-Tac dose in patients with different degrees of drug clearance capacity (low, median and high, i.e. CYP3A5 expressers and non-expressers) and under conditions of a standard or reduced target Tac exposure (aiming at C_0 of 10 ng/mL *vs.* C_0 of 3 ng/mL)(30). They concluded that a single missed IR-Tac dose can greatly affect exposure resulting in up to 49% decrease in C_0 and 70% in $AUC_{0\text{-}12h}$ for recipients with the highest clearance (60 L/h in CYP3A5-expressing ultra-rapid metabolizers)(30). As expected, the clinical impact of a single missed dose was most pronounced when aiming at reduced target Tac exposure levels. Unfortunately, for prolonged-release formulations of Tac (PR-Tac) no data are currently available. It could be conceived that comparable effects would be observed with Advagraf™, the prolonged-release formulation of

tacrolimus, as they share a similar elimination half-life. Formal testing, either *in silico* or in a clinical study setting would be required to clarify this question.

Tacrolimus intra-patient variability: consequences

Kidney transplantation (see also Table 1)

The first study examining the potential effects of high IPV on outcome was performed in renal allograft recipients treated with the olive oil-based formulation of cyclosporine A (CsA, Sandimmune™) in combination with prednisone (31). Patients (n=204) were dosed to target concentrations based on serial AUC profiles. Over a follow-up period of 5 years, the incidence of histologically proven "chronic rejection" was 24% among recipients with less variable CsA exposure (CV% $C_0 \leq 36\%$) versus 40% in patients with high variability in CsA C_0 (CV% $C_0 > 36\%$). Mean CsA exposure indices (e.g. C_0, C_{max}) did not differ between patients with and without chronic rejection (31). The definition of chronic rejection in this study (based on the presence of obliterative vascular disease, with arterial and/or arteriolar endothelial and smooth muscle cell changes and glomerulopathy) does not completely fit any of the current Banff classification defined histological entities to describe allograft injury (32). Nevertheless, the clinical triggers for performing an indication biopsy in the study of Kahan B. *et al.* are still valid today (elevation of serum creatinine and/or proteinuria) and associated with increased graft loss. So irrespective of the exact underlying histological cause of allograft dysfunction, clinical surrogate markers indicating poor outcome were thus more frequently encountered in patients with a high CsA IPV. Similar observations were made with the micro-emulsion formulation of CsA with improved oral bioavailability: Neoral™. In a cohort of 103 recipients a CV cut-off for CsA C_0 of 20-24% [identified by Receiver Operator Curve (ROC-) analysis] predicted "chronic allograft nephropathy", defined as the presence of interstitial fibrosis and tubular atrophy without specific changes suggesting chronic rejection, in a biopsy obtained because of kidney graft function deterioration (33). Importantly, the first studies that examined CsA IPV were exploratory and hence not adhering to current methodological consensus as described above : CsA C_0 values collected over time spans of many years irrespective of the immediate postoperative phase, hospitalization episodes or other potential interfering circumstances were used for IPV calculation. Definitions of histological and clinical endpoints were different.

Borra *et al.* were the first to show that high IPV in tacrolimus clearance was associated with graft failure, defined as a composite endpoint of graft loss, histologically-proven chronic allograft nephropathy and doubling of serum creatinine (8). In a cohort of 297 kidney recipients treated with

tacolimus and mycophenolate mofetil (MMF) IPV was calculated based on C_0 values that were obtained at the outpatient clinic between 6 and 12 months after transplantation. After a mean follow-up of 1849 ± 585 days (starting from month 12), 11.4% (34/297) patients reached the composite endpoint (8). Patients were divided into a group with low Tac IPV (n=148) and a group with high Tac IPV (n=149) applying the mean CV for Tac C_0 of 17% (median CV% of 14.9%) as cut-off value (8). Mean Tac C_0 CV was 9.6% in the low IPV group versus 24.2% in the high IPV group. Significantly more patients with a high IPV (70.6%) reached the composite endpoint than patients with a low IPV (29.4%)(8). In contrast, in patients who did not reach the primary endpoint, there was no difference in the proportion of recipients with a high versus low Tac IPV (47.5% *vs.* 52.5%)(7). Importantly, mean Tac C_0 were not different between cases and controls. In multivariate analysis it was demonstrated that Tac IPV was an independent predictor of the composite endpoint, together with recipient age and acute rejection in the first year post-transplantation (8). Interestingly mean IPV of mycophenolic acid (MPA), calculated over the same period, was higher than that of Tac at 28.8% but was not associated with outcome (8). Shuker N *et al*. later confirmed these finding in a larger cohort of 808 kidney recipients treated with Tac and MMF (9). The composite endpoint was slightly different from their first study and included graft loss, late biopsy-proven rejection, transplant glomerulopathy and doubling of serum creatinine concentration. Again, in multivariate analysis, the incidence of the composite endpoint was significantly higher in patients with high Tac IPV than in patients with low Tac IPV [hazard ratio: 1.42 (95% CI: 1.06-1.90), p=0.019](9). Interestingly, the risk of reaching the composite endpoint with a high IPV was dependent on Tac C_0, with lower absolute trough level values conferring a higher risk (9). The increased susceptibility for the adverse consequences of high Tac IPV (e.g. acute rejection, graft loss) of patients kept on low target Tac concentrations was confirmed in two other large retrospective studies (n=376 and n=628 respectively; 11,12). Sapir-Pichhadze R *et al*. used the standard deviation of Tac trough levels starting at 1-year posttransplant (n=356) as estimate of IPV instead of calculated CV and found a 27% increase in the adjusted hazard of the composite endpoint (late rejection, transplant glomerulopathy and graft loss) for every 1-unit increase in Tac SD [HR: 1.27 (95% CI: 1.03-1.56)](34).

