Tools to optimize the immunosuppressive treatment after renal transplantation

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Dissertation for the degree of Philosophiae Doctor (Ph.D)

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University of Oslo
2020

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo

No. 2298

ISSN 1501-7710

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

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ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the Section of Nephrology, Department of Transplantation Medicine, Oslo University Hospital Rikshospitale, during the years 2014 to 2019. I am thankful for receiving funding from The South-Eastern Norway Regional Health Authority, and for all people who have supported this work:

First, I would like to thank my supervisors, Anders Åsberg, Karsten Midtvedt, Ida Robertsen, and Hege Christensen. I have been really privileged of being a part of your research groups. Anders; thank you for sharing your broad scientific knowledge, your quick reply to all kinds of questions, for always keeping your door open, and for believing in me. Karsten; thank you for your positive energy, your great ideas and your never-ending passion to treat patients better. Ida; you have been a role model to me, thank you for your valuable help and motivation, and for sharing both frustrations and joys. Hege; thank you for your enthusiasm and encouragement. Your smile and gentle being have been deeply acknowledged

This work would not have been possible without my collaborators and co-authors. I am truly grateful to each and every one of you, and would like to thank you for your great contribution and valuable feedback during the work of the different papers.

Thanks to Kirsten, May-Ellen, Sebastian, Helga, Hanne and Lena at the Laboratory of Renal Physiology for help on data capturing and for the great social atmosphere at the lab. A warm thank to all my fellow PhD students for the good company along the way. A special thank to Veronica, Erlend, Kine, Nina, Thea, Elisabet and Kjersti for making these years unforgettable and fun.

I would like to thank all my friends and family for your chair and support. Last, but not least, I am genuinely grateful for my parents and my husband for all their love and encouraging. Thank you, Alexander, for your patience during all my frustrations.

Oslo, June 2020

Marte Theie Gustavsen

LIST OF PAPERS

I. **Gustavsen MT,** Midtvedt K, Vethe NT, Robertsen I, Bergan S and Åsberg A. Tacrolimus area under the concentration versus time curve monitoring, using home-based volumetric absorptive capillary microsampling
Therapeutic Drug Monitoring. 2020; 43(3): 407-417

II. **Gustavsen MT,** Midtvedt K, Robertsen I, Woillard JB, Debord J, Klaasen RA, Vethe NT, Bergan S and Åsberg A.
Fasting status and circadian variation must be considered when performing AUC-based therapeutic drug monitoring of tacrolimus in renal transplant recipients
Clinical and Translational Science. 2020; 0, 1-9; doi:10.1111/cts.12833

III. **Gustavsen MT,** Midtvedt K, Lønning K, Jacobsen T, Reisæter AV, De Geest S, Andersen MH, Hartmann A and Åsberg A.
Evaluation of tools for annual capture of adherence to immunosuppressive medications after renal transplantation – a single-centre open prospective trial
Transplant International. 2019; 32(6): 614-625

ABBREVIATIONS

AUC	Area under the concentration versus time curve
BAASIS	Basel Assessment of Adherence to Immunosuppressive Medication Scale
CCC	Concordance correlation coefficient
CL	Clearance
C_{max}	Maximum concentration
CP	Coverage probability
CV	Coefficient of variation
CYP	Cytochrome P450
CyA	Cyclosporine A
DD	Deceased donor
C_0	Trough concentration
dnDSA	*De novo* donor specific antibodies
IQR	Interquartile range
k_{el}	Elimination rate constant
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
LD	Living donor
LSS	Limited sampling strategy
TDI	Total deviation index
TDM	Therapeutic drug monitoring
T_{max}	Time to reach maximum concentration
Tx	Transplantation
V_d	Volume of distribution
6-MP	6-mercaptopurin
μ	Mean
σ	Standard deviation

ABSTRACT

Livelong immunosuppressive treatment is needed after renal transplantation. Tacrolimus has been the cornerstone in most transplant centers. Its narrow therapeutic window and large inter- and intra-individual variability makes tacrolimus dosing challenging and therapeutic drug monitoring is mandatory. The short-term outcome after renal transplantation is good, however, there is room for further improvement in long-term outcomes. Individually optimized dosing of current available immunosuppressive regimen, with focus on finding new therapeutic drug monitoring strategies and ensuring good medication adherence, are potential strategies to further improve both long-term patient- and graft survival.

Area under the concentration versus time curve (AUC) is the preferred measure for systemic drug exposure, and hence also in theory the best pharmacokinetic marker associated with effect. For practical reasons, trough concentrations are however used for tacrolimus dose individualization in most centers, and all efficacy studies have been done using trough therapeutic drug monitoring. Potentially AUC-targeted therapeutic drug monitoring of tacrolimus will provide better outcomes. This thesis presents novel tools that make such AUC-targeted therapeutic drug monitoring possible. In a prospective pharmacokinetic study accurate estimations of tacrolimus AUC for fasting morning dose was shown to be possible utilizing population pharmacokinetic model derived Bayesian estimators and three optimally timed blood samples using capillary microsampling. That patient themselves can obtain capillary microsamples at home, ease tacrolimus therapeutic drug monitoring in many ways.

Most population pharmacokinetic models of tacrolimus are developed on data from clinical trials; obtained in selected patients under highly controlled fasting conditions and almost exclusively only the morning dose of twice-daily tacrolimus dosing is investigated. These models do therefore not reflect real-life, as both timing of dose administration (morning, evening) and food consumption can affect tacrolimus pharmacokinetics. This thesis present a prospective pharmacokinetic study of tacrolimus morning and evening dose performed in a real-life setting with regards to food. We found that circadian variation was present after fasting dose administration, but not in a real-life non-fasting setting. Non-fasting dose administration results in flatter pharmacokinetic profiles. Data on a patient's actual behavior when it comes to dose administration are hence essential for accurately estimations of AUC with these kind of models.

Irrespectively of how accurately individualized doses are calculated, nonadherence to the immunosuppressive treatment is a major risk factor for poor clinical outcome after renal transplantation. To overcome this problem, assessment of adherence data and identification of risk factors affecting the behavior are crucial. This thesis presents an open prospective randomized controlled trial evaluating adherence tools for annual assessment of immunosuppressive adherence in a clinical routine setting.

In conclusion, the work presented in this thesis contributes with important knowledge and new tools for optimization of the immunosuppressive treatment that potentially can improve long-term outcome after renal transplantation.

1 INTRODUCTION

1.1 Renal transplantation

Renal transplantation is today the preferred treatment for patients with end stage renal disease and offers improved quality of life and longer survival compared to dialysis (1-3). The first successful renal transplantation was performed in 1954 (4, 5), but the initial success-rate measured by graft survival was poor and very few transplanted kidneys functioned after the first year. In the early 1960's the first pharmacological immunosuppression for use in transplantation, using 6-mercaptopurin (6-MP), gained attention (6). While some improvement in early graft survival was seen, only around 10% of transplant recipients were alive after 3 months post-transplant. Further on, a combination of a 6-MP derivate, azathioprine, and glucocorticoids increased one-year graft survival (6), but there was still a large room for improvement. In 1971 cyclosporine A (CyA) was discovered and isolated from a fungus (7), and further reported to have immunosuppressive qualities (8). CyA showed a remarkably improvement in graft survival in clinical trials (9-11), and the drug was approved in the early 1980's as an immunosuppressive drug used to prevent allograft rejection after solid organ transplantation. CyA is a calcineurin inhibitor, inhibiting the enzyme calcineurin, which is central in the T-cell mediated immune response. In 1994 another calcineurin inhibitor, tacrolimus, was introduced, which further reduced the acute rejection rate and increasingly became the calcineurin inhibitor of choice (12-14). In Norway almost all *de novo* renal transplant recipients are prescribed tacrolimus, and 257 out of 258 adult patients engrafted in 2019 were administered tacrolimus at the time of transplantation (15). Tacrolimus is currently the backbone in the immunosuppressive treatment worldwide in solid organ transplant recipients (16).

1.1.1 Transplantation list

Between 250 and 300 kidney transplantations have been performed annually on the National Transplant Center in Norway the last 10 years (15). Patients with end stage renal disease in need of kidney transplantation are increasing worldwide (17). The kidney transplant waiting list in Norway is also steadily increasing as seen in Figure 1, and at the end of 2019, 364 patients were enlisted (15). These patients have considerable higher morbidity and mortality when compared to the kidney transplant population (18-20).

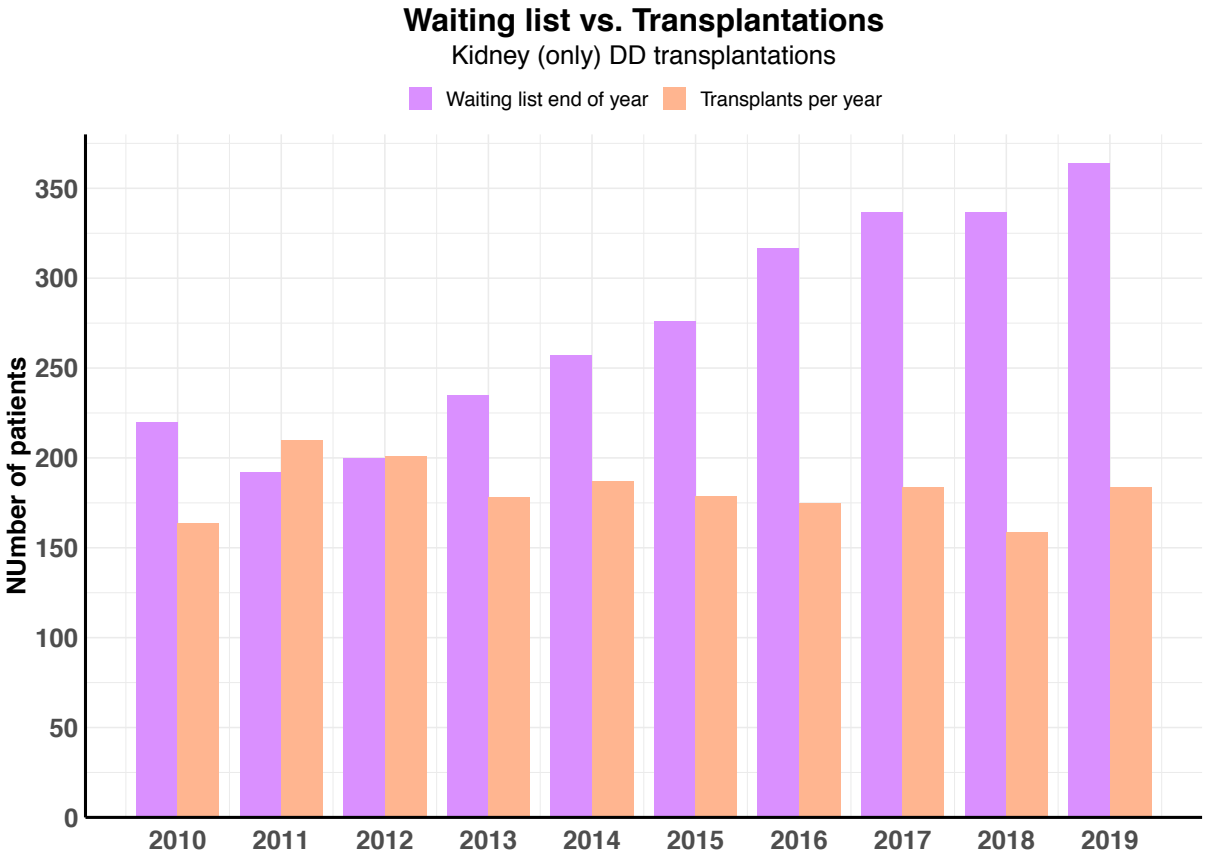

Figure 1. Number of patients on the transplantation list and the number of transplantations performed between 2010 and 2019 in Norway (Data from the Norwegian Renal Registry June 2020 (15)).

1.1.2 Graft survival

Over the past decades improvement of graft survival following kidney transplantation has mainly been due to reduction in early acute rejections (21). One-year graft survival for renal transplant recipients in Norway is today over 95%(15). Whereas acute rejection is still considered the main barrier for short term success, late graft failure is a multifactorial phenomenon. The graft survival still improves one-year post transplant, but the long-term graft survival has almost been unchanged for the last decades (21-23), with only minimal measurable progress (Figure 2) (15). This may be due to immunological damage of the graft as a result of too little immunosuppression resulting in development of *de novo* donor specific antibodies (dnDSA) leading to chronic antibody mediated rejection (24-27). To keep the immune system sufficiently depressed, adequate immunosuppression is needed. A paradox is

however that too high calcineurin inhibitor drug exposure causes nephrotoxicity (28), another contributing factor to late graft loss (29).

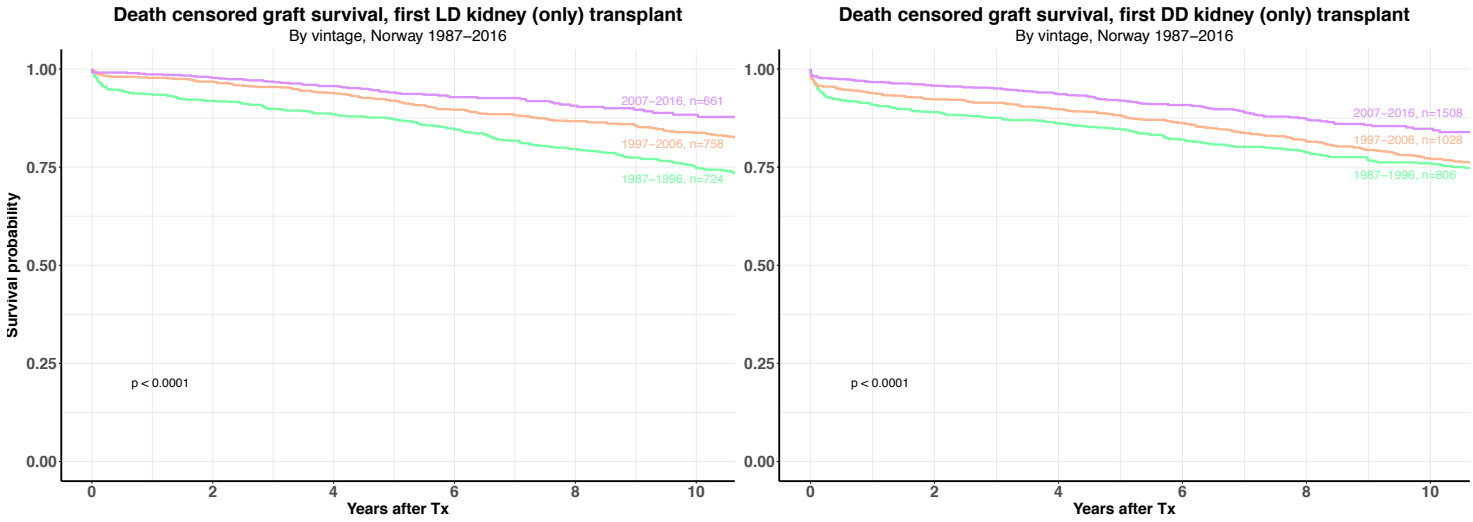

Figure 2. Effect of transplant era on death censored graft survival for first living donor (left) and deceased donor (right) transplantation (Data from the Norwegian Renal Registry June 2020 (15)).

DD; deceased donor, LD; living donor; Tx; transplantation

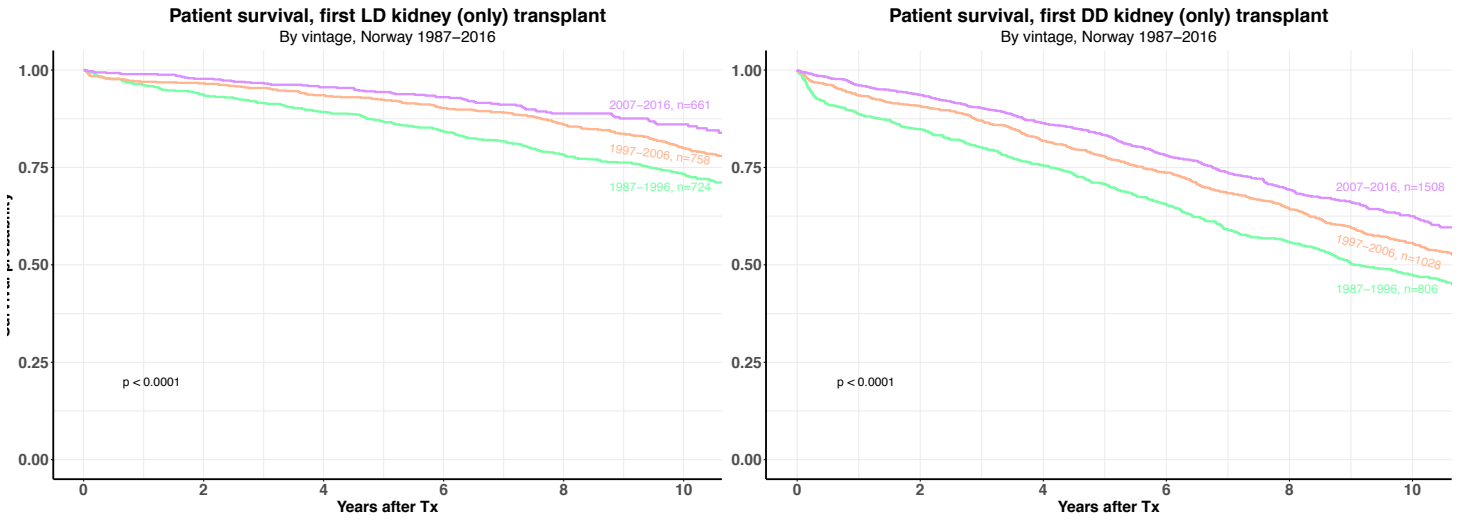

Figure 3. Effect of transplant era on patient survival for first living donor (left) and deceased donor (right) transplantation. (Data from the Norwegian Renal Registry June 2020 (15)).

