Predicting Kidney Transplantation Outcomes and Exploring Novel Matrices for Therapeutic Drug Monitoring of Tacrolimus

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Predicting Kidney Transplantation Outcomes and Exploring Novel Matrices for Therapeutic Drug Monitoring of Tacrolimus

Uitkomsten van niertransplantatie voorspellen en nieuwe manieren om de tacrolimusconcentratie te meten

Thesis

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His Majesty King Bhumibol Adulyadej (Rama IX)

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General Introduction

Based on Suwasin Udomkarnjananun, Marith I. Francke, Brenda C.M. De Winter, Midas B. Mulder, Carla C. Baan, Herold J. Metselaar, Caroline M. den Hoed, Dennis A. Hesselink.

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1. GENERAL INTRODUCTION

Kidney transplantation is the best renal replacement therapy for patients suffering from end-stage renal disease (ESRD). It is superior in terms of quality of life, cardiovascular risk, and survival compared with dialysis.[1] One of the major keys to the success of kidney transplantation has been the discovery of immunosuppressive drugs to prevent and treat allograft rejection.[2] Since the introduction of 6-mercaptopurine (6-MP), azathioprine, and corticosteroids in the 1960s, many clinical trials and experimental studies have been conducted to find the best immunosuppressive combination drug regimen ("cocktail") for kidney transplant recipients. Nowadays, the most commonly used immunosuppressive drug therapy consists of the calcineurin inhibitor (CNI) tacrolimus, mycophenolic acid (MPA), and glucocorticoids with induction therapy (either T lymphocyte-depleting antibodies or an anti-interleukin (IL)-2 antibody). With this regimen, the 5-year patient and kidney allograft survival have increased to >90% and >80%, respectively.[3] This improvement is impressive when considering that kidney allograft survival was less than 50% before the 1980s.[4, 5] However, a substantial number of patients is still experiencing rejection or suffering from immunosuppressive drug-related toxicity, emphasizing the need to further improve immunosuppressive drug regimens.

To achieve the desired therapeutic effect and prevent toxicity, therapeutic drug monitoring (TDM) is routinely performed for many immunosuppressive drugs, in particular for the CNIs tacrolimus and cyclosporine A and the mammalian target of rapamycin inhibitors (mTORi) everolimus and sirolimus. TDM is also applied for MPA in some transplant centers. The basic principles and clinical practice of therapeutic immunosuppressive drug monitoring will be discussed here.

2. PRINCIPLE OF THERAPEUTIC DRUG MONITORING

TDM refers to the practice to dose drugs based on their concentrations in biofluids, usually whole blood or plasma. TDM is most often performed for so-called narrow-therapeutic index drugs. These are drugs for which the difference between toxic and effective concentrations is relatively small, and which are therefore easily over- or under-dosed.[6, 7] TDM is now considered standard practice during the treatment with most immunosuppressive drugs after solid organ transplantation[8]. TDM is routinely performed after transplantation for tacrolimus and mTORi. However, there is ongoing debate about the benefits of TDM for MPA.[9]

TDM of a drug can be considered when the following requirements are met:[10, 11]

(a) *There exists a clear correlation between the concentration of a drug in a bodily fluid and*

the biological effect of that drug (either efficacy or toxicity);

(b) *There exists a small difference between the effective concentration and either the non-effective or toxic concentrations of that drug, i.e. the drug has a narrow therapeutic index.* The therapeutic index is generally defined as the ratio of the toxic dose (in 50% of subjects) to the effective dose (in 50% of subjects). As illustrated in Figure 1, drug A has a wide therapeutic index and TDM is not required. In contrast, drug B has a narrow therapeutic index and should be closely monitored by TDM since the margin between efficacy and toxicity is small. Most immunosuppressants are considered narrow therapeutic index drugs;

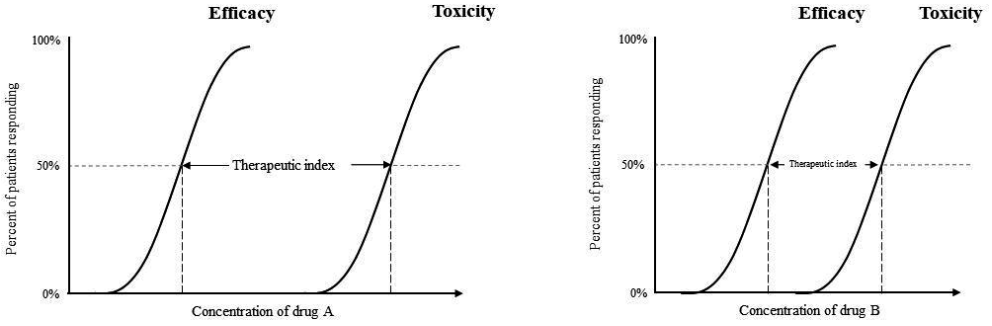

Figure 1: The therapeutic index. The therapeutic index of a drug is defined as the ratio between the toxic and effective concentration in 50% of the patients. Drug A has a wide therapeutic index, while drug B has a narrow therapeutic index. Therapeutic drug monitoring in general is not necessary for drugs with a wide therapeutic index but is recommended for drugs with a small therapeutic index.[12]

(c) *There are no other outcomes reflecting a drug's action that are easier to assess than the drug's concentration.* For example, blood pressure or the glucose concentration when monitoring the effect of anti-hypertensive drugs and glucose-lowering drugs, respectively;

(d) *There exists a high inter-patient variability in drug exposure following fixed-dosing of that drug.* The phenomenon of a high inter-patient variability following fixed-dosing is illustrated in Figure 2. Mycophenolate mofetil (MMF) was originally marketed as a fixed-dose drug.[13] However, when prescribed in a one-size-fits-all dose, the resulting MPA concentrations will vary considerably.[14] Drugs that can benefit from TDM should have both a high inter-patient variability and narrow therapeutic index. TDM would not be necessary if the therapeutic index of MPA was wider than its inter-patient variability;

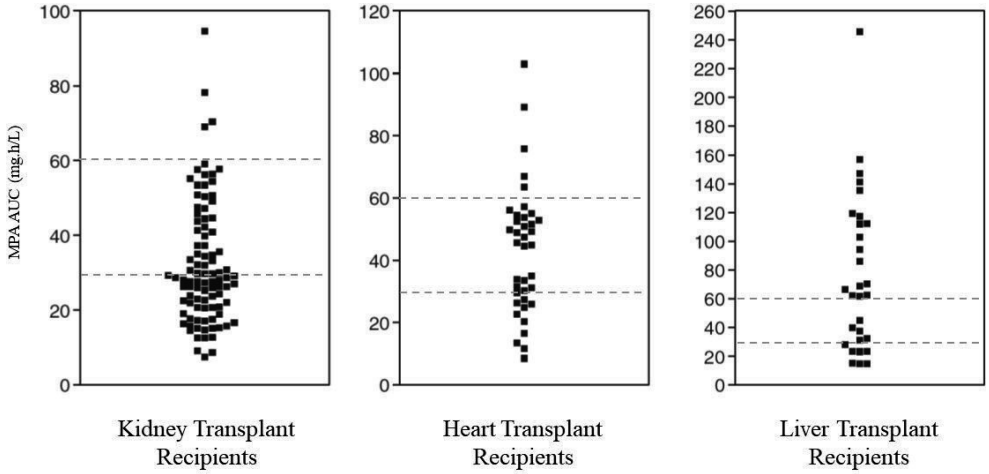

Figure 2: MPA exposure following fixed-dose MMF. Depicted is the exposure to MPA (measured as area-under the concentration *versus* time curve; AUC) in solid organ transplant recipients in response to MMF 1 g *b.i.d*. The resulting MPA exposure varies greatly between individual patients (reproduced with permission from Shaw *et al*.).[14] Dash lines represent the therapeutic index of MPA AUC (30-60 ng·h/L).

(e) *There exists a small variability in drug exposure within a single patient over time when treated with a stable dose of that drug.* This so-called intra-patient variability (IPV) can be calculated in several ways and describes the fluctuation of a drug's concentration over time when the dose is unaltered (Figure 3)[15]. TDM of a drug with a high IPV is generally not recommended as the drug concentration measured at a certain time point has little predictive value for the next time point. Patients in whom the drug concentration tends to vary little over time may be suitable candidates for TDM as the measured concentration may accurately predict the concentration on the next occasion;

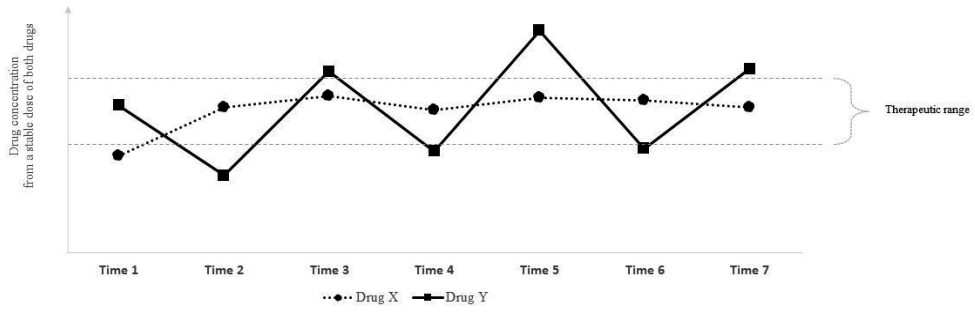

Figure 3: Intra-patient variability and TDM. Depicted are the concentrations over time of drug X and drug Y. The concentration of drug X is relatively stable over time with an unaltered dose, while the concentration of drug Y fluctuates greatly despite the patient receiving a stable dose. For drug X, TDM may be indicated and because the concentration at a certain time corresponds to the concentration at the next, this is a sensible strategy. The concentrations of drug Y however, have little predictive power for the concentration measured on the next occasion and this drug may therefore not be a suitable candidate for TDM.[12]

(f) *The duration of drug treatment must be long enough to benefit from TDM;*

(g) *The analytic methods for the measurement of the drug of interest need to be reliable and standardized.*

In the following paragraphs we will describe the evidence for TDM of tacrolimus which is used as the backbone of the immunosuppressive regimen for kidney transplantation. Areas of uncertainty and future research directions are described.