De novo donor-specific antibodies (dnDSA) and subsequent antibody-mediated allograft injury have directly and indirectly been linked to Tac variability and to Tac underexposure. In a Spanish study cohort of 310 DSA negative patients, Tac C_0 CV was calculated between 4 and 12 months after transplantation and dnDSA were prospectively monitored annually using single-antigen beads (35). A Tac IPV > 30% was independently related to the development of dnDSA [HR: 2.92 (95% CI: 1.47-5.80)] next to acute rejection in the first year [HR: 2.52 (95% CI: 1.26-5.05)] and re-transplantation

[HR: 2.17 (95% CI: 1.01-4.67)]. A Tac IPV > 30% was also associated with death-censored graft survival (35). Wiebe C *et al.* demonstrated that recipients with a high-risk HLA-DR/DQ eplet mismatch score (>11 mm) in combination with low (and/or declining) Tac C_0 (< 5 ng/mL) had a higher risk of developing dnDSA compared to patients with similar HLA-DR/DQ eplet mm score and higher time-related Tac exposure (6). These findings suggest a close interrelationship between Tac IPV, low Tac exposure, high immunological risk (or "susceptibility") and development of T-cell-mediated and/or antibody-mediated allograft injury. In contrast, Sablik KA *et al*. found no differences in mean Tac IPV (measured over 3 preceding years) between patients with a biopsy-proven diagnosis of chronic active antibody-mediated rejection (c-aABMR, n=59)) and matched controls (n=189)(36). However, in c-aABMR cases, a high IPV after the diagnosis was associated with inferior graft survival. Tac C_0 levels declined significantly over the 3 years prior to the diagnosis of c-aABMR but not in the control group which is in accordance with the observation by Wiebe C *et al* (36, 6). The lack of pre-transplantation and sequential post-transplantation DSA measurements and the lack of complete histological data for the matched controls, prevents any definite conclusions about the association (or lack thereof) between Tac IPV and the specific clinical phenotype of c-aABMR. For example, patients with a pre-existing unfavorable (allo-)immunological phenotype that cannot be reliably identified by panel-reactive antibodies alone, might be more susceptible to the adverse effects of a comparable degree of (high) Tac IPV and develop more severe or earlier clinical signs of antibody-mediated injury.

Other studies have found that MNA is an important risk factor for both (late) T-cell-mediated and antibody-mediated graft injury and can potentially play a central role in high Tac IPV-related adverse outcome (37,38). Given the dramatic effect a single missed IR-Tac dose can have on C_0 and $AUC_{0\text{-}12h}$, especially in fast Tac metabolizers (30), the association between MNA, Tac under-exposure and alloimmune activation becomes clinically recognizable despite the fact that a formal causal relationship has not yet been proven. A recent retrospective analysis including 628 kidney recipients with a mean number of 8.9 ± 3.8 tacrolimus levels taken between 6 and 12 months post-transplantation and a mean follow-up of 4.7 ± 2.1 years showed that graft loss was associated with the highest IPV group [2.51 (95%CI: 1.01-6.27), p= 0.048], mean tacrolimus level less than 5 ng/mL [4.32 (95%CI: 1.94-9.63), p= 0.0003], acute rejection [9.83 (95%CI: 4.62-20.94), p< 0.0001] and a high clinic nonattendance rate [1.10 (95%CI:1.01-1.20), p= 0.03](12). Independent risk factors for acute rejection were *de novo* DSA [3.15 (95%CI: 1.84-5.39), p< 0.0001], mean tacrolimus level less than 5 ng/mL [2.57 (95%CI: 1.27-5.19), p= 0.008], and again a high clinic nonattendance rate [1.11 (95%CI: 1.05-1.18), p= 0.0005](12). So for specific subgroups of patients the combination of MNA and high