DD; deceased donor, LD; living donor; Tx; transplantation

1.1.3 Patient survival

One-year patient survival after deceased donor transplantation in Norway is today approximately 95%, while the 5- and 10-year survival is around 85% and 63% (Figure 3) (15). Main causes of deaths are cardiovascular diseases, cancer and infections, roughly contributing with one third each. A driving force for these severe outcomes are adverse effects of the immunosuppressive treatment (12). By suppressing the immune system the risk for cancer and infections increase (30, 31), in addition tacrolimus has specific unwanted adverse effects causing hypertension, hyperlipidemia and post-transplant diabetes, all increasing the risk of cardiovascular disease (32-37).

1.2 Pharmacokinetic principles

Pharmacokinetics describes the processes of absorption, distribution, metabolism and excretion of a drug in the body over a period of time (38). These different processes are often referred to as the acronym ADME. Drug absorption from the intestine is present after oral drug administration and is determined by the rate of absorption and the amount of drug being absorbed. Several factors can potentially influence this process in the gastrointestinal tract (38). The movement of the drug in the body to various sites, like to and from the site of measurement and the peripheral tissue, is referred to as distribution (38). Normally, the pharmacological effect of a drug is determined by its unbound free fraction (39). Drug elimination from the body can take place in several pathways, either by renal elimination from the kidneys or non-renal elimination by metabolizing enzymes and drug transporters (38). Metabolism is a process where lipid-soluble drugs are chemically altered to be more water-soluble for excretion by the kidneys or the bile. Water-soluble drugs are excreted directly via the kidneys in the urine.

The pharmacokinetic processes determine the drug concentration in the body, and can be visualized by plotting a concentration versus time curve (Figure 4). Some parameters that are central in describing pharmacokinetics are (38):

- Bioavailability (F), the fraction of administered drug dose reaching systemic circulation.
- Absorption rate constant (k_a), the fractional rate of drug absorption into the body.

- Volume of distribution (V_d), an apparent volume illustrating the degree of drug distribution in the body. It is not a "real" volume, and can be calculated by dividing the total amount of drug in the body with observed plasma concentration.
- Elimination rate constant (k_{el}), the fractional rate of drug removal from the body.
- Clearance (CL), the volume of blood cleared for drug per unit of time. It describes the elimination of drug from the body, generally as a result of liver metabolism and renal excretion.

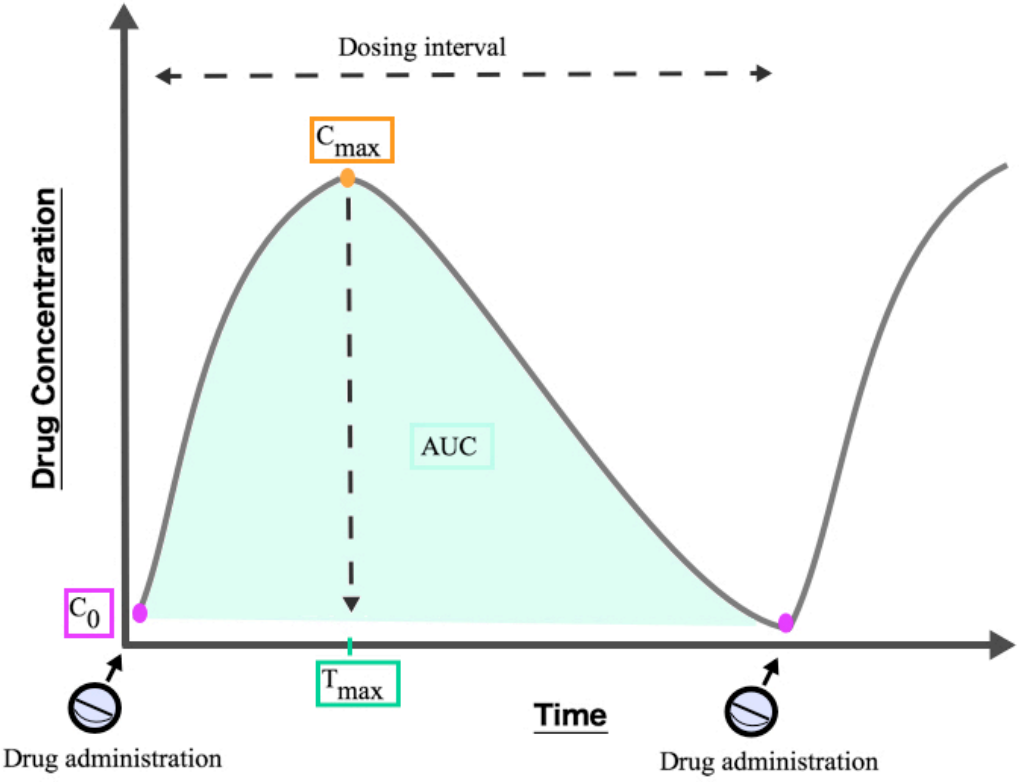

Figure 4. A concentration versus time curve showing the time course of drug concentration within a dosing interval. The trough concentration (C_0), maximum concentration (C_{max}), time to reach C_{max} (T_{max}), and AUC for respective dosing interval are illustrated.

As seen in Figure 4, the drug concentration increases as the drug is absorbed, before reaching the maximum concentration (C_{max}) at time T_{max}. A trough concentration (C_0) is the lowest concentration in a dosing interval, and is measured right before the next dose is administered. The area under the concentration versus time curve (AUC) represents the total drug exposure as a function over time. Pharmacokinetic variables like C_{max}, C_0 or AUC are often used as surrogates for determine drug efficacy or toxicity. If taking the same drug dose over a period of time, the amount of drug input will eventually equals the amount of drug eliminated from the body within a dosing regimen and reach steady state conditions. In steady state, pharmacokinetic variables like C_0 and AUC are approximately the same in proceeding dosing intervals (38).

1.3 Tacrolimus

Life-long immunosuppressive treatment is necessary after renal transplantation in order to avoid an immunological response leading to rejection of the transplanted kidney. The immunosuppressive treatment given after renal transplantation has its site of action in the lymphocytes, which play an important role in the cell-mediated immune response (40). Tacrolimus binds to the immunophilin FK506-binding protein in the cytosol and this complex inhibits the phosphatase activity of the serine-threonine phosphatase enzyme calcineurin (41). This leads to suppression of transcription factors of activated T-cells, resulting in an impaired synthesis of interleukin-2 and other cytokines important for T-cell activation (42). As a result, the proliferation and differentiation of T-cells are reduced (43, 44).

1.3.1 Pharmacokinetic properties

Tacrolimus displays large pharmacokinetic variability between patients (45, 46). It is rapidly absorbed into the bloodstream, reaching C_{max} after 1-2 hours for the oral immediate-release formulation (45). An absorption lag time of around ½ hour has been reported (47). Oral bioavailability of tacrolimus is poor and on average 25%, but can range from 5% to 90% (45, 48). Tacrolimus is a substrate of the drug transporter P-glycoprotein and the cytochrome p450 isoenzymes (CYP) 3A4 and 3A5 (49). There is considerable pre-systemic metabolism by the CYP3A enzymes in the gut wall and first-pass metabolism in liver, reducing oral bioavailability (50).

After entering the blood, tacrolimus distributes to erythrocytes in a dose dependent manner (51). The whole-blood concentration is on average 15 times higher than the corresponding plasma concentration, but is dependent on the hematocrit level, and large variation between patients is reported (51-53). In plasma approximately 99% of tacrolimus is bound to different plasma proteins (51). The unbound concentration causing the pharmacological effect is therefore only around 0.01 to 0.1% of the whole blood concentration. Tacrolimus is a low extraction-ratio drug (54), and the whole-blood concentration is expected to increase in proportion to erythrocyte-binding (proportional to hematocrit), while the unbound therapeutically active concentration will remain unchanged. Tacrolimus has a lipophilic character, and the volume of distribution (V_d) is high, but with large inter-individual variability (51).

Tacrolimus is primarily removed from the circulation through metabolism in the liver, and the drug clearance is reported to have high inter-individual variability (51). One explanatory factor is a single nucleotide polymorphism in the gene encoding CYP3A5 (55), the most important genetic covariate for tacrolimus metabolism in the liver and intestine. Patients carrying the *CYP3A5*1* allel have active enzyme activity and are called CYP3A5 expressers. The *CYP3A5*3* allele is associated with no enzyme activity, and homozygous carriers of this allele are called CYP3A5 non-expressers (56). Approximately 15% of the Caucasian population are CYP3A5 expressers (57-59). Different expression of this metabolizing enzyme plays an important role in the pharmacokinetic variability between patients, and renal transplant recipients being CYP3A5 expressers are in need of approximately two times higher tacrolimus dose compared to non-expressers (60).

Intra-patient variability

Tacrolimus can also have large pharmacokinetic variability within the same patient, causing intra-individual variability. To optimize tacrolimus dosing strategy in individual patients, detailed knowledge about elements influencing the pharmacokinetic processes and the drug response is important. Administration of oral tacrolimus is from the drug label recommended in a fasting state (61), as food consumption can decrease both the absorption rate and the bioavailability (62, 63). Decreased absorption is most pronounced for food with a high fat content (64). Time of dose administration can also cause variability, as circadian variation with a reduced absorption rate and decreased systemic exposure after the evening dose has been reported (65-68). The reason for this is not fully understood, but suggested explanatory

factors are changes in gastric emptying time, gastrointestinal transit time, organ blood flow or fluctuations in hepatic enzyme activity during the day (69, 70).

A time-dependent change in the pharmacokinetics of tacrolimus has been reported, with a gradual decrease in the dose required for maintaining the same whole blood concentration with time after transplantation (71-73). This is most pronounced during the first post-transplant year, causing frequent dose changes in this period. Co-medications that induce or inhibit CYP3A enzymes or P-glycoprotein, like glucocorticoids, some antifungals, antibiotics, calcium channel blockers and several anti-epileptic drugs can increase or reduce tacrolimus exposure (74, 75). Missed or delayed dosing of tacrolimus are obviously also important factors causing variability in the drug exposure (76).

1.3.2 Formulations

Currently there are five different formulations of tacrolimus available: an intravenous formulation, granules for oral suspension, an oral immediate-release formulation designed to be taken twice-daily and two once-daily formulations. The once-daily formulations are based on different technologies; one is a MR-4 extended release capsule formulation and the other a Meltdose tablet formulation called LCP-tacrolimus (77). The different formulations of tacrolimus are not bioequivalent and a 1:1 switch is not recommended (77-79). Generics of the various formulations of tacrolimus are also available. Many studies performed on the oral immediate-release formulation have shown that approved generics seem to be bioequivalent (80, 81). Of concern is the fact that almost all of these studies have been performed in rather young transplant recipients, usually below 50 years of age. Our average transplant population is older than 50 years, and a study by Robertsen *et al.* reported that a generic tacrolimus formulations was not bioequivalent in recipients > 60 years (82). In Norway, generic tacrolimus formulations are therefore in general not used or used with great caution, and patients are recommended not to switch between generics of tacrolimus without conferring with the treating clinician.

In the early post-transplant phase, underexposure of tacrolimus increases the risk of acute rejections and small variation in drug exposure can influence the outcome (83). The time-dependent change in tacrolimus pharmacokinetics is mostly pronounced in the early post-transplant phase (71), causing frequent dose changes. Currently all renal transplant recipients prescribed tacrolimus in Norway use the original immediate release formulation, dosed twice-

daily, the first 7-8 weeks after transplantation. After this, a switch to a once-daily formulation is determined by a joint decision between the treating physician and the patient. Around 40%-50% of the patients are switched to a once-daily formulation within 8-weeks post-transplant (15).

1.3.3 Therapeutic drug monitoring

Tacrolimus has a narrow therapeutic index, meaning that the balance between overexposure, with adverse effects and toxicity, and underexposure, with increased risk of acute rejection, is narrow. There is large variability in drug response and the dose alone is not a good marker of drug effect. Tacrolimus dosing is thus a challenging task, and requires dose adjustment according to concentration measurements (84-86).

Drug dose individualization with therapeutic drug monitoring (TDM) is mandatory to avoid both under- and overexposure of tacrolimus and to account for the large pharmacokinetic variability. Tacrolimus TDM is performed by measuring concentrations in whole-blood, the clinician evaluates the concentration and when needed adjust the dose in order to achieve a desired target concentration. Although AUC is considered the best marker for drug exposure, tacrolimus TDM is in most transplant centers performed using trough concentrations (86-88). A trough is the easiest accessible concentration in a dose interval and has been convenient to implement in a clinical routine setting. However, a trough concentration is only a surrogate marker for systemic exposure and gives little information on individual pharmacokinetics (89). While some studies have shown a good correlation between trough concentration and systemic exposure (51, 88, 90-92), others have failed to prove this (93-96), causing high variation in reported correlation coefficients. The correlation is thus not optimal, nor stable, as it changes over time (71). A study by Bouamar *et al.* (97), using pooled data from three large trials (98-100), demonstrated that tacrolimus trough concentrations did not predict the risk of acute rejections after renal transplantation. Even with whole-blood concentrations within desired trough concentration target, some renal transplant recipients experience acute rejections or toxicities (92, 97, 101). The relationship between trough concentrations and tacrolimus efficacy is not precise, and optimal target concentration of tacrolimus is still controversial. Defining appropriate tacrolimus target concentrations that both minimize toxicity and effectively prevent acute rejection episodes and development of dnDSA is a

challenge (86). The lack of strong correlation between trough-based monitoring and clinical outcome has led to attempt in finding other more useful TDM strategies.

Perred trough target has been remarkably reduced, from a high-recommended target of 15-25 μg/L in the first clinical trials (102-104), to a low trough target of 5-15 μg/L (105). The reason for the initial high trough targets was the fear of acute rejections. The impact of CNI toxicities led to reduction in trough levels and eventually combinations of immunosuppressive drugs in even lower but still effective concentrations. A large randomized controlled trial in 1645 renal transplant recipients published in 2007, the EliTE SYMPHONY-trial (98), showed that a low tacrolimus trough target between 3-7 μg/L in combination with mycophenolate mofetil and glucocorticoids was favorable in terms of renal function, acute rejections and graft survival. These findings have been reproduced in other studies (106). Of note, the actual tacrolimus trough levels measured during the EliTE SYMPHONY-trial were in the upper range with average trough level of 6-8 μg/L during the first 12 months study period. Some more recent studies found an increased risk of graft failure, and advised against such low-trough target concentrations (107, 108). This has led to large variability in reported trough concentration target among transplant centers, and some have a more conservative immunosuppressive regimen with higher target strategies, especially in the early post-transplant phase (109, 110). The preferred tacrolimus target is influenced by the patient's immunological risk, the combination of immunosuppressive therapy given and time after transplantation (105, 111).

For standard immunological risk patients in Norway initial tacrolimus dose is 0.04 mg/kg in CYP3A5 non-expressers and 0.08 mg/kg in CYP3A5 expressers. The doses are further individualized to achieve a trough target between 4-7 μg/L. For immunological high-risk patients initial dose is 0.08 mg/kg in CYP3A5 non-expressers and 1.2 mg/kg in CYP3A5 expressers and adjusted to a trough target between 8-12 μg/L the first 28 days, reduces to 6-10 μg/L during the first year and then 5-7 μg/L thereafter. The characterization of high-risk patients is the presence of donor specific antibodies at the time of transplantation.

Blood sampling procedure

Tacrolimus TDM is performed by measuring the whole-blood concentration. In the early post-transplant phase, trough concentrations are obtained by venous venipuncture up to 4 times per week, before gradually reduced to once per week by 8-weeks post-transplant. After this, blood samples are normally obtained every month to every other month, and after two

years every third month, lifelong. Patients have to come to the hospital/doctors office for blood sample collection in the morning prior to tacrolimus administration, which often is time-consuming and impractical for the patient. In a clinical setting, TDM of tacrolimus using trough concentrations is however practical for the treating physician, as it is logistically difficult to draw samples at other time points. A venipuncture can also be bothersome for patients; it demands a large blood volume, can be hurtful, and can be difficult to obtain in children, elderly or previous dialysis patients.

New innovative blood sampling technology has gained attention the last years. Dried blood spot methods on filter card (112-114) and volumetric absorptive capillary microsampling (115, 116) have been developed. Such sampling approaches are suggested to be more patient-friendly; a smaller amount of blood are needed (10-20 μL) and it enables a more flexible blood sampling procedure, as samples can be obtained at home by patients themselves. The new blood sampling technology can save both time and costs (117). TDM of tacrolimus using such new sampling approaches is intriguing (87), as it can simplify the blood sampling procedure and enable multiple sampling within a dosing interval. This also opens up the possibility of investigating other TDM strategies that potentially may improve the long-term outcome after transplantation.

1.3.4 Other immunosuppressive drugs

In Norway renal transplant recipients with standard immunological risk receive induction therapy with 2 doses of i.v. basiliximab (an interleukin-2 receptor blocker). A triple drug regimen using a combination of tacrolimus, mycophenolate mofetil and glucocorticoids are given as standard immunosuppressive maintenance therapy. Mycophenolate mofetil is an antiproliferative agent, inhibiting the proliferation of T- and B-cells (40), while glucocorticoids suppress the expression of proinflammatory cytokines (118). Both mycophenolate mofetil and glucocorticoids are normally administered as fixed dosing schedules. When given in combination with tacrolimus, mycophenolate mofetil are currently dosed 750 mg twice-daily, while the glucocorticoid prednisolon is administered in the morning according to a tapering schedule starting at 20 mg/day and tapered to a maintenance dose of 5 mg/day by day 180.