3. TACROLIMUS

3.1 Pharmacokinetics

Tacrolimus has a poor bioavailability which averages around 30%.[16] The drug is a substrate of the efflux pump ABCB1 (also known as P-glycoprotein) and the metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A5. Both ABCB1 and CYP3A are expressed in the intestine and are responsible for tacrolimus' low bioavailability by actively excreting the drug from the enterocyte and substantial first pass-metabolism, respectively. The majority of the absorbed tacrolimus binds to its receptor FK-binding protein-12 (FKBP-12) which has a high concentration in erythrocytes. Of the absorbed tacrolimus, ~80% is located inside erythrocytes, whereas ~15% is plasma-protein bound. The free fraction of tacrolimus is small and is around 0.5%.[17]

The systemic metabolism of tacrolimus is dependent on hepatic CYP3A4 and CYP3A5 expression and activity, which differs markedly between individuals. Based on their CYP3A metabolic activity, patients can be classified as poor, intermediate and fast metabolizers (discussed below). Tacrolimus is extensively metabolized by CYP3A and less than 1% of the drug is excreted unchanged in urine and feces. Approximately 95% of the tacrolimus metabolites is excreted via the biliary route, whereas only 2% is excreted by the kidneys.[18]

3.2 Monitoring strategies

For TDM of tacrolimus the pre-dose (or trough concentration (C_0)) is most widely used in every day clinical practice. Ideally, the measurement of tacrolimus exposure should be performed by measuring the 12-hour (the dosing interval) area under the concentration *versus* time curve (AUC). However, measuring a full AUC is impractical. For tacrolimus, the correlation coefficient between C_0 and AUC in general is acceptable with an *r* of 0.7 and higher.[19, 20] However, this correlation ranges between 0.34 and 0.60 in some patients.[21-23] The problem is that such patients may be considered to have an adequate exposure to tacrolimus if only a C_0 is measured, whereas the true exposure is off target. *Vice versa* some patients may have a C_0 that is outside the target range, whereas their exposure (measured by AUC) is in fact adequate. Reports have been published of patients experiencing acute rejection from inadequate exposure despite having a C_0 within the target range.[24]

In clinical practice, C_0 is generally used for TDM because of its feasibility and simplicity, although the correlation with total tacrolimus exposure is not perfect. To better estimate tacrolimus exposure, limited sampling strategies (LSS) have been developed. LSS utilize tacrolimus concentrations measured at 2-3 time points (rather than ≥8 time-points in the full 12-hour AUC), to *estimate* an AUC. The correlation coefficient between the full AUC and its estimation by LSS (using multiple linear regression) is good with $r \geq 0.90$.[25] Moreover, when LSS is combined with Bayesian estimation, which uses information from *a priori* estimated population pharmacokinetic parameters (such as drug clearance and volume of distribution), the individualized AUC for each patient can be generated after fitting the LSS to the population-based model. This enhances the predictive value of LSS for tacrolimus exposure in an individual patient to $r \geq 0.95$.[26] Ideally, the AUC should be evaluated in every patient before hospital discharge but this might not be practical in all transplant centers. Clinicians can select patients who are suspected of having a poor correlation between their tacrolimus C_0 and AUC, *i.e*. patients who develop toxicity or rejection despite an C_0 within the target concentration range, and these may have an indication for an (abbreviated) AUC measurement.

A number of studies has been conducted to identify pharmacodynamic biomarkers that can be used in combination with classic pharmacokinetic TDM of tacrolimus. These biomarkers are molecules that form part of the pathway targeted by tacrolimus, and include

the phosphatase activity of calcineurin, the nuclear translocation of the nuclear factor of activated T cells (NFAT; measured by flow cytometry), NFAT-regulated gene expression, NFAT cytoplasmic 1 (NFATc1) amplification, and IL-2 concentration (measured either by the IL-2 concentration or IL-2 messenger RNA expression). The results from these studies are limited by their relatively small study populations and the fact that these were not controlled clinical studies.[27-31] Most importantly, measuring the tacrolimus concentration is much easier to perform compared to the measurement of these pharmacodynamic biomarkers which requires sophisticated analytic procedures and which have no demonstrated clinical benefits (yet). As a result, none of the markers for pharmacodynamic TDM has currently been implemented in routine clinical practice, although this is a subject of active and ongoing research.

Recently, the enzyme-linked immunosorbent spot (ELISPOT) assay, which measures the number of cytokine-producing cells in response to specific antigen, has been proposed as a pharmacodynamic monitoring tool to predict the immunosuppressant response in terms of acute rejection after kidney transplantation.[32, 33] However, the results from studies using ELISPOT assay were conflicting and require confirmation and further study. The unanswered questions in using the donor-specific ELISPOT assay in kidney transplantation include the performance of pre-transplant *vs*. post-transplant ELISPOT assay in predicting different types of rejection (*e.g*., T cell-mediated *vs*. antibody-mediated acute rejection), and whether the ELISPOT should be better used for the exclusion or confirmation of acute rejection (*i.e*., the positive and negative predictive values of ELISPOT). In addition, the association between other transplantation outcomes such as kidney allograft function and ELISPOT should also be explored and summarized.[34-36] Besides the ELISPOT assay that measures the cytokine-producing cells in response to donor antigen, cytomegalovirus (CMV)-specific ELISPOT assay has been shown to perform well in the diagnosis and prediction of CMV infection in kidney transplant recipients.[37] Other infections, particularly BK virus (BKV) which currently lacks an immune-monitoring tool (other than quantitative polymerase chain reaction), should be investigated in this respect to find out if the ELISPOT assay can predict its clinical course or not.

Microsample-based tacrolimus concentration monitoring by dried blood spot (DBS) is becoming more popular. This method is patient-friendly, minimally invasive (only a small volume of blood (10-20 μL) is needed), and can be performed by the patient at home. DBS will allow more frequent sampling within a dosing interval and more easily enables the determination of a full AUC.[38] In addition, this method can be used by patients in whom there are contra-indications for the standard venous blood sampling, such as children, those who are difficult to sample, and those living in remote areas. During the coronavirus pandemic, when frequent hospital visits pose a risk to the patient, DBS may serve as a tool for TDM. We believe that in the near future, DBS will be more generally used for TDM.[39]

3.3 Concentration-effect relationship

The optimal tacrolimus concentration range that is associated with the lowest incidence of toxicity and rejection has been characterized poorly. Several studies have reported conflicting results and have not been able to define the exact cutoffs for the upper and lower limits of the target concentration range.[40] Kuypers *et al*. demonstrated that kidney transplant recipients with a tacrolimus AUC higher than 150 ng.h/mL on day 7 after transplantation were at lower risk of biopsy-proven acute rejection compared with recipients with an AUC lower than 150 ng.h/mL. In addition, the tacrolimus AUC in recipients with postoperative infections was significantly higher than recipients without infections (197.4±70.5 ng.h/mL *vs*. 160.5±47.9 ng.h/mL).[41]

Initially, the tacrolimus AUC was targeted at 210 ng.h/mL in the first 6 weeks then to 125 ng.h/mL, thereafter (corresponding to a C_0 of 12.5 and 7.5 ng/mL respectively, based on a model using population pharmacokinetic parameters).[26] More recent clinical trials have focused on tacrolimus C_0 rather than AUC in terms of tacrolimus exposure and clinical outcomes. However, no head-to-head study evaluating the outcomes of different target concentration ranges of tacrolimus has been conducted. The ELITE-Symphony study which targeted C_0 tacrolimus between 3 to 7 ng/mL during the first year after kidney transplantation (mean 6.4 ± 2.4 ng/mL) in combination with MPA resulted in biopsy-proven acute rejection rates as low as 12.3%, which were comparable to or even better than the acute rejection incidence in previous studies that targeted higher tacrolimus C_0. The lower target tacrolimus exposure in ELITE-Symphony did, however, result in a lower incidence of adverse effects.[42-45] The latest recommendation from the international consensus on managing modifiable risk in transplantation (COMMIT) currently recommends to target a tacrolimus C_0 of 6-10 ng/mL in the first month after kidney transplantation, which is reduced to 4-8 ng/mL thereafter.[46]

Since tacrolimus is nephrotoxic, attempts have been made to lower the tacrolimus target C_0 even further. In the large, randomized, controlled TRANSFORM trial, it was demonstrated that targeting reduced tacrolimus exposure (C_0 of 4-7 ng/mL during the first 2 months and slowly weaned to 2-4 ng/mL after 6 months) in combination with everolimus (C_0 of 3-8 ng/mL), was non-inferior to standard tacrolimus exposure plus MPA in terms of acute rejection and allograft function. However, the reduced tacrolimus exposure regimen was associated with a lower incidence of BKV and cytomegalovirus CMV infections.[47]

3.4 Inter-patient variability

Demographic factors, drug-drug interactions, and genetics are causes of inter-patient variability. Several single-nucleotide polymorphisms (SNPs) have been identified in the *CYP3A4* and *CYP3A5* genes. The most studied SNP is *CYP3A5*1/*3*. The *CYP3A5*3*

variant allele causes alternative splicing, leading to the absence of functional CYP3A5 protein and decreased CYP3A5 activity compared with the *CYP3A5*1* allele. Patients with the *CYP3A5*1/*1* and *CYP3A5*1/*3* genotype are considered CYP3A5 expressors or "rapid metabolizers" and need 1.5 to 2.0-times higher doses of tacrolimus to achieve the same target concentration compared with CYP3A5 non-expressors (individuals with the *CYP3A5*3/*3* genotype).[48]

*CYP3A4*22* is a variant allele which has been associated with decreased CYP3A4 metabolic activity.[49] Based on the combination of *CYP3A4* and *CYP3A5* genotype, rapid (*CYP3A4*1/*1* plus the *CYP3A5*1/*1* or the *CYP3A5*1/*3* genotype), and slow metabolizers (*CYP3A4*1/*22* or *CYP3A4*22/*22* plus *CYP3A5*3/*3*) can be identified. While the rest of the combinations are considered CYP3A intermediate metabolizers.[38]

Several randomized controlled trials (RCTs) in kidney transplantation have shown that *CYP3A5* genotype-guided tacrolimus (start) dosing can lead to a more rapid achievement of the target concentration compared with a standard, bodyweight-based starting dose.[50-52] However, this has not been a universal finding.[53] Possibly, more advanced dosing regimens using computerized / algorithm-based dosing can further optimize tacrolimus therapy. A *maximum a posteriori* Bayesian estimation (MAP-BE) technique is currently an accepted method to estimate the AUC that involves the use of large patient databases with concentration-time profiles.[54] Woillard *et al*. demonstrated that machine-learning algorithms can further improve the accuracy of AUC estimation from MAP-BE.[55] Moreover, the same group of authors has shown that machine-learning algorithms that used population parameters of previously published population pharmacokinetics (instead of the large patient databases used in MAP-BE) can yield a comparable estimation of tacrolimus AUC with less than 5% bias compared with the MAP-BE.[56] However even if such an algorithm-guided tacrolimus dosing strategy is shown to lead a better exposure to the drug, a clinical benefit in terms of less rejection and toxicity, remains to be demonstrated.[51, 52, 57] Moreover, since transplantation involves genotypes from 2 individuals (donor and recipient), the different combinations of donor and recipient genotypes might affect tacrolimus pharmacokinetics and outcomes of transplantation.