Tac IPV against a high risk immunological background could lead to cellular or humoral immune activation and graft injury. However the relationship between MNA and IPV is more complex than (temporary or prolonged) Tac underexposure as most studies investigating IPV have clearly found that mean/median Tac concentrations do not differ between patients with high versus low IPV (8-12). The latter observations indicate that patients with MNA and high IPV would, at least to a certain extent, compensate missed doses of Tac with delayed ingestion or increased subsequent dosing. This would explain why mean/median Tac concentration do not seem to be affected by IPV in most studies. Indeed, Goodall DL *et al.* found that in the patients with the highest IPV, the maximum absolute Tac level registered was 33.2 ng/mL (and the minimum 1.0 ng/mL) while in patients with the lowest IPV the maximum absolute Tac exposure observed was only 15.2 ng/mL (minimum 2.2 ng/mL) while mean/median Tac concentrations did not differ between groups (12).

Apart from the underlying immunological risk, other characteristics of both the donor organ and the recipient will determine the consequences of high IPV on the graft. For example regarding ethnicity, Taber DJ *et al*. showed that overall Tac trough IPV was, as expected, higher in African-American (AA, n=768) than in non-AA (n=643) patients (39.9 ± 19.8 % *vs* 34.8 ± 15.8% p< 0.001)(39). And while a 10% increase in tacrolimus IPV increased the risk of acute rejection by 20% [adjusted HR: 1.20 (95% CI: 1.13-1.28), p< 0.001] and the risk of graft loss by 30% [adjusted HR: 1.30 (95% CI: 1.23-1.37), p< 0.001], these observations were modified by race (39). African American recipients with a high IPV were at risk for both acute rejection and graft loss while non-AA with similar high IPV only experienced more graft loss as a result of increasing IPV, not more acute rejection episodes (39). Recipient factors could play a role in these differences in outcome. AA recipients are known to have characteristics associated with increased immunogenicity while they have a lower propensity to develop interstitial fibrosis and tubular atrophy (40). So it could be possible that a high Tac IPV would predominantly lead to rejection in one (pheno)type of patient triggered by episodes of underexposure while another patient would develop mainly fibrotic changes in the graft due to (repeated) episodes of overexposure. A recent study comparing a smaller number of AA-patients (n=246) with European-Americans (EA, n=1226) could find a relationship between Tac C_0 IPV, albeit measured in the initial 6 months after transplantation, and graft failure but not with acute rejection (41). Interestingly, in EA recipients Tac trough IPV decreased by 1.82% (95CI: -3.06 to -0.57; p=0.0042) per additional *CYP3A5* loss-of-function allele (**3, *6 or *7*) but not in AA, again illustrating the interplay between ethnicity, genetics and the influence of study methodology (41).

We calculated Tac IPV using C_0 CV from months 6-12 after transplantation in a cohort of 220 renal recipients with paired protocol (surveillance) renal allograft biopsies available at 3 months and 2 year post-transplantation (10). Recipients in the highest IPV tertile (CV% >22.1% with a mean CV% of 31.1 ± 7.8) had an increased risk of developing moderate to severe interstitial fibrosis and tubular atrophy by 2 years [OR: 2.47 (95% CI: 1.09-5.60), p= 0.031; and OR: 2.40 (95% CI: 1.03-5.60), p= 0.043, respectively] compared with the low-IPV tertile (CV% < 14.4%)(10). Because patients with early signs of alloimmune activation were potentially more susceptible for the effects of high Tac IPV, a possible interaction between IPV and subclinical borderline/acute rejection changes in the 3 months biopsy, in the prediction of moderate or severe fibrosis or tubular atrophy at 2 years was excluded (10). Similarly, Tac IPV tertiles were not related to any individual nor composite histologic inflammation score at 2 years. These observations imply that differences in inflammatory lesions present in 3 and 24 months biopsies could not explain the progressive fibrosis occurring significantly more frequently in the highest Tac IPV tertile. Interestingly, Tac IPV was an independent predictor of *de novo* arteriolar hyalinosis [OR: 2.29 (95% CI: 1.17-4.47); p=0.015] which can lead to reduced arteriolar blood flow, glomerular ischemia and fibrosis, and is a non-specific but characteristic histological sign seen in calcineurin-inhibitor nephrotoxicity (10).