Azathioprine is another antiproliferative agent, and was used in combination with a calcineurin inhibitor and glucocorticoids before mycophenolate mofetil became available.

Azathioprine is today more or less replaced by mycophenolate mofetil, which was proven superior in the mid 1990's (119). Cyclosporine A can be given as an alternative to tacrolimus and was considered as the calcineurin inhibitor of choice in Norway before tacrolimus became available. As cyclosporine A also is a calcineurin inhibitor, the side effect profile is quite similar to tacrolimus. While tacrolimus is considered as the first choice today, cyclosporine A can be preferred in patients with reduced glucose tolerance or in patients developing post-transplant diabetes mellitus (120). The use of mammalian target of rapamycin (mTOR) inhibitors can be given as an alternative to a calcineurin inhibitor in the maintenance immunosuppressive regimen, or it can be used in calcineurin inhibitor minimization protocols (121). In renal transplant recipients with cancer or previous cancer history mTOR inhibitors can be advantageous (12), and possibly reduce the risk of cytomegalovirus infection (122). Both cyclosporine A and mTOR inhibitors are in need of individualized dosing with TDM as for tacrolimus. Belatacept is a therapeutic protein that also can be given as an alternative to a calcineurin inhibitor (123, 124). It was approved for use in maintenance immunosuppressive regimens in 2011 and is normally given as a monthly infusion dosed from the patients weight (125). Even though some new immunosuppressive drugs have been introduced the last decades and can be favorable in some transplant sub-populations (123); none have so fare been shown to improve the long-term outcomes over tacrolimus treatment (126).

1.4 Population pharmacokinetic modeling

A population pharmacokinetic model is a mathematical model describing the dynamic relationship between drug dose and drug concentration in the body over time (127). The pharmacokinetics of a drug is examined within a patient population, and pharmacokinetic parameters and their variation in the population are studied. Environmental, demographic and drug-related factors influencing the pharmacokinetics can be incorporated in the model (128). A population pharmacokinetic model uses a discrete compartments structure to describe the drug transport in the body over time (129). Each compartment consists of a volume, and in this volume the drug is evenly dispersed instantaneously. The number of compartments, transports between compartments, and the absorption- and elimination process are defined by a fixed structural model component, including differential equations describing the transport processes. The variation in parameter values both between and within patients, and variation that cannot be explained by the model called residual error (e.g. errors in dosing time,

sampling time, assay errors), are described by a stochastic model component (129). Also a covariate component is included in the model structure, representing subject specific characteristics (e.g. drug concentrations, gender, genotype, body weight) that can be built into the model through their associations with model parameters and explain some of the between subject variability (130). An example is plasma creatinine (renal function) or a CYP genotype (liver metabolism) in relation to drug clearance. Population pharmacokinetic models can be developed for simulation of expected exposures in specific subgroups of patients (i.e. pediatrics, CYP3A5 expressers, diabetic patients), can be used to individualize dosing regimens (131), and are extensively used in the drug development process by the pharmaceutical industry (132, 133). Population pharmacokinetic modeling allows interpretation of drug concentrations also before reaching steady state conditions, and can handle both sparse and rich data (128, 132).

Two main approaches are used to estimate pharmacokinetic parameters in a population: 1) a parametric approach that assumes an underlying defined distribution of the study data (e.g. normal or log-normal) or 2) a nonparametric approach that does not assume any particular shape of the parameter distributions, but predicts a discrete distribution of the parameter values called support points with given probabilities (134). A nonparametric approach has proven to be better in detecting outliers and unexpected subpopulations (135). A parametric model is less able to give description of populations that have other pharmacokinetic parameter distributions than the model assumptions (134). This can result in low precision in estimation of predicted individual dosing regimens (136). However, a parametric approach has the ability to separate inter-individual and intra-individual variability (129), something a non-parametric model is not able to.

1.4.1 Developed models for tacrolimus dosing

To develop a population pharmacokinetic model a patient population with different characteristics must be investigated with accurate sampling and dosing time. Ideally, patients should be extensively sampled with blood samples obtained during the entire dose interval (10-12 samples) (137), but very often models are based on solely trough concentrations. Models developed from rich data provide more information about the pharmacokinetic processes than models developed with trough data (138). Numerous population pharmacokinetic models have been developed for tacrolimus in adult renal transplant

recipients the last decades (139-146). The vast majority of these models are developed for the twice-daily tacrolimus formulation, but lately also models for the two once-daily formulations have been published (144, 147, 148). Most population pharmacokinetic models of tacrolimus are either one- of two-compartment models (140). Often CYP3A5 genotype, hematocrit, steroid dose and time after transplantation are found to be important covariates (52, 140). A lag-time, a transition compartment, or more complex gamma distributions have been added to describe the absorption process of tacrolimus (59, 144, 147), and often are allometric scaling of pharmacokinetic parameters defined, to explain different physical properties and processes related to body size (149).

Many population pharmacokinetic models for tacrolimus are based on trough concentrations (138, 140), and the data are obtained from clinical trials in selected patient populations under highly controlled conditions; food consumption and co-medications are often restricted, and the data are almost exclusively obtained during the day, i.e. following the morning dose. The results from such studies do not reflect every-day life for the average renal transplant recipient, and the generalizability of using these models outside a clinical trial, in a routine setting, may therefore not be feasible. If there is any correlation between whole-blood tacrolimus exposure and long-term outcome, models reflecting the real-life scenario over the entire dosing interval are needed.

1.4.2 Bayesian forecasting

Bayesian forecasting is a method that can estimate individual pharmacokinetic parameters, by combining prior information from the population and current information on an individual level (150). The population pharmacokinetic model of the drug defines prior information, like expected variability in parameters (i.e. drug CL and V_d) between subjects and between dosing occasions, and co-variation between covariates and the parameters. Individual information is the measured drug concentrations, the dosing history and covariate values for the specific individual of interest. Prior information is balanced against the current new individual information to obtain estimations of the most likely individual pharmacokinetic parameters. As more individual drug concentrations become available, the individual information is given more weight (150).

Bayesian forecasting enables determination of individual pharmacokinetic profiles and individual dosage regimens to achieve specific target concentrations at desired times, and can

therefore be used to design optimal TDM strategies. Population pharmacokinetic model derived Bayesian estimators used for dose individualization has some superiority over standard traditional trough-based TDM, as it does not depend on steady state conditions, can use concentrations from any time during the dose interval, and can handle missed or delayed doses (151). However, there are some challenges: 1) an appropriate population pharmacokinetic model must be available, and 2) the software needed is complicated, require excessive training and can be difficult to implement in a clinical setting (137).

Different assisted programs have been developed for individualized model-based dosing (137, 152, 153). In France different population pharmacokinetic models are made available for the transplant community through a web-based immunosuppressant Bayesian dose adjustment system. The system is an important tool for dose individualization of tacrolimus and dose adaption of immunosuppressive drugs (71, 154). In Norway, a nonparametric population pharmacokinetic model of tacrolimus developed by Åsberg *et al* (59) has been validated in renal transplant recipients. The model was externally evaluated in a prospective randomized study by Størset *et al* performed in renal transplant recipients in the early post-engraftment phase (155). Utilizing the model demonstrated improved trough concentration target achievement of tacrolimus when patients were implemented in a computerized dose proposal program and compared to experienced transplant physicians performing standard trough-based TDM.

1.4.3 Estimation of AUC

AUC is suggested to be a better marker for tacrolimus dose adjustments (87), but has not been clinical applicable to perform. AUC can be calculated by full concentration-time profiling using non-compartmental analysis applying the trapezoidal method. However, this is extremely time consuming, require intensive blood sampling during a dose interval and is not suitable in most clinical routine settings. By applying population pharmacokinetic model derived Bayesian estimators and limited sampling strategies (LSS), AUC can be predicted based on a limited number of blood samples (93, 156, 157).

Even though there are some contradicting data, today's trough targets are based on several outcome studies (98, 158, 159). This is lacking for AUC as few studies have examined the relationship between AUC and clinical outcome. Specific AUC targets have thus not been consensually and formally recommended (71). What the optimal AUC-target should be

remains unknown, and will depend on many factors including time after transplantation, type of induction therapy, immunological risk (past and current) and the level and type of immunosuppressive co-medications. A recent tacrolimus TDM consensus report (87) did however propose different tacrolimus AUC targets from the trough concentration level as shown in Table 1.

Table 1. Suggested AUC targets from the trough concentration level for twice- and once-daily tacrolimus formulations. (From reference (87)).

Trough target (µg/L)	Twice-daily formulation AUC_{0-12} (µg h*L)	Once-daily formulation AUC_{0-24} (µg h*L)
3 - 7	75 - 140	150 - 275
5 - 10	100 - 190	180 - 350
8 - 12	140 - 210	260 - 400
10 - 15	180 - 270	310 - 475

1.5 Adherence to the immunosuppressive treatment

1.5.1 Definition

Medication adherence is defined by The European Society for Patient Adherence, Compliance and Persistence as "the process by which the patients take their medications as prescribed (160). According to the Ascertainig Barriers to Compliance (ABC) taxonomy of medication adherence (160, 161), adherence can be divided into three different phases: initiation, implementation and discontinuation. Suboptimal adherence called nonadherence, not taking the medications as prescribed, can occur in all of these phases. Initiation is the first phase and refers to if a patient actually starts on a prescribed dose regimen and is taking the first dose, nonadherence can occur if the medication regimen is late, incomplete or non-initiated, and is a binary event (yes/no). Implementation is the second phase and describes if the actual dosing of a drug corresponds to the prescribed dosing regimen. Nonadherence in the implementation phase can occur if a prescribed dose is not taken (if missing at least two doses in a row defined as "drug holiday"), delayed, reduced or if extra doses have been taken. Discontinuation is the last phase, and refers to if a patient discontinues the medication

regimen. The duration between initiation and discontinuation is called persistence. Nonadherence in this phase can occur if a patient completely stops taking the prescribed medications on their own initiative (160).

1.5.2 Factors affecting adherence

Adherence is a dynamic behavior influenced by multiple factors (162). The World Health Organization defined five dimensions having an impact on adherence: patient-related, treatment-related, health system/provider-related, social/economic-related and condition-related factors (163). Nonadherence can be either intentional or unintentional (164). In intentional nonadherence patients take an active choice to not follow treatment recommendations by delay, alter or skip prescribed doses (165). Intentional nonadherence is largely driven by a patient's belief and motivation (165, 166), and can be a result of patients feeling the treatment does not work or is not safe (167). Forgetfulness is often a contributing factor to unintentional nonadherence, and the risk of missing a dose is higher if a patient comes out of his/her daily routine (167, 168). Therapy related factors that can influence adherence are the side effect profile of the drug and the dose frequency (169). Simplification of the treatment with the use of once-daily formulations can potentially improve adherence (170). Patients' income and insurance status are major drive forces for nonadherence in some countries (171). However, as Norway has a full-covered healthcare system, with follow-up and provided medications free of-charge, it is unlikely that these factors affect adherence in large degrees. Some studies report minority race, male gender and time since transplantation to be risk factors for nonadherence, while others have failed to prove this (172-175). There is, however, large agreement that adolescents and young adults have poorer adherence (176-178). When using a combination of drugs, nonadherence can affect one or all drugs in the drug regimen.

Medication adherence is both complex and challenging for renal transplant recipients to implement in their everyday life (179). Patients often need to cope with frequent dose changes, emotions related to the treatment and possible drug-related side effects (180). Due to the need of lifelong immunosuppressive treatment and follow-up after renal transplantation, patients often feel they have less control over their lives (168).

1.5.3 Nonadherence and transplant outcome

In the literature there is large variation in reported immunosuppressive nonadherence after renal transplantation (range 2% to 67%) (175, 181-183). Missed or delayed doses are suggested to be the main causes leading to high tacrolimus intra-patient variability (184), a risk factor for acute rejection and anti-body mediated rejection increasing the risk of graft loss (185-188). Simulation of steady-state pharmacokinetic profiles in typically stable renal transplant recipients giving twice-daily tacrolimus dosing showed that missing a single morning dose led to an important decrease in systemic exposure (76). Also a delayed timing of dose administration significantly affect systemic exposure, in addition to increasing the risk of overexposure after the next dose (76). The development of dnDSA has been linked to both high tacrolimus variability and to underexposure of tacrolimus (e.g. missing a dose, delayed timing), and nonadherence is often considered when dnDSA are detected after engraftment (27, 189, 190). Nonadherence after renal transplantation leads to poor long-term outcomes and increased health care costs (169, 177, 183, 191, 192).

1.5.4 Adherence assessment tools

To improve adherence it is crucial to regularly monitor the behavior (193). The recent guidance report and clinical checklist by the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) group recommended medication adherence to be the "5th vital sign" in transplant recipients (194). COMMIT was formed in 2015 to prevent causes of graft loss, and provide expert practical guidance for long-term identification and management of modifiable risk factors. Nonadherence is a modifiable risk factor, and tools suitable for identification of nonadherent patients or patients at risk of nonadherence are important, but challenging. The vide variation in reported nonadherence is a result of various adherence tools with different thresholds and definitions (195). Numerous tools can be used, with different reliability and sample frequency (195, 196) (Figure 5). Indirect methods include patient self-report (interview, questionnaires, diaries, smart-phone apps), adherence evaluation from clinicians, pharmacy refill data, tablet counts and electronic monitoring (197, 198). Direct methods include direct observation of dose intake and drug concentration measurements.

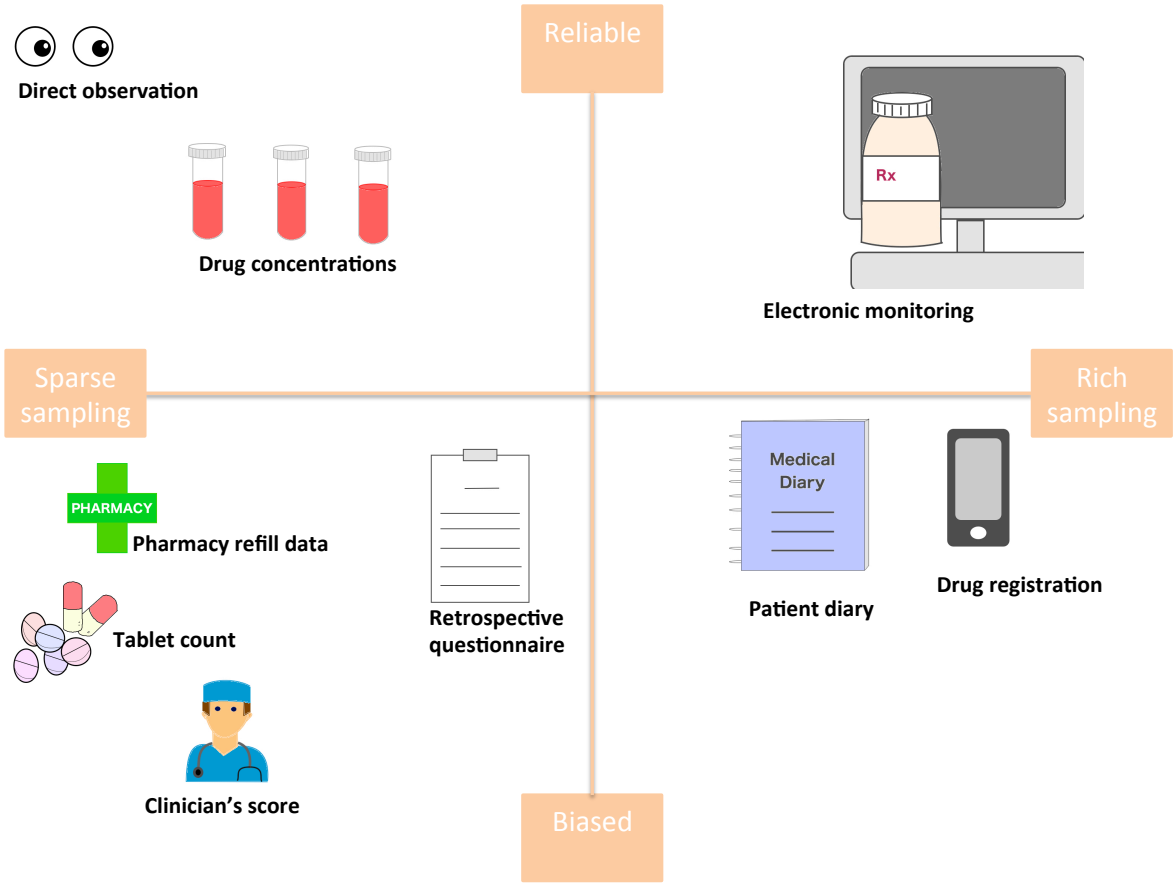

Figure 5. Illustration of the reliability and appropriate sampling frequency for adherence assessment using different adherence tools. (Modified from references (195, 196)).

Patient self-report

Self-reported adherence assessment is one of the most used adherence tools; it is practical in a clinical setting and low in cost (199, 200). A limitation is however that patients tend to underreport nonadherence as a result of social desirability and recall bias, where patients have trouble remembering their medication behavior back in time (200). Several different interviews, questionnaires and scales have been developed for use in the transplant population (201). Dobbles et al. evaluated self-report adherence tools for use in adult renal transplant recipients (199). Three different tools were recommended: the Brief Antiretroviral Adherence Index, the Medication Adherence Self-Report Inventory and the Basel Assessment of Adherence to Immunosuppressive Medication Scale (BAASIS®). An important aspect in assessing immunosuppressive adherence is the narrow therapeutic index of the drugs, and

both taking and timing of dose administration are important factors (76, 199). As many of the developed tools do not evaluate the timing perspectives, an important aspect of immunosuppressive adherence is missing. BAASIS® has established predictive validities the recent years and is today the most frequently used adherence questionnaire in transplant recipients (194, 202-206). BAASIS® follow the recent ABC taxonomy of medication adherence (160), and assess adherence using items of dose taking, timing, drug holidays, drug reduction and discontinuation.