Other factors that contribute to inter-patient variability include clinical factors and drug-drug interactions.[58] Tacrolimus is distributed widely in erythrocytes and anemia increases the concentration of unbound tacrolimus, without changing the total whole blood concentration. Patients with hepatic dysfunction have a decreased CYP3A activity and this may result in higher whole blood tacrolimus exposure. Finally, drug-drug interactions interfere with CYP3A enzyme activity and affect tacrolimus exposure as displayed in Table 1.[59]

Table 1: Significant drug-drug interaction with CYP3A enzymes

Drug class	CYP3A inducer (Decrease tacrolimus level)	CYP3A inhibitor (Increase tacrolimus level)
Antiviral	Efavirenz, nevirapine	Protease inhibitors, elbasvir
Antimycobacterial	Rifampicin	Isoniazid
Antibacterial	-	Erythromycin, clarithromycin (not azithromycin), ciprofloxacin
Antifungal	Griseofulvin	Azoles antifungal
Anticonvulsant	Carbamazepine, phenobarbital, phenytoin	-
Corticosteroid	Dexamethasone	Methylprednisolone
Others	Pioglitazone	Calcium channel blockers, omeprazole, metoclopramide, theophylline, amiodarone, ethinylestradiol, cannabidiol

3.5 Intra-patient variability

Intra-patient variability in tacrolimus exposure (IPV) denotes the variability in tacrolimus concentrations over time without changes in the dose. IPV can be calculated in several ways but the most frequently used is the coefficient of variability (CV).[15] Patients having a high IPV have large fluctuations in their exposure to tacrolimus and will likely spend less time within the therapeutic range and more time in the supra-therapeutic or sub-therapeutic concentration ranges.

Medication non-adherence is considered the most common cause of a high tacrolimus IPV, which is usually defined as a CV higher than 25-30%. Leino *et al.* showed that the tacrolimus CV was 16.8% in a cohort of kidney transplant recipients with 99.9% medication adherence.[60] This information supports the notion that the intrinsic IPV of tacrolimus is low in the absence of medication non-adherence. However, missed-doses and fluctuation of the dosing interval (*i.e.* taking the drug too early or later than scheduled) are not uncommon in the real-word, in addition to other factors that affect tacrolimus IPV, such as the hemoglobin concentration, hypoalbuminemia, gut dysmotility, and drug-drug interactions.[58]

Several studies have investigated the association between tacrolimus IPV and kidney transplantation outcomes.[15] A high tacrolimus IPV was associated with an increased risk of acute rejection, more *de novo* DSA formation, worse allograft function, more rapid evolution of chronic histologic lesions suggestive of tacrolimus nephrotoxicity, and an increased risk of allograft loss.[61, 62] These findings are in line with the hypothesis that patients with a high tacrolimus IPV will more often be subject to both under- and over-immunosuppression and the related complications of rejection and chronic tacrolimus-induced nephrotoxicity.

So far, the most effective strategy to reduce tacrolimus IPV is to improve medication adherence. This may be accomplished by remote drug monitoring and drug-dosing assist software.[62, 63] The tacrolimus IPV can also be reduced by switching from twice-daily tacrolimus to the once-daily, extended-release formulation.[64] However, this has not been a universal finding.[65] The lower tacrolimus IPV observed in patients taking the once-daily tacrolimus formulation likely results from the improvement in medication adherence rather than the pharmaceutical property of the drug itself.[63]

3.6 Analytic methods

The most frequently used analytical methods to measure tacrolimus in whole blood are liquid chromatography-tandem mass spectrometry (LC-MS/MS) which is the gold standard, or an immunoassay. LC-MS/MS is now replacing immunoassays.[66] The advantage of LC-MS/MS is its high sensitivity and specificity. However, well-trained laboratory personnel is needed and the apparatus is very expensive. An important disadvantage of immunoassays is their cross-reactivity with some of tacrolimus' metabolites which may lead to an overestimation of the true whole blood tacrolimus concentration.[38, 67]

Although whole blood is the routine matrix for TDM of tacrolimus, more evidence is accumulating that this may not be the best way to measure the drug. Since tacrolimus' site of action is within the immune cells, particularly the lymphocytes, intracellular (intra-lymphocytic) tacrolimus may better correlate with the drug's effect and transplant outcomes.[68-70] The landmark study by Capron *et al*. included 90 liver transplant recipients who received tacrolimus monotherapy. The authors found no association between the whole blood tacrolimus concentration and liver allograft histological rejection grade. However, the concentration of tacrolimus within peripheral blood mononuclear cells (PBMCs) did correlate negatively with the histological staging of acute liver transplant rejection. There was a poor correlation between the intra-PBMC and the whole blood tacrolimus concentration.[68] However, subsequent studies in liver, kidney, and heart transplantation could not confirm the association between the intracellular tacrolimus concentration and the risk and severity of rejection.[71-73] This may relate to differences in study design and analytical differences in the assays that were used. First, these studies did not incorporate the use of red blood cell lysis step before the measurement of the intracellular tacrolimus concentration. As a result, tacrolimus concentration is likely to be contaminated with tacrolimus originating from erythrocytes. Second, the timing of the intracellular tacrolimus concentration measurement was different from the timing of acute rejection episode. Ideally, this should be done on the same day or at least within 1 to 2 weeks before the acute rejection episode.[74, 75] Third, since PBMCs contain many immune cell subsets including T lymphocytes, monocytes, B lymphocytes, and NK cells, the intracellular tacrolimus concentration in one of these immune cell subsets might better correlate with

acute rejection. These limitations should be solved and assessed in future studies investigating the intracellular tacrolimus concentration.

Sallustio *et al*. explored the relationship between the intra-allograft and the whole blood concentration of tacrolimus in kidney transplant recipients.[76] They demonstrated that recipients who developed acute tacrolimus-nephrotoxicity had a significantly higher intra-allograft-to-whole blood ratio of tacrolimus. However, their finding was limited by the small sample size, low incidence of nephrotoxicity, and the different timing of blood sampling and allograft biopsies. Nonetheless, this study demonstrates that the intra-renal tacrolimus concentration may be related to its renal toxicity.

4. CONCLUSIONS

In conclusion, TDM is required for tacrolimus in kidney transplantation. The knowledge of the pharmacokinetics, pharmacodynamics, and pharmacogenetics of tacrolimus has greatly expanded over the last 20 years. We now have tools that allow for a further individualization of tacrolimus-based immunosuppressive therapy and the monitoring thereof. However, more studies to improve the TDM of tacrolimus (and other immunosuppressive drugs) are needed in order to successfully step into the era of precision medicine. The donor and recipient *CYP3A5* genotype should be considered together when assessing its association with transplantation outcomes. The ELISPOT assay has been shown to be a potential tool for pharmacodynamic monitoring but standardization of the assay is required. In addition, dynamic monitoring of a patient's immune status should help to adjust the immunosuppressive therapy to prevent acute rejection and other toxicities such as nephrotoxicity and viral infections. Finally, the measurement of the intracellular tacrolimus concentration is a promising strategy as it may better correlate with its efficacy and toxicity than the whole blood concentration. There are opportunities for improvement of the intracellular tacrolimus concentration measurement, such as eliminating erythrocyte contamination and the measurement of tacrolimus in immune cell subsets of PBMCs.

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Chapter 2

Aims of the Thesis

AIMS OF THE THESIS

Immunosuppressants are the key to the success of kidney transplantation. Adequate immunosuppression minimizes the risk of acute rejection and avoids adverse events such as nephrotoxicity and viral infections. The prediction of the response to immunosuppressive therapy would be beneficial as it may identify high-risk kidney transplant recipients who require more close monitoring of treatment. The general aim of this thesis is to explore strategies using pharmacokinetics (the intracellular tacrolimus concentration), pharmacodynamics (ELISPOT assay), and pharmacogenetics (*CYP3A5* genotype) of tacrolimus to predict kidney transplant outcomes. More specifically, the aims of the work described in this thesis was:

- To examine the effect of a *CYP3A5* mismatch between donor and recipient on tacrolimus-associated nephrotoxicity after kidney transplantation (**Chapter 3**).
- To investigate the capability of the enzyme-linked immunosorbent spot (ELISPOT) assay to predict acute rejection after kidney transplantation (**Chapter 4**).
- To study the use of BK virus-specific ELISPOT in predicting post-transplant BK virus infection (**Chapter 5**).
- To develop a prediction model that includes a combination of immunosuppressants to predict patient and kidney allograft survival in Asian population (**Chapter 6**).
- To evaluate the effect of acute rejection on the composition of peripheral blood mononuclear cells of kidney transplant recipients (**Chapter 7**).
- To explore the association between intracellular tacrolimus concentration in $CD3^+$ T lymphocytes and $CD14^+$ monocytes and acute rejection in kidney transplant (**Chapter 8**).
- To demonstrate the role of P-glycoprotein (P-gp) and FK-binding protein-12 (FKBP-12) in the intracellular pharmacokinetics of tacrolimus in $CD3^+$ T lymphocytes and $CD14^+$ monocytes (**Chapter 9**).