In summary, patients with high Tac IPV, irrespective of its cause, are more frequently exposed to episodes of sub- and supra-therapeutic (or "off-target") drug concentrations than recipients with low IPV. A high immunological risk recipient will, depending on the applied target Tac trough concentrations, be prone to experience consequences of alloimmune activation (e.g. acute rejection, DSA, …) in case of high Tac IPV. Low immunological risk patients with high Tac IPV and in whom low target Tac exposures are avoided, will potentially be more at risk of developing non-inflammatory injuries through activation of pro-fibrotic pathways, reduced allograft perfusion and ischemia (e.g. interstitial fibrosis, *de novo* arteriolar hyalinization, …) (Figure 2). Complexity is added through incompletely understood recipient characteristics [e.g. African-American ethnicity, (pharmaco-) genetics], donor organ quality (e.g. donor age, *APOL1* genetic variants, ischemia reperfusion injury), concomitant immunosuppressive drug exposure (e.g. MMF) and the degree and pattern of MNA. A positive observation is that the majority of recent studies on Tac IPV in kidney transplantation (Table 1) employ the same methodology for obtaining reliable IPV metrics and make use of established clinical outcome parameters (e.g. acute rejection) or consensus surrogate markers (e.g. DSA). Further challenges include improved standardization of Tac IPV determination, harmonization of testing for clinical co-variables that also affect outcome, better detection and quantification of MNA and the inclusion of automated (tele)monitoring of Tac C_0 IPV for interventional trials. In Table 2

recommendations for the development of a consensus standardized determination of Tac IPV in solid organ transplantation is summarized.

Non-renal transplantation

Data on Tac IPV in non-renal solid organ transplantation are sparse and different in terms of outcome parameters compared to kidney transplantation.

In a cohort of 326 adult liver transplant patients there was no difference in the primary composite endpoint between recipients with high Tac IPV (median CV 37.7%, n=164) and low IPV (median CV 20.1%, n=162) using Kaplan-Meier survival estimates (24.4% versus 18.5%, p=0.068)(42). IPV was calculated based on at least five Tac C_0 collected between 6-18 months postoperative and median CV% was 28%. The composite endpoint consisted of chronic rejection, biopsy-proven late acute rejection and suspected late acute rejection. Interestingly, a higher Tac IPV (and not high absolute trough concentrations) in combination with a low native kidney function at baseline (eGFR < 40 mL/min) was associated with a greater subsequent annual loss of renal function during follow-up (p=0.007)(42). The latter observation is even more intriguing as it demonstrates that high Tac IPV could not have led to progressive native kidney function loss through immune-mediated injury but rather by processes involved in drug renal toxicity. Other studies in adult liver recipients did show an association between high Tac IPV (or high SD) and acute rejection, poor graft survival or dnDSA despite some methodological limitations in the analysis (43-45). For example, in one study a low number of Tac C_0 samplings over a time period of 3 years was used to calculate IPV in part of the study subjects; in another study Tac C_0 CV was calculated between postoperative day 8 and 30; and in a third study the absolute number of included patients was relatively low (43-45). It is clear that using very sparsely timed Tac exposure measurements or performing IPV calculation during the early postoperative phase, are not ideal for comparison. Also in pediatric liver transplantation an association between a high Tac IPV [or in this study a high calculated Medication Level Variability Index (MLVI), (n=379)] in the first year after liver grafting and subsequent late acute rejection in the second year was observed (46). Tac IPV studies in liver transplantation are affected by the type of (composite) endpoint that is chosen by the investigators and are often more difficult to define than for kidney transplantation. Additional confounders in Tac IPV studies in hepatic transplantation are the facts that liver grafts have lower alloreactivity, generate less DSA, tolerate lower targets of immunosuppressive drugs or even complete Tac withdrawal in selected cases and are actively

involved in Tac disposition with complex time-dependent (and partly pharmacogenetically driven) interactions between the intestinal absorptive barrier (recipient-determined) and hepatic elimination (donor determined)(47-50). The overall lower alloimmune reactivity of hepatic grafts compared to kidney allografts could further attenuate potential adverse effects of MNA in liver recipients (29). Current evidence suggests that Tac IPV is a less stronger surrogate for outcome in adult liver transplantation compared to kidney transplantation. Liver grafts seem to "tolerate" higher Tac IPV and the clinical outcome parameters in liver transplantation are not only less prevalent but also not (yet) optimally characterized (e.g. DSA).