Tacrolimus concentration variability

The use of drug concentrations and calculation of intra-patient variability has been used as a method to assess adherence. Intra-patient variability is defined as the amount of fluctuation in measured trough concentrations over a certain time period with unchanged dose(184). Nonadherence is currently considered to be the most important driver for high intra-patient variability (184, 207), which leads to poor clinical outcome after transplantation (185, 186, 208). Different methods for calculation of variability can be used: a) the standard deviation, b) the coefficient of variation (CV) calculated as the ratio of the standard deviation of the mean value, or c) the time in therapeutic range. The CV has become the most common predictor of the intra-patient variability, and has been used to indicate patient and graft outcome after transplantation (184). An intra-patient variability between 10% and 35% are often observed for tacrolimus, with an average of 15% (184, 185, 209). Different CV thresholds have been used both for assessment of nonadherence and in association with outcome, but a CV >30% has been associated with poor outcomes after transplantation (210-212) and to nonadherence (213). Drug concentration measurements represent a patient's present immunosuppression and consumption, however, as tacrolimus has large pharmacokinetic variability, other factors than adherence can influence the concentrations and complicate the interpretation (185). Using tacrolimus concentration variability as a tool for adherence assessment will only provide information about that specific drug, and not the whole immunosuppressive regimen.

Other tools

Adherence evaluation from clinicians is inexpensive and easy to implement, but are dependent on the health care personnel's familiarity and interaction with the patient and have been considered unreliable as the physician often overestimate a patients adherence (214, 215). Both pharmacy refill data and tablet counts can be biased if a patient hoard or discard

drugs, it gives no information about the timing perspective and frequent dose changes can complicate the interpretation (197, 214). For safety reasons it is also common for chronically ill patients to refill the medication before running out (216).

A gold standard method is lacking, but electronic monitoring is often promoted as one of the most reliable tools used for adherence assessment (182, 195, 196). Different systems and devices are available for registration of package entry, and now also smart ingestible sensor monitoring can be used to ensure actual dose intake (217). However, such devices are often expensive, large and are almost exclusively used in clinical trials (218, 219).

The different adherence tools are all at risk of being manipulated, and the methods itself may have an impact on adherence (220). All have limitations, and a single adherence measure will have low sensitivity when used alone (195). Results from different tools for assessment of adherence can vary depending on the adherence definition, the adherence phase being investigated (implementation, persistence and/or discontinuation), what behavior domains are captured (e.g. missed dose, drug holiday, time deviation), and are all drugs in the regimen evaluated or only a single drug (221). Reported agreement between the different tools is thus variable, from poor to moderate and high (175, 183, 195, 215, 222-224). A valuable and recommended approach has been to use a combination of tools for improved adherence assessment (195, 199).

1.5.5 Current challenges

Immunosuppressive nonadherence has been suggested to be one of the main reasons for poor clinical outcome after renal transplantation (27). Adherence is however a modifiable risk factor, and identification of potential triggers and aspects of nonadherence are important to increase adherence and potentially improve long-term outcome (194). Tools suitable for assessment of adherence data in renal transplant recipients are important, and without reliable tools to measure adherence, the consequence of nonadherence are unrecognized and considered as an unexplained variance in outcome (225). In the follow-up of renal transplant recipients in Norway, medication adherence has not been formally assessed.

2 AIMS OF PRESENT STUDIES

The overall aim of the thesis was to investigate tools to optimize the immunosuppressive treatment after renal transplantation.

Specific aims of each paper:

I) Investigate the potential use of home-based capillary microsampling in combination with LSS and Bayesian estimators from a non-parametric population pharmacokinetic model for tacrolimus AUC_{0-12} estimation.

II) First, to investigate tacrolimus pharmacokinetics after the morning- and evening dose in a real-life setting with regards to food ingestion and drug timing. Secondly, to determine the predictive performance of AUC_{0-12} and AUC_{12-24} estimations using LSS and Bayesian estimators from a non-parametric population pharmacokinetic model.

III) Evaluate adherence tools suitable for annual capture of immunosuppressive adherence in a real-world setting in a renal transplant population.

3 METHODS

3.1 Study design and data capturing

Paper I

A single-center observational pharmacokinetic study was conducted in adult standard risk renal transplant recipients receiving twice-daily tacrolimus. The study was performed at the National Transplant Center in Norway (Oslo University Hospital, Rikshospitalet) between April and October 2018. Immunological high-risk patients with donor specific antibodies present at time of transplantation or patients using drugs known to interact with tacrolimus pharmacokinetics were excluded from the study. A total of 13 venous blood samples and 13 capillary microsamples were obtained at two separate 12-hour dosing intervals in the early post-transplant phase (2-8 weeks after transplantation). Patients had to be in tacrolimus steady-state condition, i.e. on an unchanged dose for the last five days prior to investigation, and the dose was kept unchanged between the first and the second investigation. $AUC_{0\text{-}12}$ was estimated from both the venous blood samples and the capillary microsamples using a population pharmacokinetic model and Bayesian estimators. Different LSS were tested, both on a population level and on an individual level. Agreement between estimated capillary microsample $AUC_{0\text{-}12}$ with full-profiled venous reference $AUC_{0\text{-}12}$ was evaluated. The final data set included 53 pharmacokinetic tacrolimus profiles from 27 patients.

Paper II

A single-center observational pharmacokinetic study was conducted in adult renal transplant recipients receiving twice-daily tacrolimus. The study was performed at the National Transplant Center in Norway (Oslo University Hospital, Rikshospitalet) between December 2015 and May 2017. Both immunological standard- and high-risk patients were eligible for inclusion. Two successive 12-hour pharmacokinetic investigations were performed after morning- and evening administration of tacrolimus in the early post-transplant phase (2-8 weeks after transplantation). Dose administration was performed either in a fasting-state (± 2 hours fasting) or under real-life non-fasting conditions (administered as in patients everyday life; no meal restrictions). Pharmacokinetic parameters were calculated and compared between the morning- and evening administration within respective dose condition (fasting/real-life non-fasting). Population pharmacokinetic modeling developed Bayesian

estimators were performed for estimation of AUC_{0-12} and AUC_{12-24} both in the fasting state and in the real-life non-fasting setting. Different LSS on a population level were determined. Estimated AUC was compared with trapezoidal determined reference AUC. The final data set included 90 12-hour pharmacokinetic profiles (45 from the morning dose and 45 from the evening dose) from 31 patients.

Paper III

A single-center open prospective randomized control trial was conducted in adult renal transplant recipients receiving tacrolimus. The study performed at the National Transplant Center in Norway (Oslo University Hospital, Rikshospitalet) between October 2014 and June 2018. Both standard- and high-risk patients were eligible for inclusion. Two thirds of the patients were included 4-weeks post-transplant (Group A) and randomized 1:1 to either intensive (A1) or single-point (A2) adherence assessment in the early post-transplant phase and at 1-year. One third were investigated through a cross-sectional investigation for intensive adherence assessment when coming for their 1-year control (Group B). Multiple methods were used for adherence assessment: patient-reported adherence using BAASIS® questionnaire, adherence evaluation from the treating clinician, tacrolimus concentration variability was calculated, and a tablet count was performed in selected patients. The adherence data were dichotomized as adherent or nonadherent. Nonadherence prevalence, response rates and agreement between the different adherence tools were evaluated. Clinical outcome data with biopsy proven acute rejection and development of dnDSA the first post-transplant year were collected from medical records. The final data set included data from 295 patients.

3.2 Ethics

All studies were performed in accordance with the Declaration of Helsinki, guidelines for Good Clinical Practice, and approved by the local ethics committee and also by the Norwegian Medicine Agency in case of the two pharmacokinetic studies (**Paper I** and **II**). The study from **Paper I** is registered at www.ClinicalTrials.gov (NCT03512431). All participants received verbal and written study information and signed an informed consent.

3.3 Statistics

Data were assessed for normality by visual inspection of histograms, Q-Q plot and boxplot, and by performing the Shapiro-Wilks test.

In **Paper I** and **II** agreement between model-estimated AUC and reference AUC was assessed using C-statistics, with concordance correlation coefficient (CCC), total deviation index (TDI) and coverage probability (CP), according to Lin *et al*. (226). CCC measure the agreement between two measures, and the values can range from 0 to 1; 0 reflects no correlation, while 1 reflects perfect correlation. In both **Paper I** and **II** a CCC $\geq$0.9 was set as acceptable level, reflecting sufficient agreement between measurements. TDI assess the proportion of data within a pre-set boundary for an allowed difference between the different measurements. CP estimates if a TDI is less than a pre-specified fixed percentage. Pre-defined agreement levels in **Paper I** and **II** were: TDI $\leq$15% and CP $\geq$0.85. If TDI is 15 and CP is sat to 0.85, it means 85% of the estimated measures have an error less than ±15% when compared to the reference measure.

Paper II

Non-normally distributed paired variables were compared using Wilcoxon signed rank test. The non-parametric Friedman test, which is the nonparametric alternative to a one-way ANOVA, was used for comparison of the trough concentrations within a dosing interval (C_0, C_{12}, C_{24}). Correlation between trough concentrations and AUC was analyzed using Spearman's rank correlation coefficient.

Almost half of included patients performed two pharmacokinetic investigations. Each investigation was analyzed separately. The numbers of patients were therefore not equally balanced in the analysis, as patients with two investigations were over-represented and patients with one investigation under-represented. We did however choose to analyze each investigation separately as the immunosuppressive doses were changed in some patients from the first to the second investigation, and due to the rapidly pharmacokinetic change in the early post-transplant phase (52, 71).

Paper III

For group comparison the two-tailed independent sample T-test was used for normally or log-normally distributed continuous unpaired variables, while Pearson's chi-square of Fisher's

exact test were used for categorical variables. Agreement between the different adherence tools was analyzed using Cohen's kappa, and the McNemars test was used to determine adherence changes over time within the same patient. A Cox regression analysis, using the coxphf package in R (227, 228) was performed to determine if adherence was a risk factor for biopsy proven acute rejections and development of dnDSA. Adherence, assessed by the different tools, was included as a time dependent variable. Each adherence assessment from different tools was investigated separately, and age, sex and study group were added in each model.

3.4 Population pharmacokinetic modeling

Non-parametric population pharmacokinetic modeling was performed in **Paper I** and **II**. The non-parametric adaptive grid (NPAG) implemented in Pmetrics® for R was used for modeling approach (135). Pmetrics® version 1.5.1 and R version 3.4.4 (228) or later versions were used. In **Paper I** the BestDose® package for R was used for estimation of individual pharmacokinetic parameters and concentration-time curves.

Tacrolimus model

A previous developed model by Åsberg et al. (59) was used in **Paper I** and **II**. In short, this is a 2-compartment model with first-order absorption from the dosing compartment into the central compartment after lag-times depending on time after transplantation, and distribution to and from a peripheral tissue compartment. The model is parameterized with apparent clearance (CL/F), intercompartment clearance (Q/F) and central and peripheral volume of distribution (V/F, Vp/F). Hematocrit is included as a covariate on clearances and volumes to describe the intracellular distribution of tacrolimus into erythrocytes. CL/F, Q/F and Vp/F are allometrically scaled by fat free mass (with fixed exponents of 0.75 and 1.0, respectively), while V/F is scaled to body mass index. A large proportion of the data used to develop this population pharmacokinetic model was morning trough concentrations of tacrolimus.

Model adaption

In **Paper II** the model by Åsberg *et al.* (59) was adapted to also handle the slower absorption and flatter pharmacokinetic profiles detected after the evening dose and after dose administration in a real-life setting (not respecting the ± 2 hours fasting rule). The adaption was limited to optimization of the parameter boundaries of the absorption rate constant and

the lag-times. The data was randomly divided into a model-adaption dataset (≈2/3 of the available pharmacokinetic profiles) and to a validation dataset (≈1/3 of the available pharmacokinetic profiles). The model-adaption dataset was cycled to convergence using the *NPrun* function in Pmetrics®, and further used as an internal validation of the model. The validation dataset was used for model-derived AUC estimations using the adapted model as Bayesian prior.

Model validation of the adapted model was performed by calculating relative bias ± standard deviation with related root mean square error for observed concentrations and estimated concentrations, and by plotting:

- Observed versus predicted plot; observed values were plotted against the model predicted values, giving an estimate of how well the predicted and observed values matched.
- Residual error plot; the difference between the model predicted values and the true observed value was plotted. The residual errors were plotted against the number of observations. The plot check for systematic patterns in the data that are not described by the model.
- Prediction corrected visual predictive checks (pcVPC); by graphically simulate if the model reproduced the central trend and variability in the observed data (229). Dose, sampling time, fat-free mass, hematocrit and time after transplantation were used for binning, and each individual was used as template for 1000 simulations. The plot estimate how well the observed values are covered by 95% confidence intervals of the predicted values.

Limited sampling strategies

In **Paper I** and **II** different LSS were used for estimation of AUC. The empirically based strategy using samples obtained at 0-, 1- and 3-hour post dose (140, 148, 157) was tested and used in both **Paper I** and **II** for estimation of $AUC_{0\text{-}12}$ in a fasting state.

To determine the best sampling strategy based on the patient data, the multiple model optimal sampling (MMopt) function was used to determine three optimally timed samples weighted for AUC. MMopt can find the sampling times that minimize the risk of misrepresenting the patient as the wrong set of support point in the model, meaning estimating the wrong set of

pharmacokinetic parameters for the patient (230). In **Paper I** the MMopt function was used both in Pmetrics®, for identification of optimal sampling times on a population level, and in BestDose®, for identification of individual optimal sampling times. MMopt sampling times is different for each individual patient due to the impact of different combinations of input data (e.g. concentrations, sex, weight, hematocrit). Each patient therefore has his/her own unique combination of optimal sampling time points. In **Paper II** the MMopt function was used only on the population level.

3.5 Bioanalytical tacrolimus methods

Tacrolimus whole-blood concentrations were measured in all papers (**Paper I-III**). The chemiluminescent microparticle immunoassay (CMIA, analysed on the Architect Instrument; Abbott Laboratories, Abbot Park, IL) was used until August 2015. From September 2015, all concentrations were measured with liquid chromatography-tandem mass spectrometry assay (LC-MS/MS). When using the immunoassay, tacrolimus concentrations are on average 18% higher when compared to concentrations measured with LC-MS/MS, which is considered the gold standard (231).

In **Paper III**, both analytical methods were used, as the study was conducted between October 2014 and May 2018. Measured concentrations were used to calculate variation coefficients for tacrolimus intra-patient variability, and as the same analytical method was used for the individual patient, it is unlikely that the overestimation of tacrolimus concentrations using the immunoassay has affected the results. In **Paper I** and **II** only the LC-MS/MS method was used.

In **Paper I** both venous blood samples and capillary microsampling using the volumetric absorptive capillary microsample device Mitra® (10 μL; Neoteryx, Torrance, CA) were obtained. All samples were analyzed with LC-MS/MS. A study by Vethe *et al.* validated the analytical performance of the sample preparation and assay of the capillary microsample procedure using the same data as in **Paper I** (232). Capillary microsampling was comparable to venous blood assay throughout the entire dose interval, it demonstrated acceptable analytical and methodological performance, and the assay sample preparation required less time than the venous liquid assay.

3.6 Adherence assessment

In **Paper III** a combination of different adherence tools were used for adherence assessment. The results from the adherence tools were dichotomized as shown in Table 2. Adherence assessment itself has the potential to affect a patient's adherence. The study therefore consisted of two patient cohorts; group A and group B, to control for the increased awareness of adherence behavior. For evaluation of how often adherence should be assessed, group A was additionally randomized to an intensive adherence assessment group with more frequently follow-up (A1), or a single adherence assessment group (A2).

Table 2. Definition of nonadherence by the used adherence tools.

Tool	Definition of nonadherence
BAASIS®	Missed one or more doses and/or a time deviation > 2 hours from prescribed time the last 4 weeks
Adherence score from clinicians	Physician/nurse scored patients adherence as suboptimal or poor (≠ excellent)
Tacrolimus concentration variability	A CV > 30% (using six concentrations at the 8-week investigation and three concentrations at the 1-year investigation)
Tablet count	A counting that corresponded to < 90% or >110% of prescribed dosing schedule during a 2 week period

CV; coefficient of variation

Self-reported

Self-reported adherence was assessed using BAASIS® questionnaire. BAASIS® assess adherence from the whole immunosuppressive regimen and consists of four different questions asking about dose taking, timing (> ±2 hour time deviation), dose reductions and discontinuation from the last 4-weeks. A "yes" to any of the four questions were considered as nonadherent. In addition, nonadherent patients were divided into either taking nonadherence (missed one or more doses the last 4-weeks) or timing nonadherence (took all prescribed doses, but had a time deviation > ± 2 hours from prescribed time the last 4-weeks).

Patients primarily answered the questionnaire online, by following a link sent via e-mail. All answers were encrypted and stored directly at the University of Oslo services for sensitive

data (TSD 2.0). The questionnaire was also available in paper form for patients not using or having access to Internet for various reasons. The completion time of BAASIS® was less than five minutes. A reminder was sent to patients not delivering their form within 3-7 days after transmission. The 8-weeks and 1-year answers were used when analyzing the data. If a patient in the intensive follow-up group did not answer BAASIS® at either 8-weeks or 1-year, the answer closest in time to respective time period was used.