The summary and main findings are discussed in **Chapter 10** and **Chapter 11**

Chapter 3

The Cytochrome P450 3A5 Non-Expressor Kidney Allograft as a Risk Factor for Calcineurin Inhibitor Nephrotoxicity

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ABSTRACT

Background

Tacrolimus is mainly metabolized by cytochrome P450 3A5 (*CYP3A5*) which is expressed in the liver. However, *CYP3A5* is also expressed in the kidney tissue and may contribute to local tacrolimus clearance in the kidney allograft. We aimed to evaluate the association between allograft *CYP3A5* genotype and transplant outcomes.

Methods

We conducted a retrospective cohort study at King Chulalongkorn Memorial Hospital, Thailand, comparing two groups of donor and recipient *CYP3A5* genotypes, the expressor (**1/*1* and **1/*3*) and the non-expressor (**3/*3*). The primary outcomes were allograft complications including calcineurin inhibitor (CNI) nephrotoxicity and acute rejection episode.

Results

Of the 50 enrolled patients, 21 donors were expressors and 29 donors were the non-expressors. Tacrolimus trough concentrations were similar between the two genotypes. The incidence of CNI nephrotoxicity was higher in recipients with non-expressor donor genotype compared with the expressor donor genotype (72.4 vs 33.3%, p=0.006). CNI nephrotoxicity incidence was not different when compared between recipient's genotypes. Multivariate analysis from Cox-regression showed a hazard ratio of 3.18 (p=0.026) for CNI nephrotoxicity in the non-expressor compared with the expressor donor. The recipient *CYP3A5* genotypes did not significantly contribute to CNI nephrotoxicity. Kaplan-Meier analysis demonstrated the lowest CNI nephrotoxicity-free survival in recipients with expressor genotype who received allograft from the non-expressor donors (p=0.005).

Conclusion

In conclusion, our results suggest that donor *CYP3A5* non-expressor genotype (*3/*3) is a risk for CNI nephrotoxicity.

INTRODUCTION

Tacrolimus is the preferred calcineurin inhibitor (CNI) drug in kidney transplantation due to less biopsy-proven acute rejection and better graft survival than cyclosporin.[1-4] High tacrolimus exposure may lead to CNI nephrotoxicity while low tacrolimus exposure poses a risk of induce acute rejection.[5] Currently, whole blood tacrolimus level is the standard method used for monitoring tacrolimus exposure in clinical practice.[6] The major determinant of the dose requirement to achieve the target blood tacrolimus level is cytochrome P450 (*CYP3A5*) which is predominantly expressed in the liver. *CYP3A5* is polymorphic.[7] The non-expressor recipient (*3/*3 genotype) requires a lower dose while the expressor (*1/*1 and *1/*3) recipient requires a higher dose to achieve the target tacrolimus level.[8-11] Therefore, the non-expressor recipient may have a higher risk of CNI nephrotoxicity while the expressor recipient may be prone to acute rejection.[7, 12, 13]

Indeed, *CYP3A5* is also found in renal tubular cells.[14] An *in vitro* study showed that inactive metabolites of tacrolimus in kidney tissue with *CYP3A5* expressor genotype were significantly greater than in the *CYP3A5* non-expressor.[15] As such, the donor (allograft) *CYP3A5* genotype could alter local tacrolimus metabolism and exposure, and therefore affect allograft outcomes, including CNI nephrotoxicity and acute rejection. Previous clinical studies using immunohistochemistry staining to detect the expression of donor *CYP3A5* in renal allograft provided conflicting results regarding the role of donor *CYP3A5* in local tacrolimus metabolism and allograft outcomes.[16, 17]

There is wide variability of *CYP3A5* among between ethnicities. More than 80% of the Caucasians are non-expressor,[18] suggesting that during transplantation there will be more than a 64% chance of recipient and donor allograft having the same *CYP3A5*. However, approximately 50% of the Asians are non-expressor, yielding less than one fourth chance of having the same *CYP3A5* between the recipient and donor allografts. As such, despite achieving the target blood tacrolimus levels, the discrepancy or mismatch of *CYP3A5* between the recipient and donor may lead to different local tacrolimus metabolism and allograft outcomes.

In the present study, *CYP3A5* genotype analysis was performed to examine the role of donor and recipient *CYP3A5* genetic polymorphism on allograft outcomes including CNI nephrotoxicity and acute rejection in kidney transplant recipients with various *CYP3A5* genetic polymorphisms.

MATERIALS AND METHODS

We conducted a retrospective cohort study of kidney transplant recipients at King Chulalongkorn Memorial Hospital, Thailand, who came for follow-up at the post-transplantation clinic from October 2015 to May 2016. The recipients enrolled in the study had to meet the following criteria, i) between 15-70 years-old, and ii) receiving a regimen of twice-daily tacrolimus (targeting trough concentration 4-7 ng/mL) with mycophenolic acid and prednisolone as maintenance immunosuppression. Patients who were receiving strong *CYP3A5* interference medications for more than one month were excluded. All recipients underwent surveillance allograft biopsy as the standard of care in our center.

CYP3A5 genotypes analysis

Kidney allograft *CYP3A5* genotypes (donor genotypes) were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). The samples were taken from paraffin block of allograft biopsy in recipients transplanted from deceased-donor. For recipients with lived-donor, the whole blood has been used for *CYP3A5* genotype analysis. Invitrogen™ kits were used for DNA extraction. Identification of the *CYP3A5* gene alleles was conducted by real-time RT-PCR using forward and reverse primer (F 5'-CAT GAC TTA GTA GAC AGA TGA-3', R 5'-GGT CCA AAC AGG GAA GAA ATA-3'). The allelic variant of *CYP3A5* (rs776746) gene was established using a fluorescent TaqMan probe. The donor *CYP3A5* genotypes were then categorized into two groups, the expressors (*1/*1 and *1/*3) and the non-expressors (*3/*3).

Immunosuppression protocol and patient follow up

The induction medications were methylprednisolone with either basiliximab or rabbit anti-thymocyte globulin. All patients included in this study received tacrolimus (Prograf) as a maintenance immunosuppressive medication (tacrolimus trough level of 4-7 ng/mL), along with mycophenolate mofetil (Cellcept) and prednisolone (2.5-5 mg/day). All recipients underwent surveillance kidney allograft biopsy from the specific time points (1st, 6th, 12th, 24th, 36th, and 48th month after kidney transplantation) and also if clinically indicated. Estimated glomerular filtration rate (eGFR) was calculated by the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration)-derived equation.[19]

The timing and incidence of both rejection and CNI nephrotoxicity from the allograft biopsy were recorded. The allograft biopsy slides were reviewed and interpreted by the same renal pathologist who was blinded to the *CYP3A5* results. The definition and histological diagnosis of CNI nephrotoxicity were defined according to a previous study,[5] including isometric tubular vacuolization, thrombotic microangiopathy (TMA) (cases with other possible causes of TMA were excluded), striped fibrosis, medial arteriolar hyalino-

sis, and tubular microcalcification. Other details of patients' clinical course including serum creatinine, therapeutic drug monitoring, and allograft rejection (diagnosis criteria according to Banff classification[20, 21]) were also recorded. The primary outcome measured was the incidence of CNI nephrotoxicity in the recipients transplanted from the donors with expressor (*CYP3A5**1/*1 and *1/*3) and non-expressor (*CYP3A5**3/*3) genotypes. The secondary outcomes also measured were acute rejection incidence and allograft function.

Ethics

Ethical considerations were reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, in compliance with the international guidelines for human research protection as set forth by the Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP) (IRB number 054/59).

Statistical analysis

The data were analyzed by SPSS statistics version 22.0 for Windows (IBM Corp., New York, NY, USA). An unpaired t-test was used for comparing continuous data and reported as mean with standard deviation (SD). A chi-square test was used for categorical outcomes. Survival analysis of CNI nephrotoxicity was estimated using the Kaplan-Meier method and probabilities were compared using log-rank analysis. The hazard ratio with 95% confidence interval (CI) for CNI nephrotoxicity was calculated by Cox-regression analysis and variables with p-value less than 0.25 from univariate analysis were included into a multivariate model. A p-value less than 0.05 and power at 80% were considered statistically significant.

RESULTS

From 255 patients who came for follow-up at the post-transplantation clinic during the inclusion period, 135 patients were receiving twice-daily tacrolimus (Prograf) with mycophenolate mofetil (Cellcept) as the maintenance regimen since transplanted, without any changes to other immunosuppressive drugs. Sixty-five patients were further excluded due to the co-administration with calcium channel blockers, statins, or benzodiazepines. Twenty patients were excluded due to inadequate allograft tissue for *CYP3A5* genotyping. Therefore, 50 patients met the inclusion criteria and were enrolled in this study. The recipients in this study were standard immunologic risk with 1 to 5 human leukocyte antigen (HLA) mismatches and low panel reactive antibody (PRA) (Table 1). Thirty-eight percent of the patients received kidney allograft from a deceased donor. *CYP3A5* distribution among donors in this study was comparable to other studies for the Asian population,[22, 23] in which *CYP3A5* *3/*3

(n=29) was the most common genotype followed by *1/*3 (n=15) and *1/*1 (n=6), respectively.