In thoracic organ transplantations few data on Tac IPV are available. Shuker N. *et al*. found no difference in the proportion of patients with high Tac IPV in the group that progressed to higher grades of cardiac allograft vasculopathy (n= 15) after 4-year follow-up versus the group without progression (n = 71; 60.0% vs 47.9%; p= 0.57)(51). No difference in the proportion of patients with high Tac IPV between patients with acute cellular rejection episodes (n=58) and without rejection (n=28; 51.7% vs 46.4%; P = .82) was observed(51). Gueta I. *et al*. collected Tac C_0 measurements between 3 and 12 months after cardiac transplantation and divided patients into high (median >28.8%) and low Tac IPV (<28.8%) groups (52). Mean tacrolimus levels did not differ between the groups (12.7 ± 3.4 ng/mL vs 12.8 ± 2.4 ng/mL, p= 0.93). Patients in the high IPV group exhibited higher late (>1 year) rejection rates (median total rejection score: 0.33 vs 0, p= .04) with no difference in rejection scores within the first year after cardiac grafting (52). Multivariate analysis showed that high Tac IPV was associated with >8-fold increased risk for rejection beyond the first year post-transplantation (p= 0.01) (52). One study in lung transplantation in 110 recipients confirmed that a high standard deviation of tacrolimus C_0 between 6 and 12 months independently increased the risk of chronic lung allograft dysfunction (CLAD) at 24 months [HR, 1.46; (95% CI: 1.23-1.73); p< 0.001] and death [HR, 1.27; (95% CI: 1.08-1.51); p= 0.005](53). A high mean tacrolimus level 6 to 12 months post-transplant independently reduced the risk of CLAD [HR: 0.74; (95%CI: 0.63-0.86); p< 0.001] but not death [HR: 0.96; (95% CI: 0.83-1.12); p= 0.65](53). In a cross-sectional study of 292 adult lung allograft recipients the Tac "time-in therapeutic range" (TTR) was calculated during the first year. An increase in TTR of 10% was associated with a significantly lower likelihood of acute rejection at 1 year (OR: 0.64, 95%CI: 0.47-0.86, p=0.003) and lower rates of CLAD (P < .001) and mortality (P < .001)(54). These observations suggest that in thoracic organ transplantation defining relevant (composite) endpoints in relation to Tac IPV is still under debate. Nevertheless high intra-patient variability in Tac exposure does seem to affect acute and chronic heart and lung allograft status despite the general use of overall strong immunosuppressive regimens.

Tacrolimus intra-patient variability: interventions

In clinical care solid organ recipients are routinely educated about circumstances and conditions that can affect Tac exposure and IPV. Patients are instructed about the timing of Tac dosing in relation to food intake, the potential interactions with particular food or herbal constituents, prescription and over-the-counter drugs and the risks of uncontrolled generic substitution (29). In addition, advice is provided by the health care team what to do in case of a missed dose or vomiting after Tac intake. After a missed dose it is recommended, based on model simulations, to take a subsequently timed dose of 1.5 times the usual dose in order to restore steady-state target ranges (30). In case of a delay in dosing it seems acceptable to take a 100% Tac dose as long as the time delay is less than 4 hours (30). Physicians should stay alert for drug-drug interactions when altering medication prescriptions and for interference from gastrointestinal illnesses. Generic substitutions should be limited and executed in a controlled setting. Tac analytical assays can differ between laboratories and change over time (29).

An important question that remains unanswered for the moment is whether switching tacrolimus formulations can intrinsically alter (i.e. improve) Tac IPV. Only one controlled PK study compared Tac IPV before and after switching from IR-Tac to PR-Tac in 40 stable renal transplant recipients (55). Five weekly Tac $AUC_{0\text{-}24h}$ profiles were measured prior to a 1:1 (mg/mg) conversion from BID Tac to QD Tac, followed by another 5 weekly $AUC_{0\text{-}24h}$ profiles on PR-Tac. Mean Tac IPV of C_0 did not change after switch to PR-Tac (15.3% *versus* 13.7%, p=0.21); mean Tac IPV of $AUC_{0\text{-}24h}$ did significantly decrease with the prolonged-release formulation of Tac (14.1% *versus* 10.9%, p<0.012)(55). This unique study demonstrated that given the strictly controlled conditions, the improvement in Tac $AUC_{0\text{-}24h}$ IPV with the PR-formulation was limited, unlikely to be clinically relevant and not observable in Tac C_0. Secondly, this study confirmed a "baseline" Tac C_0 IPV for the immediate release formulation of around 15%, confirming the findings of Leino AD et al (15). These PK observations also suggest that additional improvements in Tac C_0 IPV after switching from IR-Tac to PR-Tac formulation, as noticed in some but not all of the recent single-center clinical trials, are predominantly the result of improvement in MNA rather than caused by intrinsic pharmaceutical characteristics of the extended-release formulation (56-58). Indeed, experimental data in healthy volunteers demonstrate that (using a Tac in polyethylene glycol 400 solution) tacrolimus exposure ($AUC_{0\text{-}12h}$) is not significantly affected by its intestinal site of absorption (stomach, proximal and distal small bowel and ascending colon)(59). So clinical studies that did observe an improvement in Tac IPV (or even improvement in clinical outcome) after changing from IR-Tac to a PR-Tac formulation, are,

at least in part, biased by increased adherence due to intrinsic study circumstances and simplification of the drug dosing regimen. We have clearly demonstrated in a prospective randomized controlled study in 219 kidney recipients that switching from IR-Tac to PR-Tac formulation is associated with improved medication adherence measured by MEMS (60). The once-daily LCP-Tacrolimus formulation (LCP-Tac, Envarsus™ XR) has a higher bioavailability (MeltDose™ drug delivery technology, Veloxis Pharmaceuticals) than IR-Tac and PR-Tac (30 to 36% lower dose requirements respectively) but no comparative data are available on LCP-Tac IPV (61).