Adherence score from clinicians

The treating health care personnel that followed the patient scored patients individual immunosuppressive adherence on a 3-point scale: "poor", "suboptimal" or "excellent". Only "excellent" was interpreted as adherent. The score was obtained both in the early post-transplant phase (Group A) and at the 1-year investigation.

Tacrolimus concentration variability

The intra-patient variability in tacrolimus concentrations was calculated using CV:

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

σ is the standard deviation and μ is the mean concentration. A CV% > 30 was interpreted as nonadherent. The blood samples used for calculations were trough concentration measurements drawn immediately before the morning dose. In the early-post transplant phase (6-9 weeks after transplantation) six different TDM concentrations were used for CV calculation. At the 1-year investigation the last three TDM concentrations during the last 3 months were used. For a concentration to be used in the calculation, the patient had to be on a stable tacrolimus dose for the last three days prior to measurement. At the 1-year calculation, the last three tacrolimus concentrations back in time were used, as long as the dose had been unchanged. In this post-engraftment phase most patients visit their physician to monitor the tacrolimus concentration every month.

Tablet count

A manual tablet count was performed in the intensive adherence assessment group in the early post-transplant phase (group A1) on tacrolimus, mycophenolate mofetil and prednisolone. The count was performed between two clinical visits separated by 2-weeks. A count that corresponded < 90% or > 110% of prescribed dosing schedule was interpreted as

nonadherent: i.e. missing 2 doses of twice-daily formulations or 1 dose of once-daily formulations. For a tablet count to be valid, patients had to bring all their immunosuppressive medication containers to the two visits.

3.6.1 Clinical outcome

In addition to adherence assessment in **Paper III**, biopsy-proven acute rejections were obtained from 4-weeks to 1-year post-transplant. Early rejections before week 4 were disregarded as this was before time of study inclusion, and rejections before this time period are most likely not caused by nonadherence, but rather other factors. Graft loss and mortality were obtained from patient chart for all included patients during the study follow-up. At the 1-year investigation detection of dnDSA and glomerular filtration rate measured by 2-point iohexol plasma clearance were determined.

4 SUMMARY OF RESULTS

Paper I

Tacrolimus area under the concentration versus time curve monitoring, using home-based volumetric absorptive capillary microsampling

Using a single trough concentration to estimate $AUC_{0\text{-}12}$ showed unacceptable low agreement when compared to the venous full-profiled reference $AUC_{0\text{-}12}$. LSS with sampling times 0-, 1- and 3-hour post-dose using capillary microsamples showed acceptable agreement (CCC 0.941, TDI 14.9, CP 0.85). The current findings suggest that three capillary microsamples concentrations can be used for $AUC_{0\text{-}12}$ estimation, and is suitable for use in TDM of tacrolimus. However, prospective clinical trials to determine optimal AUC-targets are needed.

Paper II

Fasting status and circadian variation must be considered when performing AUC-based therapeutic drug monitoring of tacrolimus in renal transplant recipients

Circadian variation was observed under fasting conditions, with 45% higher peak-concentration and 20% higher AUC following the morning dose ($P<0.006$) (Figure 6, A and B). Under real-life non-fasting dose administration circadian variation was not present: the pharmacokinetic-profiles were flat and comparable after the morning- and evening dose (Figure 6, C and D). The absorption rate and AUC were lower when compared to the fasting morning administration. LSS with samples obtained at 0-, 1-, and 3-hour predicted $AUC_{0\text{-}12}$ well for the fasting morning dose. Using a previously developed population pharmacokinetic model, adapted to fit the slower absorption, LSS with sampling times 1-, 3- and 6-hour predicted AUC well for the fasting evening dose ($AUC_{0\text{-}12}$) and the real-life non-fasting dose administration ($AUC_{0\text{-}12}$ and $AUC_{12\text{-}24}$). This study demonstrates that monitoring tacrolimus in a real-life setting produce flatter pharmacokinetic profiles and no circadian variation. For a population pharmacokinetic model to estimate AUC during both conditions, data on the real-world behavior are essential.

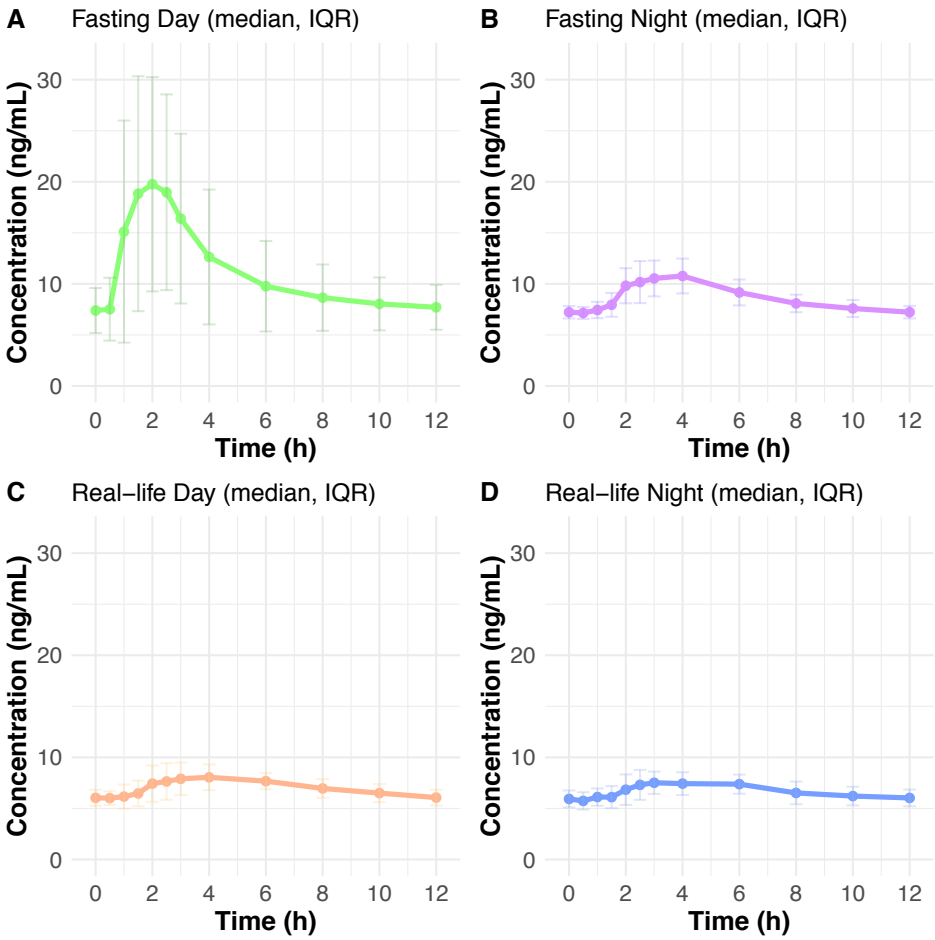

Figure 6. Individual time-corrected concentrations were used to make median concentration versus time curves with related inter quartile range (IQR) for the four different dose scenarios: A) fasting morning dose (n=11 12-hour pharmacokinetic profiles), B) fasting evening dose (n=11 12-hour pharmacokinetic profiles), C) real-life non-fasting morning dose (n=34 12-hour pharmacokinetic profiles), and D) real-life non-fasting evening dose (n=34 12-hour pharmacokinetic profiles). (Adapted from Figure 2 in Paper II).

Paper III

Evaluation of tools for annual capture of adherence to immunosuppressive medications after renal transplantation – a single centre open prospective trial

The response rates and data capturing were good when using BAASIS®, adherence score from clinicians and tacrolimus concentration variability, with a total response rate and sufficient data above 80%. Tablet count was difficult in a clinical outpatient setting, as patients did not bring all their immunosuppressive medications to the visits. With only 43% evaluable tablet

counts; the method was not a tool suitable for annual adherence assessment in a routine setting. Adherence assessment using the BAASIS® questionnaire, adherence score from clinicians and tacrolimus concentration variability captured different patients as nonadherent, the overlap was low and only a weak agreement was observed between the tools. The nonadherence prevalence at one-year ranged from 7% to 38% depending on the assessment method. Overall, nonadherence was due to sub-optimal implementation of the immunosuppressive medications during the first post-transplant year. More intensive adherence assessment captured more nonadherent patients, but did not improve the adherence behaviour *per se*. Nonadherence did not increase the risk of biopsy proven acute rejection, but patients with nonadherent BAASIS® evaluations had an increased risk of developing dnDSA the first post-transplant year. The present study suggests that a combination of BAASIS®, adherence score from clinicians and tacrolimus concentration variability is feasible for annual routine adherence assessment. When including these tools in a national quality register, they can identify factors influencing adherence in the long-term post-transplant phase.

5 DISCUSSION

The short-term outcome after renal transplantation is good, however, there is still room for improvements in long-term outcome. Utilizing the current immunosuppressive regimes, optimization and individualization of the immunosuppressive treatment is warranted to further extend long-term patient- and graft survival (87). Finding safe methods to reduce tacrolimus-related adverse effects without compromising the immunosuppressive effects, leading to an increased risk of acute rejection and dnDSA development are crucial (233). Even though plausible, it has not been proven that dose adjustments of tacrolimus using trough concentrations actually improve long-term outcome (86). The EliTE SYMPHONY-trial showed that low-dose tacrolimus trough target resulted in a good balance between acute rejection rate and renal function the first year after transplantation (98). It is however, challenging keeping patients within this concentration range (234). This has lead to an increased focus on finding new TDM strategies or new biomarkers for optimization of tacrolimus dosing (87, 235). However, finding the optimal dose is meaningless if patients are not adherent. Nonadherence to the immunosuppressive regimen after renal transplantation is a major challenge and an independent risk factor for poor clinical outcome (27, 169). Assessment of immunosuppressive adherence and identification of factors affecting the behavior are important if trying to overcome this problem.

This thesis has contributed with important strategies and tools that can advance the follow-up after renal transplantation and optimize the immunosuppressive treatment. Potentially this will result in improved long-term outcome for this patient population.

5.1 Modern therapeutic drug monitoring

5.1.1 Real-life systemic exposure

To prevent immunological damage it is crucial to keep tacrolimus exposure sufficiently high. Conflicting reports exist regarding correlation between trough concentration, systemic exposure and tacrolimus efficacy (87). In **Paper II**, time of dose administration affected tacrolimus pharmacokinetics in a fasting state. Circadian variation was present, with a slower absorption and flatter pharmacokinetic profile with a lower AUC and C_{max} when administered in the evening. The difference between $AUC_{0\text{-}12}$ and $AUC_{12\text{-}24}$ was however not reflected by respective trough concentrations (C_0, C_{12}, C_{24}) when administered under fasting conditions.

Only fasting morning dose administration produced the well known tacrolimus pharmacokinetic profile with a sharp peak around 20 μg/L after 1-2 hours (82). In the real-life non-fasting setting, circadian variation was not present, and the pharmacokinetic profiles both after morning and evening administration produced relatively flat pharmacokinetic profiles. One may therefore speculate that the level of immunosuppression and definitely the proposed therapeutic AUC levels actually required are overestimated. As all outcome studies are based on trough levels (97, 98, 158), perhaps it is satisfactory to keep tacrolimus whole-blood concentrations right above the trough target over the full dose interval, as observed in the real-life non-fasting setting? Side effects of tacrolimus are often suggested to be linked to high tacrolimus whole-blood concentrations (236-238), but as C_{max} is much lower in a real-life non-fasting setting, this association may also be overestimated.

Given the flat pharmacokinetic curves in the real-life non-fasting setting, one can hypothesize that patients are more vulnerable to nonadherence, and a missed dose or a time deviation can cause extremely low concentrations. In **Paper III**, nonadherence did not increase the risk of biopsy proven acute rejection, but was associated with an increased risk of dnDSA development the first post-transplant year. This is unfortunate, since dnDSA are associated with higher rates of graft failure and complicates re-transplantation (27, 239). Nonadherence early after transplantation can affect the likelihood of graft loss and death the next decade (240). As over 30% of patients were assessed as nonadherent when using a combination of tools in **Paper III**, this proves the importance of including adherence assessment in the follow-up of renal transplant recipients. Once detected, prevention studies must be applied. How this can affect long-term clinical outcome requires further investigations.

In **Paper II**, the correlation between trough concentrations and AUC was dependent on the fasting/non-fasting status. The trough concentration reflected systemic tacrolimus exposure better in the real-life non-fasting setting, considering the flat curves and the strong correlation between C_{24} and $AUC_{0\text{-}24}$. One can then argue that there is not so much to gain by performing AUC-monitoring in a real-life setting, and it might be that trough-based TDM actually is as good as you can get it in the clinic. This is somewhat supported by the low acute rejection rate with todays trough-based monitoring. The optimal total daily tacrolimus exposure is unknown, and with lack of validated AUC-targets, it is difficult to interpret the correlation between trough and AUC, as the AUCs are very different between dosing situations. Dose recommendations of tacrolimus administration either in a fasting or non-fasting state cannot

be given based on data from **Paper II** before clinical outcome on this aspect is assessed. But keeping in mind that the evening dose is the one preceding the clinically used trough concentration measurement, one could argue that also in the morning, drug intake might accompany breakfast, since this would tend to give similar exposure following morning- and evening doses.

The newest once-daily LCP-tacrolimus is a delayed absorption formulation released and absorbed more distally throughout the digestive tract (241). LCP-tacrolimus might therefore be less susceptible to variations induced by timing of dose intake and food ingestion. Patients receiving LCP-tacrolimus formulation have lower peak to trough fluctuations within dosing intervals and a lower C_{max} when compared to immediate- and extended-release tacrolimus formulations when administered in a fasting state (77). A recent study performed in healthy volunteers receiving LCP-tacrolimus reported that the timing of dose administration did not affect the pharmacokinetic of tacrolimus, however, concomitant food intake reduced AUC_{0-24} and C_{max} (242). This proves that also for other tacrolimus formulations, real-life behavior of the patients is important to account for when monitoring tacrolimus to optimize the dosing and especially if aiming towards an AUC-target.

5.1.2 Sampling strategies

With the use of modern sample approaches, as the volumetric absorptive capillary microsample procedure performed in **Paper I**, blood samples for tacrolimus concentration measurements can be obtained from home by patients themselves. This sampling approach can easily be implemented as a supplement in tacrolimus TDM routine and enables a closer follow-up of renal transplant recipients with more intensive and flexible blood sampling. Other TDM strategies using alternative concentration targets and time points can be investigated, and collection of data for use in clinical trials or rich data for development of appropriate population pharmacokinetic models can more effortlessly be obtained.

AUC-monitoring

Population pharmacokinetic model derived Bayesian estimators in combination with LSS can estimate tacrolimus AUC, using only three samples within a dosing interval. Normally samples are obtained within the first 3-4 hours of the dosing interval for practical reasons (140). In **Paper I**, the empiric sampling scheme 0-, 1- and 3-hour post-dose showed

acceptable AUC_{0-12} predictions for the fasting morning dose. This was also confirmed in **Paper II**. However, other sampling time points were needed for appropriate estimations of AUC after the evening dose (AUC_{12-24}) and dose administration in the real-life non-fasting setting (AUC_{0-12}, AUC_{12-24}): sampling times at 1-, 3- and 6-hour post-dose were needed. Different optimal sampling times are thus required for appropriate estimations of AUC, depending on the time of dose administration and the fasting state.

It has not been proven that AUC-based TDM of tacrolimus actually improve patient- and graft survival over standard trough-based TDM, as no randomized clinical trials have been performed with this aim (87). Both in **Paper I** and **II** large variations in AUC was observed in the early post-transplant phase. In **Paper I**, only including immunological standard risk patients, AUC_{0-12} ranged from 70 to 226 µg h/L. The highest values being way outside currently suggested AUC-range (87). Validated AUC-targets must be defined before prospective studies comparing trough- and AUC-based monitoring can be performed. Further on, comparison of dose recommendations from different TDM-strategies and how this possibly translates into clinical meaningful improved outcome are needed.

5.1.3 Population pharmacokinetic modeling

When performing current standard trough-based TDM on twice-daily dosed tacrolimus, a similar pharmacokinetic is assumed after the morning- and the evening dose. Population pharmacokinetic models that assume similar pharmacokinetic-profiles following the morning- and evening-dose of tacrolimus will induce biased AUC_{0-24} predictions due to circadian variation and the food effect on the pharmacokinetics observed in **Paper II**. In **Paper I** and **II** the same structural model was used, but in **Paper II,** the model was also adapted to describe the pharmacokinetics after the evening dose and the real-life non-fasting dose administration. AUC-based monitoring of tacrolimus is thus more complex than first anticipated. If used to optimize tacrolimus treatment, it is important that the population pharmacokinetic model describes the real-world behaviour of patients. Data from **Paper II** demonstrates the importance of knowing a patient's behavior in dose administration and food timing. This can be achieved by simply asking the patient and registering the answer. In Norway renal transplant recipients are instructed to be consistent with their immunosuppressive dosing-schedule, both in terms of concomitant food intake and in timing of dose administration of tacrolimus and other co-medications. However, assuming stable behavior patterns in patients

is not warranted. A patient's habit will most likely change and alter over time according to different situations, and it is difficult to assume and fix certain covariates affecting tacrolimus pharmacokinetics. This is in agreement with findings in **Paper III**, where adherence behavior was not stable within patients, and the most common cause of immunosuppressive nonadherence was a time deviation in dose intake. This affects and challenges the use of population pharmacokinetic modeling, as both the timing of dose and food clearly have large impact. One possible solution is to use a smart-phone app to register both exact dosing times and food intake, with information of fasting/non-fasting status. Further on, for the model to be really user friendly, it must be able to use that information directly to distinguish between different dosing times and fasting/non-fasting situations, for predictions of pharmacokinetic parameters and curve patterns.