Table 1: Baseline characteristics

Variables	Expressor donor (CYP3A5*1/*1 and *1/*3) (n=21)	Non-expressor donor (CYP3A5*3/*3) (n=29)	p-value
Recipient age (year), mean (SD)	43.4 (9.7)	38.7 (12.6)	0.147
Recipient gender, n (%)			0.815
Male	13 (62%)	17 (59%)	
female	8 (38%)	12 (41%)	
HLA mismatch, n (%)			0.307
0	4 (19%)	2 (7%)	
1-5	15 (71%)	26 (89%)	
6	2 (10%)	1 (4%)	
Panel reactive antibody (PRA), n (%)			0.078
0	16 (75%)	18 (62%)	
1-79	3 (15%)	11 (38%)	
≥ 80	2 (10%)	0 (0%)	
Donor age (year), mean (SD)	40.7 (9.6)	42.5 (11.0)	0.556
Donor terminal creatinine (mg/dL), mean (SD)	1.86 (3.22)	1.65 (1.26)	0.840
Recipient CYP3A5 genotypes, n (%)			0.927
***1/*1 and *1/*3**	14 (67%)	17 (65%)	
***3/*3**	7 (33%)	9 (35%)	

There were 21 patients in the expressor donor group (*CYP3A5* *1/*1 and *1/*3) and 29 in the non-expressor donor group (*CYP3A5* *3/*3). There were no differences in tacrolimus trough levels and tacrolimus dosages (Table 2).

Table 2: Results according to donor *CYP3A5* genotypes.

Outcomes	Donor CYP3A5 genotypes		
	*1/*1 and *1/*3 (n=21)	*3/*3 (n=29)	p-value[a]
C0 tacrolimus (ng/mL)[b] , mean (SD)	7.23 (1.29)	6.48 (1.95)	0.128
Tacrolimus dosage per day (mg), mean (SD)	6.82 (2.97)	6.18 (3.57)	0.508
C0 per dosage tacrolimus ((ng/mL)/mg), mean (SD)	1.27 (0.60)	1.51 (1.01)	0.349
C0 per dosage tacrolimus per kg ((ng/mL)/(mg/kg)), mean (SD)	0.022 (0.012)	0.027 (0.016)	0.259
eGFR at 1 year post kidney transplantation (mL/min/1.73 m^2) [c], mean (SD)	**66 (14)**	**54 (14)**	**0.005**
eGFR at 3 years post kidney transplantation (mL/min/1.73 m^2) [c], mean (SD)	59 (15)	53 (22)	0.297
Follow-up time (months), mean (SD)	19.1 (12.5)	20.2 (26.6)	0.856
CNI nephrotoxicity [d], n (%)	**7 (33.3%)**	**21 (72.4%)**	**0.006**
T cell mediated rejection [e], n (%)	5 (23.8%)	5 (17.2%)	0.567
Antibody-mediated rejection [e], n (%)	4 (19%)	6 (20.7%)	0.886

[a] P-value was calculated using unpaired t-test for continuous data and chi-square test for categorical data.

[b] Tacrolimus trough concentration

[c] estimated GFR was calculated by CKD-EPI creatinine equation (reference in text).

[d] Pathological diagnosis of CNI nephrotoxicity included isometric tubular vacuolization, thrombotic microangiopathy (cases with other possible causes of TMA were excluded), striped fibrosis, medial arteriolar hyalinosis, and tubular microcalcification.[5]

[e] Rejection was diagnosed according to Banff 2007 classification with update on antibody-mediated rejection in 2013.[20, 21]

According to the recipient *CYP3A5* genotype, both expressor and non-expressor recipients achieved the same target tacrolimus levels. However, the expressor recipient group required a higher dose of tacrolimus (p = 0.013) (Table 3). The acute rejection rate was similar in both cellular and antibody-mediated subtypes. Twenty-eight patients (56%) experienced biopsy-proven CNI nephrotoxicity which had significantly lower eGFR at 1 year and 3 years when compared to patients without CNI nephrotoxicity (53±14 vs 67±12 mL/min/1.73 m^2, p-value <0.001 and 50±20 vs 64±16 mL/min/1.73 m^2, p-value=0.01, respectively). CNI nephrotoxicity incidence was comparable between the two genotype groups of the recipients (Table 3). Patients receiving kidney allograft from the donors with non-expressor genotype had higher incidence of CNI nephrotoxicity (72.4 vs 33.3%, p = 0.006), compared with the expressor genotype donors (Table 2). From 176 total biopsies, 104 biopsies were from the non-expressor allografts (3.6 biopsies per patient), and 72 biopsies were from the expressor

allografts (3.4 biopsies per patient). CNI nephrotoxicity features were found significantly higher in the non-expressor allograft (42 biopsies) compared with the expressor allograft (12 biopsies) (40.4 vs 17.7%, p-value <0.001).

Table 3: Results according to recipient *CYP3A5* genotypes.

Outcomes	Recipient CYP3A5 genotypes		
	***1/*1 and *1/*3 (n=31)[f]**	***3/*3 (n=16)[f]**	**p-value[a]**
C0 tacrolimus (ng/mL) [b], mean (SD)	6.53 (1.86)	7.18 (1.47)	0.200
Tacrolimus dosage per day (mg), mean (SD)	**7.46 (3.24)**	**4.95 (3.00)**	**0.013**
C0 per dosage tacrolimus ((ng/mL)/mg), mean (SD)	**1.08 (0.61)**	**1.94 (1.01)**	**0.005**
C0 per dosage tacrolimus per kg ((ng/mL)/(mg/kg)), mean (SD)	**0.020 (0.012)**	**0.032 (0.016)**	**0.012**
eGFR at 1 year post kidney transplantation (mL/min/1.73 m^2) [c], mean (SD)	61 (15)	57 (16)	0.496
eGFR at 3 years post kidney transplantation (mL/min/1.73 m^2) [c], mean (SD)	59 (19)	47 (19)	0.062
Follow-up time (months), mean (SD)	16.0 (13.5)	29.8 (31.4)	0.110
CNI nephrotoxicity [d], n (%)	14 (45.2%)	11 (68.8%)	0.125
T cell mediated rejection [e], n (%)	4 (12.9%)	4 (25.0%)	0.296
Antibody-mediated rejection [e], n (%)	4 (12.9%)	5 (31.3%)	0.130

[a] P-value was calculated using unpaired t-test for continuous data and chi-square test for categorical data.

[b] Tacrolimus trough concentration

[c] estimated GFR was calculated by CKD-EPI (reference in text).

[d] Pathological diagnosis of CNI nephrotoxicity included isometric tubular vacuolization, thrombotic microangiopathy (cases with other possible causes of TMA were excluded), striped fibrosis, medial arteriolar hyalinosis, and tubular microcalcification.[5]

[e] Rejection was diagnosed according to Banff 2007 classification with update on antibody-mediated rejection in 2013.[20, 21]

[f] Data of recipient *CYP3A5* was missing in 3 patients due to patient's death and loss to follow up.

To calculate the hazard ratio of CNI nephrotoxicity from our potential risk factors in this study, Cox-regression analysis was performed. Multivariate analysis showed the significant contribution of donor *CYP3A5* genotypes to CNI nephrotoxicity (Table 4). Kidney allografts with the non-expressor genotype were at 3.18-fold risk for developing CNI nephrotoxicity compared with the expressor genotype. A higher tacrolimus dosage also increased the risk of CNI nephrotoxicity, while the recipient *CYP3A5* genotypes and tacrolimus levels were not associated.

Table 4: Hazard ratio of CNI nephrotoxicity from Cox-regression analysis.

Variables	Univariate analysis		Multivariate analysis	
	Unadjusted hazard ratio (95% CI)[a]	p-value	Adjusted hazard ratio (95% CI)[b]	p-value
HLA mismatch (per 1 MM increase)	1.00 (0.78-1.27)	0.967	-	-
PRA (per 1% increase)	1.01 (0.99-1.02)	0.420	-	-
Donor age (per 1 year increase)	1.05 (1.00-1.10)	0.048[+]	1.03 (0.98-1.08)	0.260
Donor terminal creatinine (per 1 mg/dL increase)	1.01 (0.84-1.22)	0.924	-	-
Tacrolimus dosage (per 1 mg increase)	1.23 (1.09-1.39)	0.001[+]	**1.24 (1.07-1.42)**	**0.003**
Trough tacrolimus concentration (per 1 ng/mL increase)	0.82 (0.62-1.08)	0.159[+]	1.01 (0.77-1.31)	0.960
Recipient *CYP3A5* genotype (non-expressor vs expressor)	0.96 (0.62-1.49)	0.864	-	-
Donor *CYP3A5* genotype (non-expressor vs expressor)	2.31 (0.96-5.54)	0.061[+]	**3.18 (1.15-8.77)**	**0.026**

[a] Unadjusted hazard ratio for calcineurin inhibitor nephrotoxicity

[b] Adjusted hazard ratio for calcineurin inhibitor nephrotoxicity

[+] Variables with p-value less than 0.25 from univariate analysis were included into a multivariable model.

CNI nephrotoxicity-free survival was determined according to donor *CYP3A5* genotypes using Kaplan-Meier analysis (Figure 1). The non-expressor donor *CYP3A5* genotype was significantly associated with a higher rate of biopsy-proven CNI nephrotoxicity compared with the expressor genotype. The median time-to-CNI nephrotoxicity were 11 and 25 months in the non-expressor and the expressor groups, respectively (log-rank test, p-value = 0.048).

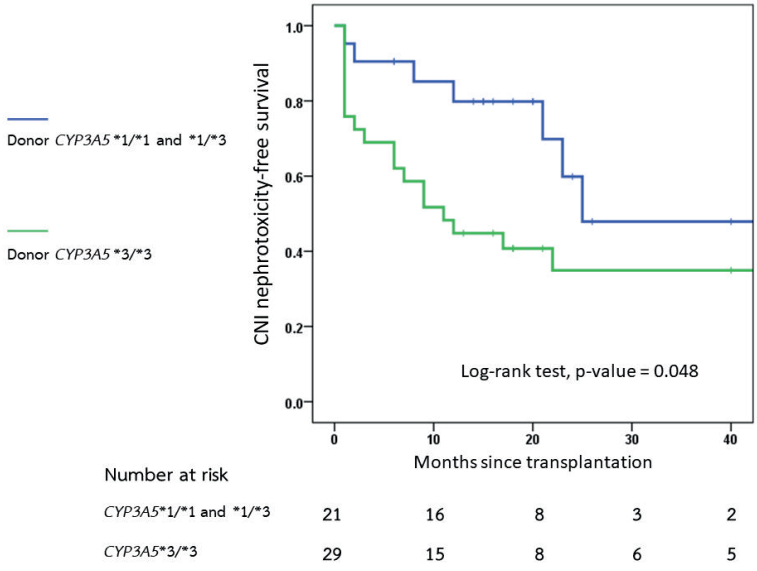

Figure 1: Kaplan-Meier analysis of CNI nephrotoxicity-free survival according to donor *CYP3A5* genotypes.