Although direct causal evidence is lacking, (unintentional) medication non-adherence (MNA) is the single most important clinical cause of a high Tac IPV. MNA can take on different clinical phenotypes ranging from complete discontinuation of immunosuppressive medication, drug holidays, occasionally missed doses to variable dose timing, dosing errors and changing dosing conditions (e.g. food intake)(29). Clinicians should be alerted for the presence of MNA in case a high Tac IPV is detected and all other (modifiable) causes of increased IPV (Figure 1) have been excluded. Computerized (online) reporting systems that alert health care professionals in case of high Tac IPV can help to trigger interventions aimed at reducing Tac intra-patient variability, as was recently demonstrated in Taiwanese transplant patients (62). MNA diagnosis, identifying the barriers to medication adherence and subsequently providing help to patients with drug regimen simplification in combination with educational and support measures, requires a multi-disciplinary team effort (e.g. doctors, nurses, pharmacists) (Figure 3). These therapeutic strategies for dealing with MNA in solid organ transplantation have been described extensively by others and are outside the scope of this review (29, 63,64). To demonstrate a direct benefit of interventions that improve MNA on outcome after solid organ transplantation remains an important challenge (65).

An interesting question is whether using a computerized dosing aid in clinical practice would result in lower Tac IPV and better long-term outcome after transplantation. Computer-assisted Tac dosing has proven to be superior to concentration-controlled dose adjustments in achieving target Tac C_0 concentration ranges early after transplantation. In a randomized-controlled prospective study Størset E et al. used a software program that integrated updated patient-specific characteristics (fat-free mass, hematocrit, time after transplantation, Tac dosing history and previous Tac C_0 measurements) with information obtained from a Tac population PK model (66). The software evaluated a range of doses and provided the dosing regimen with the highest probability of achieving the middle value of the target concentration range for that patient. The proportion of Tac

C_0 per patient within the preset target range was significantly higher with computerized dosing than with conventional dosing in the first 8 weeks post-transplantation (66). In high risk patients (targeted at higher Tac C_0 ranges) the CV% of Tac C_0 was 22% in the computerized dosing group versus 31% in the control group (66). The input from nonparametric functional regression models could be used to predict and estimate the variance in the complex relationships between Tac C_0 and Tac dose for further fine-tuning of computer-assisted dose recommendations (67). Whether computerized dosing assistance can reduce Tac IPV in stable steady-state maintenance recipients is more difficult to demonstrate. In adherent stable patients "baseline" Tac IPV is estimated around 15% which implies that even in case of a hypothetical -50% IPV reduction, further dosing regimen adjustments with the currently commercially available tacrolimus doses, would be challenging (15,55). Performing more frequent Tac C_0 measurements by patients in a home setting, for example by using a volumetric absorptive capillary microsampling (VAMS) technique (68), could provide more insights in the day-to-day fluctuations in Tac IPV. In addition, as shown be Stifft F *et al*., significant (small) differences in Tac AUC IPV (in this case between formulations) were not detected in the corresponding trough levels, illustrating the potential importance of obtaining more reliable Tac exposure data; for example by using limited sampling strategies (LSS) in combination with VAMS. However, it would remain very difficult to show a clinical benefit from more frequent C_0/AUC-triggered dose fine-tuning. Given that MNA is the main cause of high Tac IPV, home-based Tac exposure monitoring could prove a supportive tool for patients with adherence problems, both as a diagnostic and a therapeutic aid.

Tacrolimus intra-patient variability: conclusions

High tacrolimus IPV has emerged as an important prognostic risk factor after solid organ transplantation. High IPV leads to cumulative injuries to the allograft including (late) acute T-cell-mediated rejection, development of donor-specific anti-HLA antibodies which can lead to humoral types of acute and chronic rejection (e.g. transplant glomerulopathy), vascular changes (e.g. arteriolar hyalinization) in kidney grafts causing chronic ischemia and ensuing fibrotic irreversible damage. Graft function (e.g. chronic lung allograft dysfunction) and graft survival decline as a result of this. The main clinical driver of high Tac IPV is medication non-adherence which is highly prevalent among solid organ recipients. Improving MNA requires a persistent supportive multi-disciplinary approach by a team of health care providers focused on educational, motivational and practical help and simplification of maintenance (immunosuppressive) drug regimens. Important progress in characterization and standardization of Tac IPV metrics in clinic has been achieved and provides the

necessary basis for development of remote drug monitoring and drug dosing assist software that could help to simultaneously improve Tac IPV and MNA and in solid organs recipients.

Table 1: Studies of tacrolimus intra-patient variability in kidney allograft recipients: clinical outcome.