Nonadherence, fasting/non-fasting status and time of dose administration are all factors that can cause pharmacokinetic variability (62, 67, 76). These factors have been challenging to measure, are not easily implemented in a population pharmacokinetic model and are often considered as unexplained variability. This can now be investigated by using the population pharmacokinetic model used from **Paper II** to simulate what impact either a single missed morning-/evening dose or a delayed dosing time have on tacrolimus exposure both in a fasting- and a real-life non-fasting setting.

5.2 Immunosuppressive adherence

In **Paper III**, nonadherence was a result of sub-optimal implementation of the immunosuppressive medications during the first post-transplant year. Nonattendance to healthcare appointments has been associated with immunosuppressive nonadherence (162). Multilevel interventions, beyond the patient level, are therefore important when trying to improve adherence. The organization of the transplant follow-up care is a factor that can have an impact (181, 243, 244). Utilizing capillary microsampling from **Paper I** in an at-home setting can reduce the number of necessary clinical appointments in the follow-up of renal transplant recipients. By further exploiting technology with the use of a smart-phone app as a dose reminder, for registration of data (i.e. dosing time, time of food consumption), and as a platform to give patients feedback on their adherence status, their concentration levels and future dose changes, the follow-up of renal transplant recipients can be simplified. None of the adherence tools used in **Paper III** can distinguish between intentional or unintentional

adherence, and we cannot specify any reason behind the behavior, besides a missed dose or a time deviation. Nonadherence was not a constant behavior during the study period, we therefore do believe most nonadherence was unintentionally and sporadic, and an extra reminder of the medications intake might help (245). This will also allow the patients to be more responsible and "included" in their every-day medical treatment, factors suggested to potentially improve adherence (246).

Previous studies have reported simplification of drug regimen to improve intra-patient variability and medication nonadherence, both important factors influencing long-term outcome (27, 185). It is however important to remember that renal transplant recipients have a high pill burden, as the immunosuppressive treatment today normally consists of a triple drug regimen. After a switch to a once-daily tacrolimus formulation most patients will still be on a twice-daily mycophenolate mofetil schedule (247). A missed dose of a once-daily formulation will cause a reduced drug exposure the next 24 hours, which might be more severe than missing one out of two doses of a twice-daily formulation only affecting the next 12 hours (248). We did not find any difference in nonadherence one-year post-transplant when comparing patients on a twice- or once-daily tacrolimus dosing regimen in **Paper III**. The focus in the study was however to assess adherence of the whole immunosuppressive regimen, and it was not designed to look specifically for differences between twice- and once-daily formulations. BAASIS® assess adherence of the whole immunosuppressive regimen, and as mycophenolate mofetil is dosed twice-daily, comparing nonadherence between the two tacrolimus formulations is challenging when using BAASIS®. However, patients using once-daily tacrolimus formulation did not have lower tacrolimus concentration variability, as previously reported (249). The immunosuppressive dose regimen should be tailored to each individual patient, and the choice of formulation should be decided in collaboration with the patient.

5.2.1 Annual assessment

For annual assessment of adherence data, the ideal adherence tool is low in cost, user-friendly, easy to implement, reliable and practical (250, 251). Based on data from **Paper III**, more patients were assessed as nonadherent with a more intensive assessment method. Repeated measures should therefore be applied when using these tools for adherence assessment in a clinical routine setting, and one can speculate if annual assessment of

adherence is too infrequent. It is however not clinically applicable to collect responses as often as BAASIS® was assessed in the present study, and it will not be favorable, as we did see a reduced response rate with time in the frequent assessment group. What the optimal compromise would be cannot be determined. For registries to collect 2 responses separated by 4-weeks each year should be possible and affordable in a clinical routine setting. Adherence score from clinicians was found to be applicable in a clinical setting, however, as the interaction between patients and clinicians is limited in the long-term follow up, this tool is most likely the less informative. This is supported by the findings in **Paper III**, where nonadherence assessed by clinicians was considerably lower than what was assessed by the other tools. Calculation of tacrolimus concentration variability was possible to implement on an annual basis by using traditional TDM trough concentrations. TDM of tacrolimus in the long-term follow-up are more spaced in time, which can increase the risk of other factors affecting the concentrations. With the use of the validated capillary microsample method used in **Paper I**, collection of tacrolimus concentrations over a defined period for calculation of intra-patient variability can be more flexible.

The increased focus on adherence in the early-post transplant phase did not improve adherence one-year later. The tools are suitable in identifying patients that need assistance with their immunosuppressive medications, but for further improvement of adherence, intervention studies are needed (194, 252, 253).

5.3 Methodological considerations

A limitation in **Paper I** and **II** is the lack of pharmacokinetic profiles long time after transplantation. The findings may not be representative for later post-transplant years and extrapolation should be performed with care due to pharmacokinetic changes with time after transplantation. Secondly, only the twice-daily tacrolimus formulation was investigated in both studies. The population pharmacokinetic models used in **Paper I** and **II** have not been developed or validated for use in patients receiving once-daily tacrolimus formulation. It is unlikely that models are interchangeable between different formulations without any adaptions.

Paper I

High-risk patients were excluded from inclusion in the present study. A study criterion was that patients had to be on an unchanged tacrolimus dose from the first to the second pharmacokinetic investigation. As high-risk patients have a higher possibility of needing a dose change, especially in the early post-transplant period, they were excluded.

The MMopt function in BestDose® was used to determine individual optimal sampling times for AUC_{0-12} estimation. Three individual optimal sampling times weighted for AUC were determined for each patient by implementing individual data from the first pharmacokinetic investigation. These individualized MMopt sampling time points were applied during the second investigation. This was performed in an attempt to provide the optimal sampling times that provide the least biased predictions, and to overcome restriction in study design and prespecified sampling scheme (140, 254, 255). Estimated AUC_{0-12} using the individualized MMopt sampling times did however not result in accepted agreement with respective reference AUC_{0-12}. An explanation may be the rapidly change in tacrolimus pharmacokinetics early after transplantation (52, 71, 73), making individually determined optimal sampling times not valid a week ahead in this early post-transplant phase. This is a clear limitation and can limit the use of population pharmacokinetic models in tacrolimus TDM in the early post-transplant phase. At least models need to better describe the pharmacokinetic change with time after transplantation.

Agreement between venous blood samples and capillary microsampling was, as reported by Vethe *et.al*. (232), within the acceptance limit according to the European Medicine Agency throughout the entire dose interval. However, some samples showed moderate variability, resulting in different LSS estimated AUC_{0-12} for the venous sampling and capillary microsampling procedure. There is an increased risk for bias in a single capillary microsample measure, and it is important that patients receive systematically training and guidance of the sampling procedure. A reason that can explain some of the variability in single samples is that there was on average two to five minutes between when the venous sample and the capillary microsample were drawn. This can introduce biologically true changes in concentrations, especially in the steep absorption and distribution phases, underestimating the agreement between samples.

Paper II

Tacrolimus circadian variation is a controversial, as some publications suggest reported variation is only a result of study design and the time interval between food and tacrolimus dosing (242, 256). In **Paper II** we observed circadian variation of tacrolimus pharmacokinetic in the fasting state, with a slower absorption rate and lower systemic exposure after the evening dose. However, due to the study design, patients fasted for a longer time prior to the morning dose than the evening dose. The morning dose was administered after an overnight fast, and breakfast was consumed 2 hours after dosing. The evening dose was administered after a ± 2 hour fasting rule. This difference in fasting time may have contributed in the observed circadian variation. However, a ± 2 hour fasting rule is considered sufficient to avoid the influence of food (61) and therefore we do not believe this has affected our results.

Validation of a population pharmacokinetic model should be performed in a separate dataset (140). In the model adaption process in **Paper II**, data was divided into a model-adaption dataset and a validation dataset. This provides more evidence for generalizability, but results in a smaller number of patients. Both in the model adaption dataset and the validation dataset the number of patients were low, limiting the internal- and external validation of the model. The findings must therefore be confirmed in a larger cohort of patients.

Paper III

Selection bias can occur if included participants are systematically different from non-participants. In **Paper III** we cannot exclude selective non-participation, as patients with poor adherence are more likely to refuse study participation. In addition, group B consisted of patients actually meeting at the transplant center for their 1-year control. Patients with clinic nonattendance might have a higher risk of poor transplant outcome (257, 258). In year 2013, 2014 and 2015; 83%, 76% and 85% of all living renal transplant recipients with a functioning graft met for their 1-year investigation, respectively.

Adherence assessment

There is a high possibility that patients not responding to self-reported adherence assessment are nonadherent. We did not evaluate non-responders as nonadherent, and we did not remove them from the analysis, as these patients had adherence evaluation assessed by the other tools. BAASIS® response rates were overall 87% at 8-weeks and 82% at the 1-year investigation.

The “true” self-reported nonadherence prevalence is therefore most likely higher than what was reported in the study.

The lack of a gold-standard assessment method for identification of the “true” nonadherent prevalence is a limitation. Identification of the optimal tool or combination of tools for assessment of adherence is therefore hard to interpret, as the study was not designed or powered for this. The present study aimed to investigate what adherence tools are possible to implement in a clinical routine setting in order to get annual adherence data into a registry. When the tools are implemented in a registry, future analyses will hopefully elucidate the potential association each tools has on long-term outcome.

Tacrolimus intra patient variability was calculated using CV. Patients with a CV >30% were considered nonadherent. This threshold has previously been associated with poor clinical outcome after renal transplantation (211). However, it must be kept in mind that no standardized threshold of tacrolimus CV has been recommended (184, 222). This complicates the comparison of results between studies. Also, the timing of when tacrolimus concentrations and dosing information are collected is important for comparison of data between trials. The first 3 months after transplantation is not considered an optimal period to assess tacrolimus intra-patient variability, as a lot of other factors, rather than adherence, can cause variation (184). After the first 6 months following transplantation patients are considered to be in a stable phase, where the intra-patient variability is a more relevant marker for tacrolimus efficacy and toxicity (208, 211). The results from the adherence assessment using tacrolimus concentration variability in the early-post transplant phase should therefore be interpreted with caution, as high variation may not be nonadherence, but normal early post-transplant variability.

Adherence assessment using tablet counts were hard to perform in the trial, and were not further analyzed due to biased data collection. We concluded that tablet count is not a suitable tool for annual adherence assessment in a clinical routine setting. The main problem was that patients forgot some medications and packages at home when coming back for the control measurement. The fact that tablet count is not applicable in continuous routine monitoring is in agreement with previous findings reporting tablet counts to be disadvantageous as a result of time constrains and restricted resources (198).

6 CONCLUSION

The objective of the thesis was to evaluate tools and strategies for optimization of the immunosuppressive treatment after renal transplantation. Based on the different papers the following conclusions have been made:

I) By applying LSS with sampling times 0-, 1- and 3-hour post-dose, in combination with population pharmacokinetic model derived Bayesian estimators, accurately estimation of AUC_{0-12} during fasting dose administration is possible. Capillary for determination of tacrolimus concentrations is suitable for this strategy and is possible to implement in routine TDM.

II) Circadian variation of tacrolimus pharmacokinetics was observed when administered in a fasting state. Dose administration in a real-life non-fasting setting showed no circadian variation and produced relatively flat pharmacokinetic profiles. Using different LSS and population pharmacokinetic model derived Bayesian estimators, estimations of AUC_{0-12} and AUC_{12-24} were possible both in a fasting and in a real-life non-fasting setting, but data on the real-world behavior of the patients are essential.

III) Different adherence tools were evaluated for implementation in a quality register. The BAASIS® questionnaire, adherence score from clinicians and tacrolimus concentration variability were applicable for annual routine capture of adherence data. The tools identified to a large degree different patients as nonadherent, and they should be used in combination for identification of potentially high-risk patients.

7 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Capillary microsampling, used in **Paper I**, can easily be implemented to supplement routine TDM of tacrolimus. The method enables closer follow-up with more flexible and intense blood sampling. Further research should include renal function parameters in addition to drug concentration measurements in each microsample (117). This will simplify and potentially improve the care of renal transplant recipients by reducing patient burden and costs.

LSS and population pharmacokinetic model derived Bayesian estimators can give accurately estimations of AUC, as shown **Paper I** and **II**. Before AUC-based monitoring of tacrolimus can be used for tacrolimus dose recommendations, optimal AUC-targets needs to be validated. Clinical superiority of AUC-based monitoring of tacrolimus has not been demonstrated and a direct comparison of the different exposure targets of tacrolimus is needed. Randomized controlled trials comparing AUC-based monitoring of tacrolimus against standard trough-based monitoring and how this affects clinical outcome is required. By utilizing home-based capillary microsampling to obtain concentration measurements, such clinical trials can more easily be performed within reasonable cost and effort from both patients and investigators.

Further research should look on the possibility of including a pharmacodynamic measure in combination with pharmacokinetic to build a pharmacokinetic-pharmacodynamic model, for a better estimation of the actual effect of tacrolimus (87). Also inclusion of kidney-specific biomarkers for detection of kidney damage as a result of too low immunosuppression or drug toxicity may result in earlier detection and intervention to repair the damage (259), and reduce the need of invasive tissue biopsies.

The adherence tools evaluated in **Paper III** have been implemented in the routine follow-up, and annual adherence data are now registered in the Norwegian Renal Registry. Each year renal transplant recipients answer the BAASIS® questionnaire (on two occasions separated by 4-weeks), perform six capillary microsamples of tacrolimus from home every other week for calculation of tacrolimus concentration variability, and the local clinician score patient's adherence. As we now have diagnostic tools to identify and assess nonadherence, we will soon be able to describe how this affect long-term outcome. This will in turn open for evaluating prospective intervention studies to improve adherence in specific populations in a proper way. Multidisciplinary and personalized interventions with intentions to help and

identifying barriers of the behavior are crucial in overcoming immunosuppressive nonadherence

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Citation: Clin Transl Sci (2020) 0, 1–9; doi:10.1111/cts.12833

ARTICLE

Fasting Status and Circadian Variation Must be Considered When Performing AUC-based Therapeutic Drug Monitoring of Tacrolimus in Renal Transplant Recipients

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Therapeutic drug monitoring (TDM) is mandatory for the immunosuppressive drug tacrolimus (Tac). For clinical applicability, TDM is performed using morning trough concentrations. With recent developments making tacrolimus concentration determination possible in capillary microsamples and Bayesian estimator predicted area under the concentration curve (AUC), AUC-guided TDM may now be clinically applicable. Tac circadian variation has, however, been reported, with lower systemic exposure following the evening dose. The aim of the present study was to investigate tacrolimus pharmacokinetic (PK) after morning and evening administrations of twice-daily tacrolimus in a real-life setting without restrictions regarding food and concomitant drug timing. Two 12 hour tacrolimus investigations were performed; after the morning dose and the following evening dose, respectively, in 31 renal transplant recipients early after transplantation both in a fasting-state and under real-life nonfasting conditions (14 patients repeated the investigation). We observed circadian variation under fasting-conditions: 45% higher peak-concentration and 20% higher AUC following the morning dose. In the real-life nonfasting setting, the PK-profiles were flat but comparable after the morning and evening doses, showing slower absorption rate and lower AUC compared with the fasting-state. Limited sampling strategies using concentrations at 0, 1, and 3 hours predicted AUC after fasting morning administration, and samples obtained at 1, 3, and 6 hours predicted AUC for the other conditions (evening and real-life nonfasting). In conclusion, circadian variation of tacrolimus is present when performed in patients who are in the fasting-state, whereas flatter PK-profiles and no circadian variation was present in a real-life, nonfasting setting.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Circadian variation of tacrolimus (Tac) is controversial. Most Tac population pharmacokinetic (PK) models are based on fasting-day data.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ It investigated circadian variation in Tac PK and the effect on Tac PK-profiles when administered in a real-life setting with regard to food and concomitant drug timing.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ In a real-life nonfasting setting, the PK-profiles were flat without circadian variation. The study supports circadian variation of Tac under fasting conditions. Data on the real-world behavior of the patients are needed for a population PK model to predict area under the concentration curve (AUC) during both conditions.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Proposed Tac AUC-target levels need to be redefined due to circadian variation and flat real-life nonfasting PK-profiles. The association between high peak concentrations and side effects of Tac may be overestimated given the flat real-life nonfasting PK-profiles. The effect of real-life dosing of Tac may very well be present for other drugs and should be investigated for drugs where TDM is indicated.

Following organ transplantation, there is a need for life-long immunosuppressive therapy. For the last 10–15 years, the calcineurin inhibitor tacrolimus (Tac) has been the cornerstone in most transplant centers.[1] The narrow therapeutic index and large pharmacokinetic (PK) interindividual and intra-individual variability makes therapeutic drug monitoring (TDM) of Tac mandatory,[2] and is normally performed using morning trough concentrations.