The recipients were stratified into 4 groups according to the donor and recipient *CYP3A5* genotypes: the expressor donor/expressor recipient, the non-expressor donor/non-expressor recipient, the expressor donor/non-expressor recipient, and the non-expressor donor/expressor recipient. We analyzed these *CYP3A5* genotypes matching by Kaplan-Meier methods with CNI nephrotoxicity-free survival as the outcome (Figure 2 and Table 5) and found that, the non-expressor donor/expressor recipient group had the highest incidence of CNI nephrotoxicity (p = 0.005 vs expressor donor/expressor recipient group).

Table 5: Median time to CNI nephrotoxicity according to donor-recipient *CYP3A5* genotypes matching.

Donor-recipient CYP3A5 genotypes	Median time (months) to CNI nephrotoxicity	p-value (log-rank test)
Expressor donor/Expressor recipient	N/A	Reference
Non-expressor donor/non-expressor recipient	72	0.504
Expressor donor/non-expressor recipient	23	0.090
Non-expressor donor/Expressor recipient	6	0.005

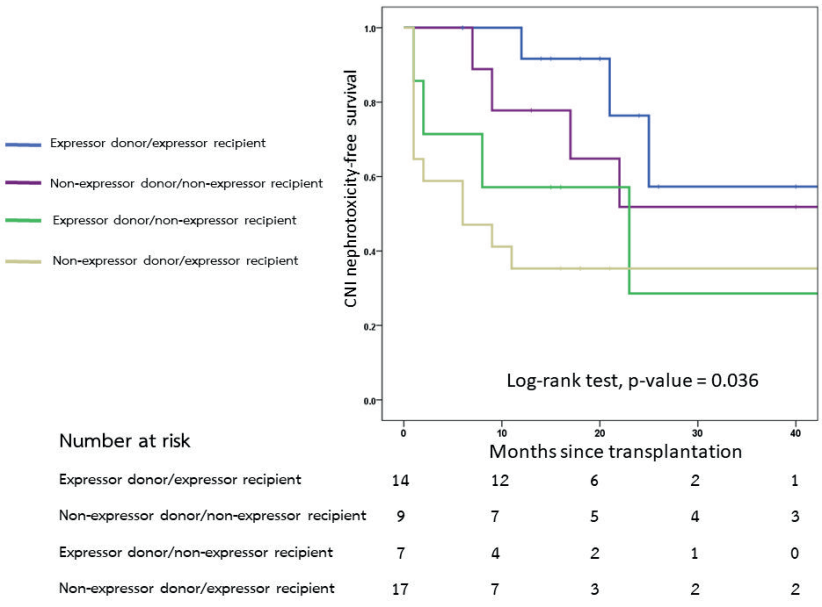

Figure 2: Kaplan-Meier analysis of CNI nephrotoxicity-free survival according to the donor and recipient *CYP3A5* genotypes.

DISCUSSION

CYP3A5 is the major enzyme responsible for tacrolimus metabolism and clearance. The wild type sequence of human *CYP3A5* is *CYP3A5**1 but the expression is highly variable. Individuals with at least one allele can synthesize a functional *CYP3A5* protein. The *CYP3A5**3 polymorphism (*CYP3A5* 6986 A>G) affects RNA splicing, resulting in premature termination codons and inactivation of *CYP3A5*.[24, 25] As such, there are two groups of *CYP3A5* genotypes; expressor (*1/*1 and *1/*3) and non-expressor (*3/*3). As stated previously, human *CYP3A5* is mainly expressed in the liver but can also be detected in the kidney. Therefore, systemic clearance of the drugs and the degree of systemic immunosuppression are likely related to the recipient *CYP3A5*[11, 18] while the local tacrolimus metabolic clearance, accumulation, and potential risk of CNI nephrotoxicity may be associated with the donor allograft *CYP3A5*.

The results in the present study have demonstrated that, regardless of the *CYP3A5* genotypes of both recipients and donors, the concentration of tacrolimus in recipients had achieved the recommended levels and the optimal degree of systemic immuno-

suppression (Table 2 and 3). Thus, recipients who obtained allografts with expressor (*CYP3A5**1/*1 and *1/*3) and non-expressor (*CYP3A5**3/*3) donor genotypes had comparable tacrolimus levels (Table 2). Acute rejection rates between donor allografts with expressor and non-expressor genotypes were similar (Table 2). Despite these comparable parameters, the non-expressor donor genotype induced a higher and earlier incidence of CNI nephrotoxicity together with lower eGFR than the expressor donor genotype (Table 2). The recipients with non-expressor donor allograft were at 3 times higher risk to develop CNI nephrotoxicity (Table 4). Increased tacrolimus dosage also enhanced the risk of CNI nephrotoxicity while the recipient *CYP3A5* genotypes and tacrolimus levels did not affect the risks of CNI nephrotoxicity (Table 4).

Due to the non-association with CNI nephrotoxicity, tacrolimus blood concentration may not be a good indicator for local tacrolimus level in the kidney allograft. This study highlights the role of the donor *CYP3A5* genotype in developing CNI nephrotoxicity (Table 4 and Figure 1). The decreased local metabolism and clearance of tacrolimus in the non-expressor donor allograft might result in elevated tacrolimus concentration and CNI nephrotoxicity. This hypothesis is supported by an earlier study of tacrolimus metabolic clearance in human kidney microsomes. The inactive metabolite of tacrolimus (13-*O*-desmethyl tacrolimus) was 13.5-fold higher in kidney tissue with *CYP3A5* *1/*3 genotype compared with *CYP3A5**3/*3 genotype.[15] The local tacrolimus clearance is not yet confirmed by *in vivo* study. Measuring the inactive metabolite of tacrolimus in the allograft tissue or in urine might reveal the evidence to support the hypothesis.

Previously, three studies have evaluated the role of donor *CYP3A5* in the kidney transplant patient. However, the results were conflicting. Joy et al. demonstrated the association of donor *CYP3A5* and CNI nephrotoxicity with 59 patients in a previous case-control study.[16] *CYP3A5* expression in the renal tubular cells was detected by immunohistochemistry staining. The CNI nephrotoxicity group had less *CYP3A5* expression compared with the control group. Metalidis et al. also used immunohistochemistry staining to identify the expression of *CYP3A5* on the brush border of renal tubules in 103 kidney transplanted recipients.[17] *CYP3A5* expression in the distal tubules was lower in the CNI nephrotoxicity group than the control (10 vs 39%, $p<0.01$). However, the expression of *CYP3A5* in the proximal tubules was more commonly found in the CNI nephrotoxicity group than the non-nephrotoxicity group (47 vs 14%, $p<0.01$). A cohort of 209 French kidney transplantation patients studied by Glowacki et al. found no correlation between donor or recipient *CYP3A5* polymorphisms and tacrolimus toxicity.[9]

Two of the aforementioned studies used immunohistochemistry staining to detect the expression of *CYP3A5* protein. A weakness of this method is the semi-quantitative nature

and the sensitivity of staining and grading. Furthermore, protein expression may vary over time and may not correlate with function. The present study use *CYP3A5* genotyping which is an intrinsic property of patients, and the correlation between *CYP3A5* genotype and function has been previously shown in many studies.[8, 11, 18]

Earlier studies that attempted to determine the association between the recipient genotype and CNI nephrotoxicity yielded inconclusive results.[7, 12, 13, 24, 26-28] In this study, multivariate analysis did not show a direct association between the recipient *CYP3A5* genotype and CNI nephrotoxicity (Table 4). However, the recipient *CYP3A5* genotype may have an indirect role on CNI nephrotoxicity. The expressor recipient would require a higher tacrolimus dose to achieve the therapeutic level, causing the higher tacrolimus dose to be an independent factor of CNI nephrotoxicity (Table 4).

Of interest, the highest risk of CNI nephrotoxicity occurred when expressor recipients were matched with donor non-expressor allografts compared with other genotype combinations (Figure 2 and Table 5). This finding can be explained by the slow tacrolimus clearance from the donor allograft with non-expressor genotype and the high tacrolimus dosage needed in the recipient with expressor genotype.

In addition to the *CYP3A5* genotype, other genes that involve with the metabolism and clearance of tacrolimus might contribute to the CNI nephrotoxicity.[29-32] These include the multidrug resistance protein 1 (*MDR1*) which transport nephrotoxic CNI out of cells, and the caveolin 1 (*CAV1*) which prevent the interstitial fibrosis by inhibiting the transforming growth factor beta signaling. Further study of these genes might add more information to guide and tailor the immunosuppressive drugs in the concept of precision medicine.

As mentioned earlier, the proportion of non-expressor is 84% in the Caucasians compared with 51% in Asians.[18, 23] This proportion results in 13% and 25% of kidney transplanted patients being at a high risk for CNI nephrotoxicity (the expressor recipient matched with the non-expressor allograft) in Caucasians and Asians, respectively. Of note, the recent case report of severe CNI nephrotoxicity in a Caucasian kidney transplanted patient despite low tacrolimus trough level[33] may be explained by "*CYP3A5* mismatch". Interestingly, 85% of Blacks is *CYP3A5* expressor genotype.[23] African-American who live in US might be at a 71% risk for CNI nephrotoxicity when matched with Caucasian allograft, compared with 13% risk if receive allograft from Blacks. However, the supported data in this speculation is lacking.