Author, year of publication, reference	n	Tac IPV assessment period	Tac IPV cut-off(s)	Outcome parameter	Main results	p-value
Borra L *et al*. 2010 (8)	297	6-12 months	Mean 17% Median 14.9%	Composite endpoint: graft loss, chronic allograft nephropathy and doubling plasma creatinine concentration	Composite endpoint: High IPV: 70.6% vs. low IPV: 29.4%	.011
Shuker N *et al*. 2016 (9)	808	6-12 months	Median 16.2%	Composite endpoint: graft loss, transplant glomerulopathy, late rejection and doubling plasma creatinine concentration	Composite endpoint: High IPV: 49.5% vs. low IPV: 41.8%	.018
Rodrigo E *et al*. 2016 (35)	310	4-12 months	Mean 30%	*De novo* DSA, death-censored graft loss	*De novo* DSA development [HR (95%CI)]: Acute rejection: 2.52 (1.26-5.05) Re-transplantation: 2.17 (1.01-4.67) CV>30%: 2.92 (1.47-5.80)	 .009 .045 .002
Whalen *et al*. 2017 (11)	376	6-12 months	Median 15%	Acute rejection, graft loss and graft function	Acute rejection [HR (95%CI)]: High IPV: 1.95 (1.23-3.09) Graft loss [HR (95%CI)]: High IPV: 4.34 (1.25-15.10) eGFR better in low IPV group at 1,2,3 and 4 years	.0054 .0207 <.0001
Gooddal *et al*. 2017 (12)	628	6-12 months	Quartiles: Lowest:<13.45% Low:≥13.45-18.15% High:≥18.15-25.27% Highest:>25.27%	Patient survival, graft loss and acute rejection	Death-censored graft survival [HR (95%CI)]: Highest IPV quartile: 2.51 (1.01-6.27) Mean Tac C_0 < 5 ng/mL: 4.32 (1.94-9.63) High clinical nonattendance rate: 1.10 (1.01-1.20) Rejection: 9.83 (4.62-30.94)	 .048 .0003 .03 <.0001
Taber DJ *et al*. 2017 (39)	1411	1 month until	Cut-point of 40%	Acute rejection, graft	10% increase Tac CV [adjusted risk=aHR (95%CI)]:	

		clinical event		loss	Acute rejection: 1.20 (1.13-1.28) Graft loss: 1.30 (1.23-1.37)	<.001 <.001
Siebert *et al*. 2018 (41)	1226 EA 246 AA	0-6 months	Quartiles	Acute rejection, graft failure	Graf failure [HR (95%CI)]: Highest IPV quartile AA (49%): 2.95 (1.67-5.23) Highest IPV quartile EA (38%): 1.53 (1.06-2.20)	 .0002 .024
Vanhove T *et al*. 2016 (10)	220	6-12 months	Tertiles: Low:<14.4% Middle:14.4-22.1% High:≥22.1%	(Progression of) IF/TA score between 3-24 months in protocol biopsies	Mean increase chronicity score 3-24 months: Low IPV tertile: 1.12 ± 1.80 Middle IPV tertile: 1.18 ± 2.44 High IPV tertile: 1.97 ± 2.03	 .023 .016
Süsal C *et al*. 2019 (14)	6638	1-3 years	Tertiles: Low:<30% Middle:30-44% High:≥45%	Graft survival, death-censored graft survival	Death-censored graft survival [HR (95%CI)]: IPV <30%: 1 (ref) IPV 30-44%: 1.42 (1.11-1.82) IPV ≥ 45%: 2.11 (1.61-2.77)	 .0005 <.0001
Sapir-Pichhadze R *et al*. 2014 (34)	356	Starting 1 year posttransplant	Standard deviation of Tac C_0 levels (SD)	Composite endpoint: late rejection, transplant glomerulopathy and graft loss	Composite endpoint [HR (95%CI]: SD >2.5 vs. ≤2.5: 1.84 (1.04-3.25) SD >3.0 vs. ≤3.0: 2.56 (1.42-4.62)	 .04 <.001

Legend Table 1: DSA: donor-specific antibodies. HR: Hazard Ratio. 95%CI: 95% Confidence Interval. eGFR: estimated Glomerular Filtration Rate. EA: European American. AA: African American. IF/TA: Interstitial Fibrosis/Tubular Atrophy. *See Text for details*.

Table 2: Recommendations for the development of a consensus standardized determination of Tac IPV in solid organ transplantation.