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Received: April 9, 2020; accepted: May 27, 2020. doi:10.1111/cts.12833

When Tac was introduced in transplant protocols, importance of avoiding acute rejections led to TDM targeting high Tac trough concentrations. High concentrations induce nephrotoxicity and development of other side effects, like hypertension, post-transplant diabetes mellitus, neurotoxicity, and cancer.[3,4] In combination with mycophenolate mofetil (MMF) and modern induction therapy, the recommended Tac trough concentration target has gradually been reduced.[5,6] There is still room for improving long-term outcomes following renal transplantation,[7,8] and improved tailoring of the Tac dosing may be an important contributor.[9] The area under the concentration vs. time curve (AUC), reflecting total systemic Tac exposure, should theoretically be a more relevant measure for both efficacy and side effects compared with trough concentrations.[10] A recent consensus report also recommended AUC thresholds and advocates the need for prospective AUC-dosed studies.[10] By utilizing limited sampling strategies (LSS), preferably by capillary microsampling, in combination with population PK model-derived Bayesian estimators have made AUC-targeted dosing of Tac applicable in clinical practice.[11,12] However, data used to develop most Tac population PK models are based on data from clinical trials.[13,14] Such data are generally obtained in selected patients under highly controlled conditions (i.e., fasting, without concomitant drugs at time of Tac dose administration); hence, these results may not reflect a real-life situation of individual transplant recipients. In addition, the majority of AUC data are obtained during the day (i.e., following the morning dose of Tac). Because Tac has shown circadian variation, with higher drug exposure after the morning dose,[15–18] using models that assume a similar PK-profile following the morning dose and evening dose will introduce biased Tac exposure 0–24-hour AUC ($AUC_{0–24}$) predictions. In addition, Tac PK is also affected by food consumption.[19] If there is a correlation between systemic Tac exposure and long-term outcomes, models reflecting the real-life scenario over the entire dosing interval may prove advantageous.

The primary aim of this study was to investigate Tac PK after the morning and evening administration of twice-daily Tac in a real-life setting with regard to food and concomitant drug timing. Second, we aimed to determine the predictive performance of Tac AUC predictions using LSS and Bayesian estimators from a nonparametric population PK model.

METHODS

Study design

A prospective, open, nonrandomized PK study was performed at the National Transplant Center in Norway, Oslo University Hospital – Rikshospitalet, from December 2015 to May 2017. Renal transplant recipients older than 18 years using twice-daily Tac (Prograf; Astellas Pharma Ltd., Chertsey, UK) without concomitant drugs known to interact with Tac PK were included.

The study was conducted in accordance with ethical principles in the Declaration of Helsinki, guidelines for Good Clinical Practice, and was approved by the Norwegian Medicine Agency (EudraCT number: 2015-004734-10) and the local ethic committee (reference number 2015/2098). All patients received verbal and written information and signed an informed consent before entering the study.

Immunosuppressive treatment

Maintenance therapy consisted of a combination of Tac, MMF, and steroids. Tac was initiated on the day of transplantation, given a starting dose of 0.04 mg/kg for immunological standard-risk patients and adjusted to a trough (C_0) target range of 3–7 μg/L. For immunological high-risk patients (presence of donor specific antibodies at time of engraftment), Tac starting dose was 0.05 mg/kg and dose adjusted to a C_0 target of 8–12 μg/L. MMF was given at a fixed dose of 750 mg twice-daily from the day of transplantation and dose adjustments were only performed in case of side effects. Prednisolone was administered according to a fixed tapering schedule starting at 20 mg/day (80 mg/day in high-risk patients) the day after transplantation and tapered to a maintenance dose of 10 mg/day by weeks 4–8. All patients received induction therapy with basiliximab 20 mg on day 0 and day 4 after transplantation, and intravenous methylprednisolone 250 mg (standard-risk) or 500 mg (high-risk) on day 0. High-risk patients also received intravenous humane immune globulins 0.4 g/kg daily on days 0 and 4 and rituximab 375 mg/m^2 on day 0.

Tacrolimus analysis

Tac whole-blood samples were collected using vacutainers with spray-coated potassium EDTA acid (4 mL Vacuette K_2EDTA; Greiner Bio-One, Monroe, NC). The analysis was performed using liquid chromatography tandem mass spectrometry, as previously reported.[20] Lower limit of quantification was 0.6 μg/L, with imprecision coefficients of 9.0% at 2.3 μg/L and 6.0% at 7.0 μg/L.

Pharmacokinetic investigation

In the early post-transplant phase (2–8 weeks after transplantation), two 12-hour PK investigations were performed in succession (following morning and evening doses). In almost half of the participants, the PK investigations were repeated within 1 month (**Figure 1**).

Blood samples were collected predose (0 hour) and 11 times postdose; approximately after 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 hours. Exact sampling times in h:min were recorded. At the time of transplantation, patients were instructed to take their Tac doses at 9 AM and 9 PM, and this was also applied in the study. For inclusion, the Tac dosage had to be unchanged for at least 5 days prior to the PK investigation.

Patients were investigated either after administering their immunosuppressive medications as in their everyday life (i.e., both with regard to food consumption) in association to the Tac dose and concomitant drug administration (i.e., "real-life" nonfasting dose administration), or they were restricted to fast 2 hours before and after the Tac dose administration (i.e., no food, drinks, caffeine, or tobacco); concomitant drugs were administered simultaneously with Tac also on these occasions (i.e., "fasting" dose administration).

With this study design, 12-hour Tac PK were investigated following 4 different dosing scenarios: (1) fasting morning dose, (2) fasting evening dose, (3) real-life nonfasting morning dose, and (4) real-life nonfasting evening dose.

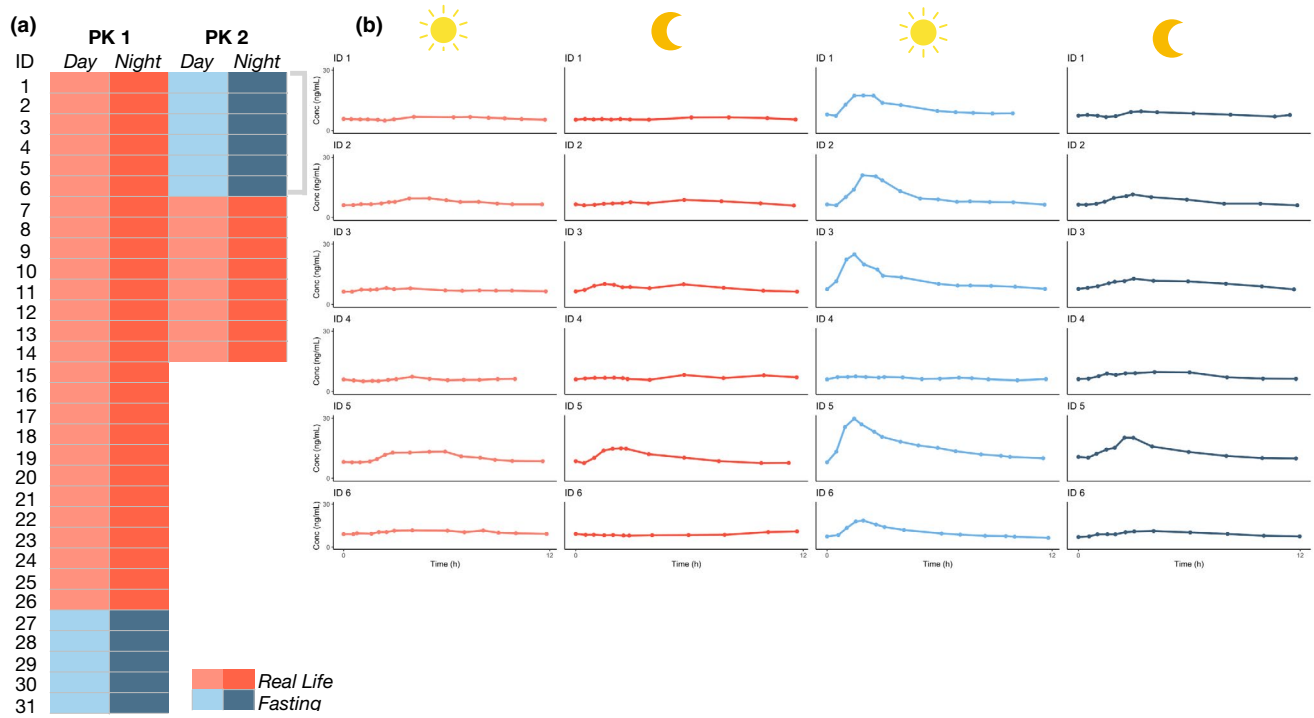

Figure 1 Overview of the pharmacokinetic (PK) investigations. (**a**) A table representing the PK investigations after the morning and the evening doses performed in a real-life nonfasting setting and under fasting conditions. The various colors represent the four different dose scenarios. The headings "Day" represents the morning dose and "Night" the evening dose. All 31 patients performed the PK investigation number 1 (PK1), and 14 patients performed the second investigation (PK2). (**b**) Six patients performed the PK investigations both in a real-life nonfasting setting and under fasting conditions, giving 12 hour PK-profiles with paired data from the 4 different dose scenarios. The various colors represent the four different dose scenarios, whereas the "sun" and the "moon" symbols represent the morning and the evening doses, respectively.

Pharmacokinetic calculations

The trapezoidal method was used to calculate AUC for the dose intervals after the morning dose ($AUC_{0–12}$) and the evening dose ($AUC_{12–24}$). In each dose interval, the maximum concentration (C_{max}) was determined as the highest observed concentration. The actual observed time of C_{max} (T_{max}) in relation to the respective dose administration was also determined. Three different trough concentrations were assessed on the investigation days: prior to the morning dose (C_0), prior to the evening dose (C_{12}), and 12 hours after the evening dose (C_{24}).

AUC determined by limited sampling strategies

Different LSS were used to predict individual Tac AUC using a previously developed and validated nonparametric population PK model as Bayesian estimator.[21,22] The model was adapted to also handle the flatter real-life nonfasting and evening-time PK profiles obtained in the present study (see **Supplementary Material**). The *makeAUC* function in the Pmetrics package for R (linear model) was used to calculate model-derived $AUC_{0–tau}$ values over respective dose interval.[23] The predictive performance for AUC determination when using the Bayesian estimator derived from the adapted model, used in combination with different LSS, was evaluated in a validation dataset, not previously used for developing the adapted model, by comparing the different LSS-derived AUCs with respective trapezoidal determined AUCs.

The LSS tested included the validated sampling times of 0, 1, and 3 hours, as previously published[12] and single trough concentrations (C_0 for $AUC_{0–12}$ and C_{12} for $AUC_{12–24}$). In addition, the multiple model optimal sample time function (MMopt) in Pmetrics,[23] weighted for AUC, was used to determine the best LSS using three optimal sampling times for the real-life nonfasting and evening PK-profiles. The MMopt function in the Pmetrics package for R was used to determine the sampling times that minimize the risk of misrepresenting the patients as the wrong set of support points in the model (i.e., estimating the wrong set of individual PK parameters).[24]

Statistical analyses

Population characteristics are summarized as median (range). For comparison between the paired PK variable following the morning and evening doses of Tac, the Wilcoxon signed rank test was used. The different trough concentrations were compared using nonparametric Friedman test, and correlation between AUC and trough concentrations were calculated using Spearman's rank correlation coefficient. A two-tailed P value < 0.05 was considered significant.

Agreement between the respective LSS derived AUCs and the trapezoidal determined reference AUC were assessed by C-statistics, with concordance correlation coefficient (CCC), total deviation index (TDI), and coverage probability (CP),

as previously described.[25] CCC is a correlation coefficient measuring the agreement between two measurements; the values can range from 0 to 1, where 1 reflects perfect correlation and 0 no correlation. TDI is a measure of the proportion of data within a pre-set boundary for an allowed difference between the reference and the estimations. CP can range from 0 to 1, and is an estimate of whether a given TDI is less than a prespecified fixed percentage. Predefined accepted agreement levels were determined to be: CCC $\geq$ 0.9, TDI $\leq$ 15%, and CP $\geq$ 0.85.[12]

RESULTS

Patients

Thirty-one stable renal transplant recipients (74% men) were prospectively enrolled in the study between 13 and 54 days post-transplant. Demographic data and patient characteristics are presented in **Table 1**, and were considered representative of our kidney transplant population. All included patients, except one, received concomitant MMF. Four (13%) were immunological high-risk patients and three patients (10%) were CYP3A5 expressers (all *CYP3A5*1/*3*). No overall difference in Tac PK was observed between CYP3A5 expressers and nonexpressers.

All patients performed 2 successive 12-hour PK investigations (morning and evening doses), and 14 patients (45%) also repeated these PK investigations within 7–28 days (median 14 days; **Figure 1**). A total of ninety 12-hour PK profiles: 45 from the morning dose and 45 from the successive evening dose were obtained in the present study. In 11 of these morning-evening dose investigations (i.e., twenty-two 12-hour PK investigations), Tac was administered in fasting conditions, as defined in Methods. In the other 34 morning and evening dose investigations (sixty-eight 12-hour PK investigations), Tac was administered as in a real-life setting (**Figure 1**). Patients were told to do "as normal." Patient-reported time of food consumption for breakfast was < 0.5 hours before/after the morning dose, dinner 3–4.5 hours before the evening dose, and supper < 0.5 hours before/after the evening dose.

Table 1 Patient characteristics and demographic data (*n* = 31)

	Number (%)	Median (range)
Male	23 (74)	
Living donor	15 (48)	
First transplant	28 (90)	
Pre-emptive transplantation	13 (42)	
Standard immunological risk	27 (87)	
CYP3A5 genotype		
**1/*1*	0 (0)	
**1/*3*	3 (10)	
**3/*3*	26 (84)	
Unknown	2 (6)	
Age, years		62 (22–78)
Height, cm		175 (159–192)
Weight, kg		79 (52–103)
Donor age, years		55 (6–73)
Time since transplantation to PK1, days		22 (13–54)
P-creatinine, μmol/L		122 (70–192)
Hematocrit, %		36 (29–44)
Tacrolimus dose, mg/day		5 (3–14)
Prednisolone dose, mg/day		15 (7.5–20)
Mycophenolate mofetil dose, mg/day		1,500 (720–1,500)

CYP3A5, cytochrome P450 3A5; PK1, first pharmacokinetic investigation.

Chronopharmacokinetics

Fasting dose administration. In fasting conditions, Tac PK displayed circadian variation (**Table 2**) with slower absorption and reduced exposure following the evening dose (**Figure 2**): AUC and C_{max} (median [range]) were significantly higher following the morning dose ($AUC_{0–12}$: 127 [77–200] μg h/L, C_{max}: 20.6 [7.4–31.8] μg/L) compared with the evening dose ($AUC_{12–24}$: 102 [84–155] μg h/L, C_{max}: 11.5 [9.4–20.3] μg/L), $P < 0.006$. Additionally, T_{max} was significantly shorter after the morning dose (1.5 [1.3–2.0] hours vs. 3.9 [2.0–10.1] hours), $P = 0.003$. However, there were no significant differences between the three respective trough levels: C_0 (7.5 [5.4–9.2] μg/L), C_{12} (7.1 [6.0–10.7] μg/L), or C_{24} (7.2 [6.0–9.9] μg/L), $P = 0.761$. The correlations among $AUC_{0–12}$, $AUC_{12–24}$, or $AUC_{0–24}$ with C_0, C_{12}, or C_{24} were only moderate and not statistically significant (**Table 3**).

Real-life nonfasting dose administration. Administering Tac in a real-life nonfasting setting showed slow absorption PK profiles without indication of circadian variation on PK

Table 2 Chronopharmacokinetics of tacrolimus under fasting and real-life nonfasting dose administration

	Fasting (*n* = 11) Median (range)	Real-life (*n* = 34) Median (range)
Tacrolimus total daily dose, mg	6 (3–11)	5 (3–14)
AUC, μg h/L		
Morning dose	127 (77–200)	82 (55–128)
Evening dose	102 (84–155)	80 (53–129)
Comparison	***P* = 0.006**	*P* = 0.083
C_{max}, μg/l		
Morning dose	20.6 (7.4–31.8)	8.9 (5.3–18.4)
Evening dose	11.5 (9.4–20.3)	8.4 (5.8–15.2)
Comparison	***P* = 0.008**	*P* = 0.334
T_{max}, hours		
Morning dose	1.5 (1.3–2.0)	4.0 (0.7–9.2)
Evening dose	3.9 (2.0–10.1)	4.1 (1.0–11.7)
Comparison	***P* = 0.003**	*P* = 0.077
C_{12}, μg/l		
Morning dose	6.6 (5.4–10.7)	5.9 (3.4–9.5)
Evening dose	7.2 (4.9–9.9)	5.6 (4.0–11.1)
Comparison	*P* = 0.286	*P* = 0.912

Data shown as AUC (calculated using the trapezoidal method), the observed C_{max} and T_{max}, and the measured concentration 12 hours after the dose (C_{12}).
Comparison between the morning dose and evening dose calculated using nonparametric Wilcoxon signed rank test.
Bold type indicates significant difference between the morning and evening doses.
AUC, area under the concentration vs. time curve; C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; C_{12}, the concentration 12 hours after dose administration.