Taken together, this study supports the concept of tailoring immunosuppression not only to drug level, but also by the genotypes of both recipient and donor allograft. The proposed algorithm for targeted tacrolimus trough concentrations according to the donor and

recipient *CYP3A5* genotypes is shown in Figure 3. Further study should aim to validate the algorithm, and to verify the appropriate tacrolimus trough concentration. In addition, the *CYP3A5* genotypes testing could benefit in better allograft function and saving the cost of unnecessary biopsy.

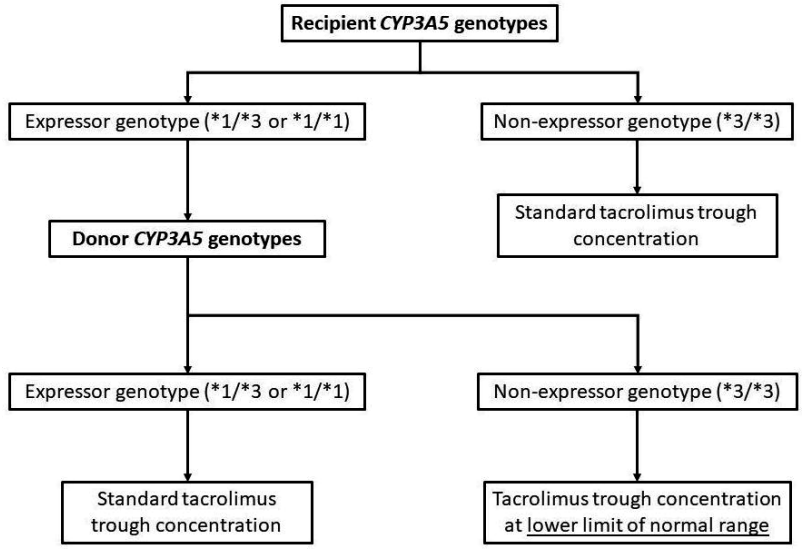

Figure 3: Proposed algorithm for the targeted tacrolimus trough concentrations according to the donor and recipient *CYP3A5* genotypes

CONCLUSION

The donor *CYP3A5* non-expressor genotype is an independent risk factor for CNI nephrotoxicity. In addition, the expressor recipient with non-expressor allograft has the highest risk of CNI nephrotoxicity. The present study reveals the benefit of *CYP3A5* genotyping both recipient and donor kidney allograft for tailoring immunosuppression.

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Chapter 4

Donor-Specific ELISPOT Assay for Predicting Acute Rejection and Allograft Function after Kidney Transplantation: A Systematic Review and Meta-Analysis

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ABSTRACT

Acute rejection remains an important problem after kidney transplantation. Enzyme-linked immunosorbent spot (ELISPOT) assay has been investigated extensively and has shown promising results as a predictor of allograft rejection. The objective of this study was to systematically review and analyze the predictive value of the donor-specific ELISPOT assay to identify recipients at risk for acute rejection. Electronic databases were searched for studies reporting donor-specific ELISPOT and kidney transplantation outcomes. Odds ratio (OR) for acute rejection was calculated, along with standardized mean difference (SMD) of cytokine producing-cells between recipients with and without acute rejection. Pooled estimates were calculated using random-effect models. The positive ELISPOT cutoff frequencies were extracted from each study. From 665 articles found, 32 studies were included in the meta-analysis. IFN-γ was the most investigated cytokine (30 out of 32 studies). Patients with positive pre-transplantation donor-reactive IFN-γ ELISPOT had an OR of 3.3 for acute rejection (95%-CI 2.1 to 5.1), and OR of 6.8 (95%-CI 2.5 to 18.9) for post-transplantation ELISPOT. Recipients with rejection had significantly higher frequencies of pre- and post-transplantation cytokine producing-cells (SMD 0.47, 95%-CI 0.07 to 0.87 and SMD 3.68, 95%-CI 1.04 to 6.32, respectively). Pre-transplantation ELISPOT had a positive predictive value of 43% and a negative predictive value of 81% for acute rejection. A positive ELISPOT result was associated with a lower estimated glomerular filtration rate (SMD -0.59, 95%-CI -0.83 to -0.34). In conclusion, patients with a high frequency of donor-reactive IFN-γ ELISPOT are at higher risk for acute rejection. The donor-specific IFN-γ ELISPOT assay can serve as an immune-monitoring tool in kidney transplantation.

INTRODUCTION

Acute kidney transplant rejection remains a major barrier to allograft longevity.[1-3] With improvements in immunosuppressive treatment, the incidence of acute rejection in the first year after transplantation has decreased to 10-20%, depending on the recipient's immunological risk.[2, 4-6] Acute rejection is associated with poor outcomes, including an increased risk of acute graft loss, *de novo* donor-specific anti-human leukocyte antigen (HLA) antibody (DSA) formation, and allograft loss in the long-term.[1, 2, 4, 7]

An immune-monitoring tool that reliably predicts an individual patient's rejection risk would allow clinicians to intervene earlier and to personalize immunosuppressive therapy. Patients with a high-immunological risk profile could receive more potent immunosuppression, for example with T lymphocyte-depleting agents, whereas patients with a low risk of rejection could be given standard or less intense immunosuppression. This would prevent over-immunosuppression and may therefore reduce complications such as malignancy and infection.[8, 9]

In the acute rejection process, foreign (*i.e.,* donor-derived) HLA is presented to recipient T and B lymphocytes by antigen-presenting cells (APCs). APCs can present donor antigens in 3 ways, the so-called direct, indirect, and semi-direct pathway. Direct antigen presentation occurs in the early post-transplantation period, when intact donor HLA molecules on the surface of donor APCs are recognized directly by recipient T lymphocytes. In indirect antigen presentation, donor HLA molecules are internalized and processed in recipient APCs, and are then presented as peptide fragments in the context of recipient HLA. The semi-direct pathway is the process in which intact donor HLA is acquired on the surface of recipient APCs.[10] The presentation of intact donor HLA or fragments thereof, leads to an aggressive effector T lymphocyte response, which includes $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T helper lymphocytes (Th).[11, 12] Upon alloantigen activation, these effector T lymphocytes produce large amounts of pro-inflammatory cytokines, such as interferon-γ (IFN-γ), interleukin (IL)-17 and IL-21. In brief, IFN-γ is a pleiotropic cytokine which enhances both innate and adaptive immune responses against the donor organ, by increasing the expression of donor HLA, promoting leukocyte migration to the allograft, and modulating the cytotoxic functions of T lymphocytes and NK cells.[13-15] IL-17 is the hallmark cytokine of Th17 lymphocytes, and stimulates and recruits neutrophils and monocytes to the site of inflammation, thereby facilitating the acute rejection process.[16-19] IL-21 stimulates the expansion of $CD8^+$ T lymphocytes and enhances their cytolytic potential.[20, 21] Moreover, both IL-17 and IL-21 are involved in germinal center formation and modulate antibody production, which is associated with antibody-mediated rejection (ABMR).[22-25] In addition to the aforementioned pro-inflammatory cytokines, the recipient's immune cells also release IL-10, a cytokine that

has anti-inflammatory and immunosuppressive functions, and inhibits the release of pro-inflammatory cytokines from antigen-presenting cells.[26, 27]

The enzyme-linked immunosorbent spot (ELISPOT) assay is a highly sensitive and specific test that quantifies cytokine-producing T lymphocytes reactive to a specific antigen. In the setting of organ transplantation, peripheral blood mononuclear cells (PBMCs) of the transplant recipient are co-cultured with donor cells (either donor PBMCs or spleen cells). These donor cells present antigens via the direct antigen presentation pathway. The recipient PBMCs respond by producing cytokines which can be captured in an ELISPOT plate pre-coated with an anti-cytokine antibody. Subsequently, the cells are lysed and washed away. Areas in which cytokine has been captured are detected by a biotinylated anti-cytokine detecting and staining procedure (Figure 1). This method allows the quantitative measurement of cytokine-producing cells at the single cell level.[28-30]

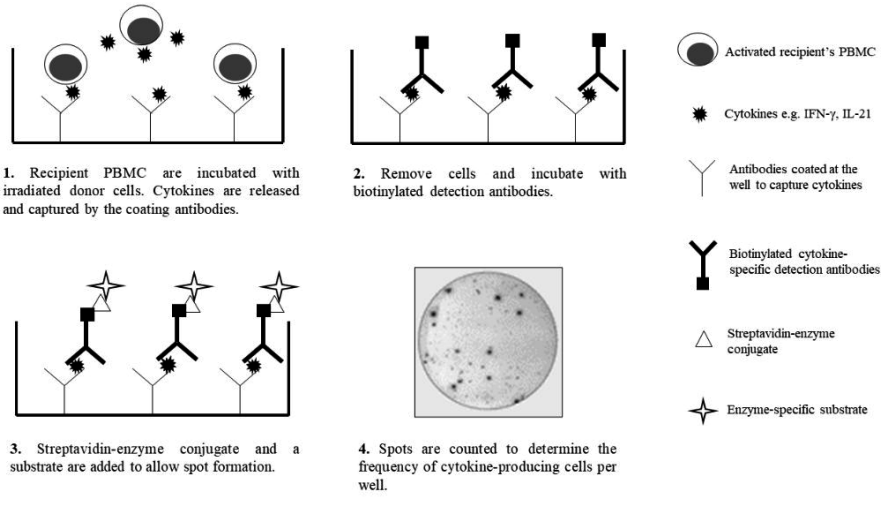

Figure 1: ELISPOT method

Previous ELISPOT studies reported that kidney transplant recipients with high numbers of donor-reactive, cytokine-producing cells were at high risk of developing acute rejection and had inferior allograft function.[31-34] However, these studies differed in terms of the type of cytokine measured, the timing of measurement (pre-transplantation or post-transplantation), and the type of acute rejection (acute T cell-mediated rejection (aTCMR) *versus* ABMR). Moreover, a previous meta-analysis included only pre-transplantation ELISPOT studies and IFN-γ was the only cytokine of interest.[35]

The objective of this study was to determine the extent to which donor-specific ELISPOT assays can be used to predict the risk of acute rejection and graft function after kidney transplantation. A systematic review with meta-analysis was performed of all studies using the ELISPOT assay as an immune-monitoring tool in kidney transplantation. ELISPOT studies before and after transplantation, and measuring all types of cytokines were included.