Tac IPV Parameter	Recommendations	Comments
Definition of Tac IPV: '*fluctuations in tacrolimus blood concentrations in an individual patient over a certain time period in which the tacrolimus dose was not changed*'	- Tac C_0 values during stable Tac dose	If Tac dose changes occur, dose-corrected C_0 values are recommended Avoid Tac IPV measurements: -during hospitalization -at the time of clinical events in outpatient setting (e.g. diarrhea, anemia, infections) -during temporary use of concomitant drugs that interfere with Tac disposition (drug-drug interactions) -at the time of transplant organ dysfunction (e.g. biliary stricture after liver transplantation) -during switch to different Tac formulation -during generic substitution
Timing of Tac IPV determination	- 6-12 months post-transplantation	Stable clinical situation, relatively frequent follow-up, potential onset of unintentional MNA, opportunity for early (preventive) intervention Avoid timing of Tac IPV determination: -between 0-4 months: not clinically stable (e.g. acute rejection, delayed graft function), adaptations immunosuppressive drug regimen, changes in Tac disposition (e.g. corticosteroid tapering, changes in liver allograft function) - > 1 year*: less Tac C_0 sampling with prolonged follow-up, relatively late for alloreactivity indicators (e.g. DSA, subclinical acute rejection), relatively late for IPV-targeted preventive interventions *in case of Tac C_0 home monitoring (DBS, VAMS) IPV determination

		can be continued beyond the first year as diagnostic (and therapeutic) tool for MNA
Tacrolimus exposure parameter	-Tac C_0 / predose trough concentration value	-Ideal: estimation of dose-interval Tac exposure by using a LSS, either in outpatient clinic or home monitoring (DBSM, VAMS) -Full dose-interval Tac AUC profile is practically less feasible -Avoid using different Tac analytical assays interchangeable
Number of Tac C_0 values for IPV calculation	- 4 to 6 Tac C_0 values over 6 months	-Avoid < 3 Tac C_0 values -Check for interference by, for example, clinical events in case of > 6 Tac C_0 values available over a period of 6 months
Tac IPV calculation	-Coefficient of variation (CV, CV%)	See also Shuker N *et al*. (7) for different formulas Alternatives: -Standard deviation (SD) or Medication Level Variability Index (MLVI): less useful than CV for comparison between studies Avoid: -"Time outside target Tac concentration (C_0) range": bias due to center-specific target Tac C_0 ranges (e.g. "low" versus "standard" exposure ranges), dependent on frequency and timing of Tac C_0 sampling
Target Tac IPV	- Aim for target CV Tac C_0 < 20%	Avoid CV Tac C_0 ≥ 30% If Tac IPV > 30%: -Elimination of common causes of Tac IPV (e.g. Tac dosing with food) -Assessment of MNA: - MNA diagnostic work-up (e.g. patient interview) - MNA intervention (e.g. simplification drug regimen, education, supportive measures)

		Lower target Tac C_0 CV (<20%) require intensified follow-up strategies, (preferably) including home monitoring (DBSM, VAMS) and/or use of LSS

Legend Table 2: DBSM: Dried Blood Spot Monitoring.

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Figure Legends

Legend Figure 1.

Known causes of intra-patient variability in tacrolimus exposure are represented according to their impact on tacrolimus intra-patient variability from high to low and the extent to which they are modifiable by clinical interventions ((i.e. therapeutic interventions, Tac dose adaptations, patient education and instructions). The clinical appreciation expressed in Figure 1 as to what degree variables that affect Tac IPV are modifiable in daily practice, is not based on the results of comparative clinical trials and is (partly) modified from references 7 and 29. *See text for details*.

Legend Figure 2.

The theoretical risk of allo-immune activation versus allograft ischemia and fibrosis associated with off-target tacrolimus trough concentrations according to target Tac concentration ranges and patient immunological risk.
Panel A. A low immunological risk patient dosed to standard target Tac trough concentrations (8-12 ng/mL).
Panel B. A high immunological risk patient dosed to standard target Tac trough concentrations (8-12 ng/mL).
Panel C. A low immunological risk patient dosed to low target Tac trough concentrations (3-7 ng/mL).
Panel D. A high immunological risk patient dosed to low target Tac trough concentrations (3-7 ng/mL).
See text for details.

Legend Figure 3.

Summary from Neuberger JM *et al*., Myaskovsky L *et al*. and Nevins TE *et al*. (29,63,64). **Diagnostics:** it is advised to use a combination of diagnostics to identify MNA according to available resources . Validated questionnaires include the BAASIS (Basel Assessment of Adherence to Immunosuppressive Medications Scale©) and TAQ (Transplant Adherence Questionnaire). **Barriers:** Socio-economic include poor socio-economic status, low educational level/illiteracy, medication costs/health insurance status/health care access, poor social support or isolation, low self-efficacy. Condition-related include depression, distress, cognitive problems, substance abuse, higher comorbidity, low self-care ability, physical limitations. Treatment-related include complex medication regimens, side-effects, taste/size, frequent medication changes, total number of medication and dosing frequency. Patient-related includes past MNA, forgetfulness, low health literacy, health beliefs, (busy) lifestyle. Demographics include younger age, transition (from pediatrics), (minority) ethnicity, time since transplantation, rural residence, living donor transplantation. **Interventions:** requires a multidisciplinary support team consisting of medical and nursing staff, pharmacists, psychologist, social worker and dietician. CV%: Coefficient of Variation %.