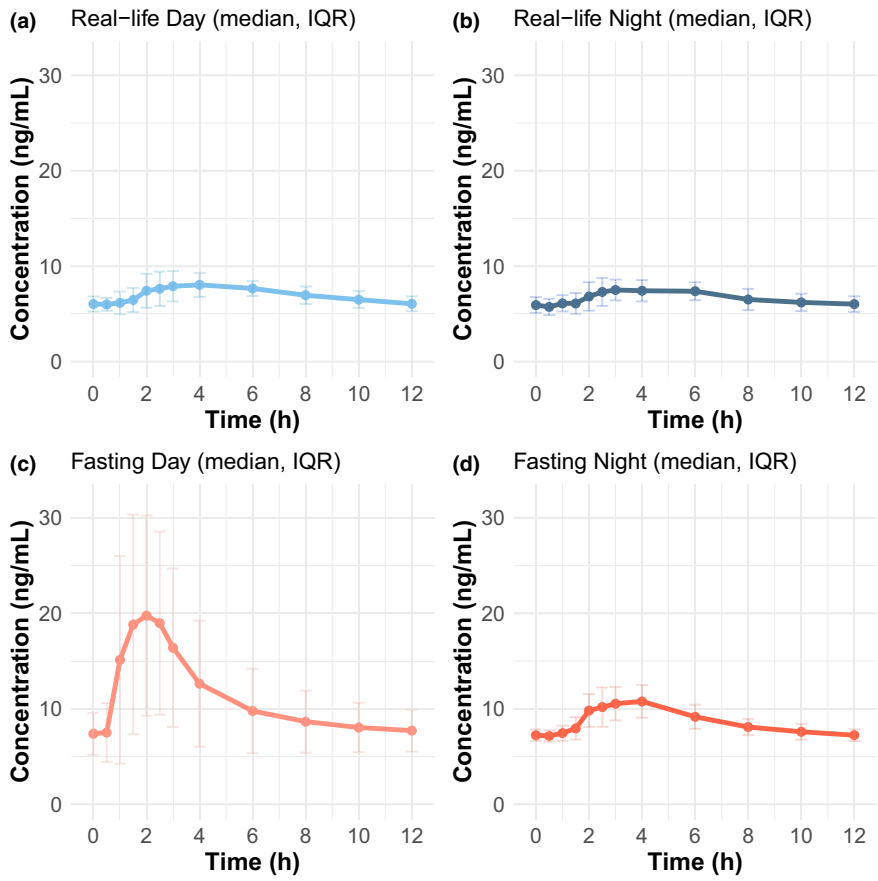

Figure 2 Median curves for the four different dose scenarios. Individual time-corrected concentrations were used to make median curves with related interquartile range (IQR) for the four different dose scenarios: (**a**) real-life nonfasting morning dose (n = 34 in the 12-hour PK-profiles), (**b**) real-life nonfasting evening dose (n = 34 in the 12-hour PK-profiles), (**c**) fasting morning dose (n = 11 in the 12-hour PK-profiles), and (**d**) fasting evening-dose (n = 11 in the 12-hour PK-profiles.

parameters (**Figure 2**). There were no differences in AUC, C_{max}, or T_{max} between morning and evening doses (**Table 2**). In addition, trough levels (median [range]) did not vary during the 24-hour dosing interval: C_0 (5.9 [3.5–9.2] µg/L), C_{12} (5.9 [3.4–9.5] µg/L), or C_{24} (5.5 [4.0–11.1] µg/L), P = 0.262. The correlations among $AUC_{0–12}$, $AUC_{12–24}$, or $AUC_{0–24}$ with any of the trough values C_0, C_{12}, or C_{24} were strong. The highest correlation coefficient was found for $AUC_{0–24}$ and C_{24} (**Table 3**, Spearman's rho 0.866, $P < 0.001$).

In 6 of the 14 patients repeating the PK investigations, both fasting and real-life nonfasting dose conditions were investigated. The paired PK-profiles from all four 12-hour dose intervals showed high variation (**Figure 1**). The population PK

Table 3 Correlations between AUC and trough concentrations under fasting and real-life nonfasting dose administration

	$AUC_{0–12}$		$AUC_{12–24}$		$AUC_{0–24}$	
	Fasting (n = 11)	**Real-life (n = 34)**	**Fasting (n = 11)**	**Real-life (n = 34)**	**Fasting (n = 11)**	**Real-life (n = 34)**
C_0	0.345 P = 0.298	0.820 **$P < 0.001$**	0.282 P = 0.401	0.801 **$P < 0.001$**	0.309 P = 0.355	0.857 **$P < 0.001$**
C_{12}	0.527 P = 0.096	0.807 **$P < 0.001$**	0.509 P = 0.110	0.818 **$P < 0.001$**	0.518 P = 0.102	0.859 **$P < 0.001$**
C_{24}	0.573 P = 0.066	0.799 **$P < 0.001$**	0.464 P = 0.151	0.838 **$P < 0.001$**	0.509 P = 0.110	0.866 **$P < 0.001$**

Reported Spearman's rank correlation coefficient.
AUC calculated using the trapezoidal method.
Bold type indicates significant correlation.
AUC, area under the concentration vs. time curve; $AUC_{0–12}$, area under the concentration vs. time curve after the morning dose; $AUC_{12–24}$, area under the concentration vs. time curve after the evening dose; $AUC_{0–24}$, total daily area under the concentration vs. time curve; C_0, trough concentration right before the morning dose; C_{12}, trough concentration right before the evening dose; C_{24}, trough concentration 12 hours after the evening dose.

Table 4 Population PK model derived parameter values for the four different dose scenarios

	Fasting morning dose[a] (*n* = 11)		Fasting evening dose[b] (*n* = 8)		Real-life morning dose[b] (*n* = 22)		Real-life evening dose[b] (*n* = 22)	
Parameters	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Absorption rate constant, Ka hours	1.50	0.33	0.79	1.35	0.70	0.84	0.21	1.43
Apparent clearance, L/h	12.9	11.2	22.0	5.8	18.3	16.1	25.7	11.5
Apparent intercompartment clearance, L/h	44.1	56.6	85.6	41.6	95.4	94.2	17.3	123.8
Apparent central volume of distribution, L	107	31	269	208	339	217	305	439
Apparent peripheral volume of distribution, L	866	1094	13457	9290	21626	16936	29956	16717
Lag time week 2–4 post-transplant, hours	0.52	0.40	2.02	0.47	1.86	2.46	1.27	1.23
Lag time after first month post-transplant, hours	0.58	0.26	2.32	2.45	1.28	1.89	1.52	2.61

IQR, interquartile range; PK, pharmacokinetic.
[a]Used the previous developed population PK model. [b]Used the adapted version of the previous developed population PK model (derived from the model-adaption dataset; see **Supplementary Digital Content, Methods page 1–2**).

parameters of the model are presented in **Table 4**. The absorption rate was higher and the apparent clearance lower for the fasting morning dose when compared with the fasting evening dose and the real-life nonfasting morning and evening doses.

Limited sampling strategy determined AUC

Fasting dose administration. The previously validated LSS of Tac with samples obtained at 0, 1, and 3 hours postdose predicted $AUC_{0–12}$ with high accuracy and precision (**Table 5**). CCC was 0.922 (95% confidence interval (CI): 0.800–1.0), reflecting high precision and accuracy for the LSS-predicted AUCs. TDI was 13.4 (95% CI: 6.5–20.3), which means that 85% of the predicted AUCs showed an error ranging from –13.4% to +13.4% compared with reference (trapez) AUC. CP was 0.854 (95% CI: 0.607–1.0), which indicates that < 15% of the predicted AUCs had an error greater than ±15%. Using MMopt sampling times (1, 3, and 6 hours postdose) for the slow-absorption profiles (fasting evening dose) resulted in predicted $AUC_{12–24}$ of accepted agreement (**Table 5**) in the validation dataset (*n* = 3). A single trough concentration did not predict neither $AUC_{0–12}$ nor $AUC_{12–24}$ within the acceptance limit.

Real-life nonfasting dose administration. The LSS with samples obtained at 0, 1, and 3 hours postdose or a single trough concentration did not show acceptable agreement for real-life nonfasting AUC predictions (**Table 5**). Using the MMopt determined sampling times (1, 3, and 6 hours postdose) for predictions of both $AUC_{0–12}$ and $AUC_{12–24}$ showed overall better agreement with trapezoidal $AUC_{0–12}$ and $AUC_{12–24}$ compared with LSS 0, 1, and 3 hours for fasting conditions: CCC was 0.946 (95% CI: 0.897–0.995),

Table 5 Agreement between population PK estimated AUC, applying different number of samples, compared with reference AUC

Sampling times	CCC (95% CI)	TDI (95% CI)	CP (95% CI)
$AUC_{0–12}$ – fasting morning dose (*n* = 11)[a]			
Full-profiled, 12 samples	**0.991** (0.975, 1.0)	**4.6** (2.5, 6.7)	**1.0** (0.965 1.0)
3-sample LSS, 0, 1, and 3 hours	**0.922** (0.800, 1.0)	**13.4** (6.5, 20.3)	**0.854** (0.607, 1.0)
Trough only	0.482 (0.023, 0.914)	52.5 (18.8, 86.3)	0.331 (0.217, 0.445)
$AUC_{12–24}$ – fasting evening dose (*n* = 3)[b]			
Full-profiled, 12 samples	**0.988** (0.735, 1.0)	**3.2** (0, 15.0)	NA
3-sample LSS, 0, 1, and 3 hours	**0.938** (0.196, 1.0)	**7.7** (0, 30.4)	NA
Trough only	0.874 (0.165, 1.0)	12.5 (0, 42.0)	NA
3-sample LSS,[c] 1, 3, and 6 hours	**0.944** (0.340, 1.0)	**7.2** (0, 23.4)	NA
$AUC_{0–12}$ and $AUC_{12–24}$ – real-life morning dose and evening dose (*n* = 24)[b]			
Full-profiled, 12 samples	**0.974** (0.951, 0.997)	**7.7** (5.2, 9.9)	**0.994** (0.954, 1.0)
3-sample LSS, 0, 1, and 3 hours	0.788 (0.621, 0.955)	25.3 (17.2, 33.4)	0.608 (0.284, 0.455)
Trough only	0.424 (0.236, 0.612)	81.5 (54.0, 108.9)	0.227 (0.160, 0.294)
3-sample LSS,[c] 1, 3, and 6 hours	**0.946** 0.897, 0.995)	**11.2** (7.9, 14.5)	**0.934** (0.823, 1.0)

Reference AUC calculated using the trapezoidal method.
Bold type indicates better agreement than the prespecified boundaries: CCC ≥ 0.9, TDI ≤ 15, and CP ≥ 0.85.
AUC, area under the concentration vs. time curve; $AUC_{0–12}$, area under the concentration vs. time curve after the morning dose; $AUC_{12–24}$, area under the concentration vs. time curve after the evening dose; CCC, concordance correlation coefficient; CI, confidence interval; CP, coverage probability index; LSS, limited sampling strategy; NA, not available (too few samples – see **Supplementary Digital Content 1**); TDI, total deviation index.
[a]AUC calculated using the previous developed population PK model. [b]AUC calculated in the validation dataset using the adapted version of the previous developed population PK model. [c]LSS using sampling times closest to the multiple model optimal sampling times determined by the MMopt-function in Pmetrics.

and TDI and CP were 11.2 (95% CI: 7.9–14.5) and 0.934 (95% CI: 0.823–1.0), respectively.

DISCUSSION

In a real-life nonfasting setting, Tac does not show the well-known PK profile with a C_{max} about 20 μg/L after about 1–2 hours.[26] Instead, the PK profiles are flat, with a very slow absorption rate. C_{max} was less than half, and the systemic exposure about two-thirds of that obtained following morning dose administered under fasting conditions in the present study. It is indeed important to point out that this comparison was performed against fasting morning doses because Tac showed circadian variation when administered in a fasting state (but not in the real-life nonfasting setting); with slower absorption and flatter PK-profiles after the evening dose. When fasting, the AUC was on average 20% and C_{max} 45% higher after the morning dose compared with the evening dose. The circadian variation that was consistently observed after fasting Tac dose administration did, however, not influence the various trough levels investigated (C_0, C_{12}, and C_{24}). In the literature, there are some conflicting reports with respect to circadian variation of Tac exposure,[15,17,27,28] but there is a tendency in support of circadian variability under fasting conditions. After the evening dose and under real-life nonfasting dose administration, the absorption rate constant was lower, reflecting a slower absorption when compared with the fasting morning dose. In addition, apparent clearance was higher when compared with the fasting morning dose, most likely reflecting a decreased oral bioavailability rather than higher clearance. With adaptions of the parameter boundaries for absorption constant and lag time of a previous developed population PK model, AUC determinations were possible both for the fasting and the real-life nonfasting setting, but other optimal sampling time strategies were required.

Our data raise several important questions regarding current and future Tac TDM recommendations and evaluations. Some studies have demonstrated a satisfying correlation between Tac trough concentrations and AUC.[29,30] However, this has not been reproduced in other studies, and as in agreement with our fasting-day data, the general view is that the correlation between trough and AUC is relatively poor.[31–33] Although AUC is regarded the optimal measurement of drug exposure, for practical reasons, morning trough concentrations are today widely used in the routine for Tac dose individualization. According to the present results, it may, however, be that the correlation is greater in the real-life nonfasting setting, considering the flat curves and the strong correlation between C_{24} and $AUC_{0–24}$ (r = 0.866). Hence, one may argue that there is not so much to gain by doing AUC-monitoring, as trough in this setting better reflects the systemic exposure of Tac. It should also be kept in mind that the actual AUC in the fasting and nonfasting conditions are very different. If performing AUC-monitoring, data reflecting the real-life situation are needed to develop more clinically appropriate population PK models for dose individualization, because most PK models presented in the literature will not perform well on real-life data.

There is a lack of studies addressing the optimal total daily Tac exposure ($AUC_{0–24}$).[34] The proposed $AUC_{0–24}$ target ranges have to be redefined because fasting day and night AUCs are not similar, and the fasting-day AUC cannot just be doubled. For the last decades, we have been following the low-dose Symphony protocol from the time of transplantation.[5] With this approach, the 24-hour Tac AUC is in the lowest range of the suggested target,[10,35] mainly as a result of either the circadian variation when fasting or dose administration performed relatively close to food consumption. As almost all available PK studies and population PK models are based on fasting-day data, further research involving prospective studies investigating Tac $AUC_{0–24}$ in patients at different immunological risk and time after transplantation, performed during nonregulated conditions, where patients eat and take their medications as in their everyday routine, is strongly warranted. With the use of capillary microsampling and patients performing blood sampling at home, it might be possible to perform such clinical trials, within reasonable cost and effort boundaries for both patients and investigators.[12]

An important drawback of the clinical implementation of AUC-guided dosing of Tac is that blood samples are not convenient to obtain following the evening dose, and accurate predictions of the full 24-hour Tac exposure is thus not feasible. Based on the current data, we evaluated potentially clinical applicable sampling strategies for predictions of AUC following the evening dose ($AUC_{12–24}$; data not shown). Samples closest to the MMopt sampling times (1, 3, and 6 hours) were tested, but samples during sleep were avoided (between 11 PM and 7 AM). In this regard, the best strategy with the highest agreement in C-statistics was to use samples 1, 2, and 10 hours after the evening dose (e.g., at 10 PM and 11 PM and again at 7 AM the next morning when utilizing a 9 AM to 9 PM dosing scheme (CCC was 0.895 (95% CI: 0.795–0.995), TDI 16.6% (95% CI: 12.0–21.1), and CP 0.816 (95% CI: 0.620–1.0)).

Once-daily Tac is suggested to increase adherence.[36,37] An additional hypothesized clinical benefit of using once-daily Tac formulations has been to avoid the high peak concentrations and the large peak-to-trough variation, which is present with the twice-daily formulation (when administered in a fasting state).[38–40] Most of the patients at our transplant center take their Tac dose without respecting the ±2-hour fasting rule, and as clearly shown in the present study, the high peaks following administration of the twice-daily Tac formulation will then be avoided. This raises the question of the actual need and benefit of giving a prolonged-release formulation, as a close to similar PK-profile can be achieved by administering the twice-daily formulation closer to food consumption.

The main strength of the present study is the rich sampling obtained following both the morning dose and the evening dose of Tac. In total, 1,187 Tac samples have been investigated in the present study, on average, 26 per 24-hour PK investigation. This ensures detailed individual description of Tac PK during the full 24-hour interval. Second, the study was performed in a real-life setting: patients took their medications as in their everyday routine. This study obviously also has some limitations. First,

this study is performed in the early post-transplant phase, and because Tac PK change during the first 6–12 months after transplantation,[15,35,41] the results from the present study should be extrapolated with care to the long-term follow-up situation. Second, even though the validation metrics of the adapted population PK model were convincing, relatively few PK-profiles of the different dose scenarios were included in the development and validation datasets (see **Supplementary Material**), so the results have to be interpreted with caution. The numbers of patients with complete dual data, performing both fasting and real-life nonfasting investigations, are very low (n = 6). Finally, only the immediate-release formulation of Tac was investigated. It will be important to also investigate if these effects are present with prolonged-release formulations.

These findings raise several questions pertaining to the optimal monitoring of Tac in a standard clinical setting. If the exposure of Tac following an evening dose is less influenced by intake of food, such restrictions are unnecessary and can be omitted when advising patients on their drug habits.

In summary, our results demonstrated that dosing Tac in real-life, without respecting the ±2-hour fasting rule, showed rather flat PK-profiles and no circadian variation. Dosing Tac under fasting conditions in the morning produced the well-known Tac PK-profile, with a sharp peak after ~ 1–2 hours. Circadian variation was present with fasting administration and the profiles after the evening dose were flat and quite similar to the real-life nonfasting profiles. Following real-life nonfasting dose administration, the correlation between trough (C_{24}) and total daily exposure (AUC_{0-24}) was high. LSS in combination with population PK model-derived Bayesian estimators was able to accurately predict AUC for both fasting and real-life nonfasting dose administration, but different optimal sampling times for predictions of AUC were required. Data on the real-world behavior of the patients are needed for a population PK model to predict AUC during both dose scenarios. Whether this will improve long-term outcome needs to be verified in a large prospective clinical trial.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Acknowledgments. The authors thank all patients who participated, and the great technical assistance from Sebastian Müller, Helga Grimstad Sørhøy, Hanne Ravnskog Moldskred, and May-Ellen Lauritsen in the process of capturing data.

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

Funding. No funding was received for this work.

Author Contributions. M.T.G. and A.Å. wrote the manuscript. K.M. and A.Å. designed the research. M.T.G. and K.M. performed the research. I.R., J.B.W., J.D., R.A.K., N.T.V., and S.B. analyzed the data.

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