METHODS

Data sources and searches

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement was used for this systematic review.[36] The search was performed in Scopus, MEDLINE, and the Cochrane Central Register of Controlled Trials to identify eligible studies on 28 December 2020. The references listed in the retrieved articles were also reviewed and manually added if deemed appropriate. The following search term was used for Scopus: TITLE-ABS-KEY (ELISPOT AND Transplantation), and the Medical Subject Heading (MeSH) terms ("Enzyme-Linked Immunospot Assay"[MeSH]) AND "Kidney Transplantation"[MeSH] were used in the MEDLINE search. The MeSH descriptors which explored all trees of [Enzyme-Linked Immunospot Assay] and [Kidney Transplantation] were applied to the Cochrane Central register of Controlled Trials.

Study selection

Prospective and retrospective studies of the donor-specific ELISPOT assay for cytokine-producing cells were included. Regarding the ELISPOT assay procedure, the included studies had to use donor PBMCs or spleen cells and incubate these with recipient PBMCs, and had to measure the frequency of cytokine-producing T lymphocytes. This systematic review and meta-analysis included all cytokines measured and ELISPOT assays performed both the pre- and post-transplantation phase. Only studies that reported the association or correlation between the ELISPOT test results and acute rejection in kidney transplant recipients were selected; studies had to provide detailed information about the number of patients with positive and negative ELISPOT results, or the actual frequencies of the cytokine-producing T lymphocytes, in the rejection and non-rejection group. In addition to the risk of acute rejection, we investigated the association between ELISPOT, *de novo* DSA formation, and kidney allograft function. The Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015 were followed as a guidance for study reviews.[37] Two authors (S.U. and S.K.) independently screened the titles and abstracts from the electronic databases, and full-text articles were retrieved for comprehensive review. Disagreement was resolved through the consensus and judgement by C.B. and D.H.

Data extraction and quality assessment

The following information was extracted from each study: authors' name, year of publication, country of origin, timing of the ELISPOT assay (before or after transplantation), cytokine measured, total patients included in the study, the number of patients with acute rejection, and the type of acute rejection. If the studies did not distinguish between TCMR and ABMR, the total number of rejections was used for the analysis. ELISPOT cutoff values that discriminated between patients having either a positive or negative ELISPOT result were obtained as presented in each study. These threshold values varied according to each study's protocol. The actual frequencies of donor-specific cytokine-producing cells in the ELISPOT assay were also extracted, if available. For studies that measured ELISPOT assays at multiple time points post-transplantation, the mean ± standard deviation (SD) of the post-transplantation ELISPOT frequencies was used as a representative value. Kidney allograft function was extracted in the studies and recorded as estimated glomerular filtration rate (eGFR). The risk of bias was assessed by the Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2).[38] This tool evaluates 4 domains including patient selection, index test, reference standard, and flow and timing. These domains are used to classify the risk of bias and applicability to the population of interest, and can be categorized into "low", "high", and "unclear".

Data synthesis and analysis

The pooled diagnostic OR of predicting acute rejection was calculated from patients with positive donor-specific ELISPOT and compared with patients with a negative ELISPOT test result. A continuity correction was applied to all cells in studies with any zero-cell count.[39] Pooled sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were analyzed using bivariate models that account for the correlation between these parameters. SMD was analyzed to represent the difference between the frequencies of donor-specific cytokine-producing cells between patients with and without acute rejection. In an effort to standardize the different number of recipient PBMCs used in the ELISPOT assay in each study, the cytokine-producing cells were normalized to 3 x 10^5 recipient PBMCs. Allograft function is presented as eGFR and was compared between those who had a positive or negative ELISPOT assay result. The mean and SD were estimated by the method of Wan *et al*. if not provided in the study.[40] All pooled estimates were calculated using random-effects models. A funnel plot was used to investigate publication bias, and Egger's test was used to test for asymmetry of the funnel plot.[41] The existence of heterogeneity among study effect sizes was analyzed using Cochrane's Q-test and the I^2 index. A low Q-test p-value indicates the presence of heterogeneity. An I^2 index higher than 75% indicates high heterogeneity. The analyses were performed using Stata Statistical Software (Release 16.1) with the user written

commands midas, metandi, and metadta (StataCorp LLC, College Station, TX) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA).

Ethical considerations

This meta-analysis and systematic review did not directly obtain data from human or animal subjects. All of the included studies' information was published in scientific journals without the possibility to identify individual patients.

RESULTS

Characteristics of the studies

A total of 665 citations was identified in the initial search. Duplicate and irrelevant studies were excluded, leaving 71 studies for full-text review, of which 32 studies were included in the meta-analysis.[31-34, 42-69] The flow diagram of study selection is depicted in Figure 2. The summary of study characteristics is illustrated in Table 1. In brief, 16 studies investigated pre-transplantation donor-specific ELISPOT,[33, 34, 42, 46, 50-52, 54, 56, 58, 60-62, 66, 67, 69] 8 studies described post-transplantation ELISPOT,[43, 45, 49, 53, 55, 59, 63, 64] and 8 studies investigated ELISPOT both before and after transplantation.[31, 32, 44, 47, 48, 57, 65, 68] The exact timing of the post-transplantation ELISPOT measurements varied between studies, ranging from an exact time point in the first few months after transplantation, whereas in other studies, the ELISPOT assay was measured at non-fixed time points. With regard to the cytokine measured by ELISPOT, most studies measured IFN-γ-producing cells, three studies measured IL-10-producing cells, one study measured IL-17-producing cells, and one study evaluated IL-21-producing cells. Each study used different cutoff values to discriminate between a positive and negative ELISPOT test result (Table 1).

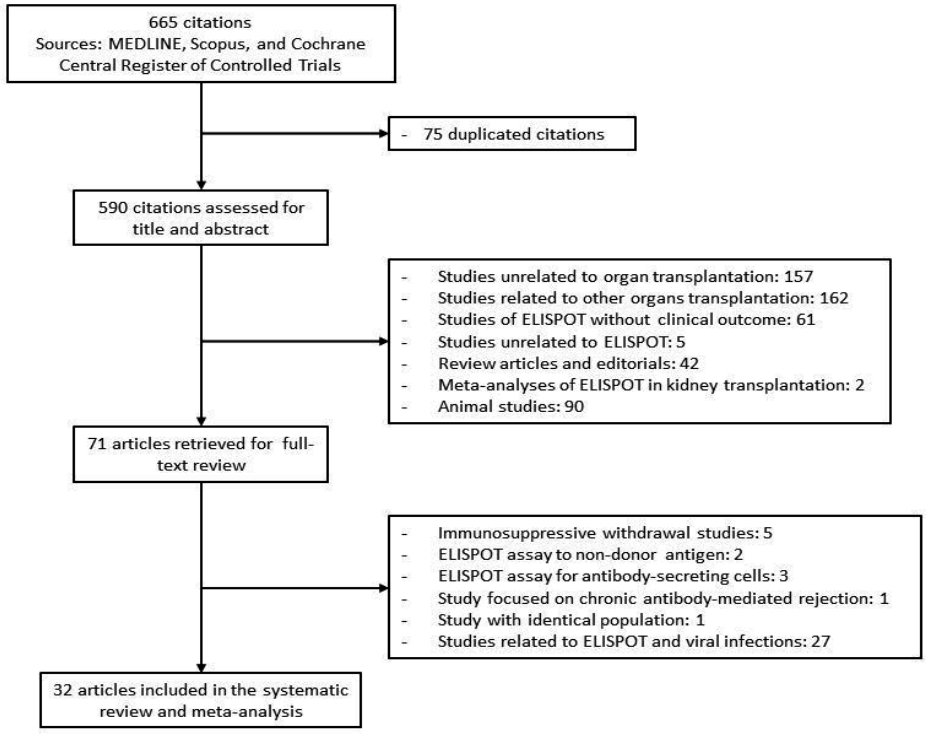

Figure 2: Flow diagram of study selection

Table 1: Summary characteristics of included studies

References	Authors and year of publication	Country of origin	Pre-transplantation ELISPOT	Post-transplantation ELISPOT	Cytokine measured	Cutoff of ELISPOT after subtraction of negative control	Total patients with available ELISPOT results	Patients with acute rejection	Timing of acute rejection	Patients with aTCMR	Patients with ABMR
41	Heeger et al. 1999	USA	Yes	No	IFN-γ	20 pc per $3x10^5$ PBMCs	19	7	N/A	7	0
42	Najafian et al. 2002	USA	No	Randomly after 6 months post-KT	IFN-γ	60 pc per 10^6 PBMCs	27	15	N/A	15	0
30	Hricik et al. 2003	USA	Yes	Randomly in the first 6 months post-KT	IFN-γ	10 pc per $3x10^5$ PBMCs	55	5	Up to 6 months pot-KT	5	0
43	Nickel et al. 2004	Germany	Yes	Randomly in the first 6 months post-KT	IFN-γ	200 pc pre-KT and 10 pc post-KT per $3x10^5$ PBMCs	52	18	Up to 6 months pot-KT	18	0
44	Poggio et al. 2004	USA	No	Randomly post-KT	IFN-γ	15 pc per $3x10^5$ PBMCs	20	11	N/A	11	0
45	Augustine et al. 2005	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	37	11	N/A	11	0
46	Bellisola et al. 2006	Italy	Yes	Protocol: 5 times in 2 months post-KT	IFN-γ	N/A (per $2x10^5$ PBMCs)	8	3	N/A	3	0
47	Nather et al. 2006	Germany	Yes	Protocol: 2 times in 6 months post-KT	IFN-γ	21 pc pre-KT and 13 pc post-KT per $2x10^5$ PBMCs	23	12	N/A	12	0
48	Van Den Boogaardt et al. 2006	Netherlands	No	Randomly in the first 4 months post-KT	"IFN-γ, IL-10"	150 pc pre-KT and 30 pc post-KT per $1.5x10^5$ PBMCs	16	8	Up to 4 months post-KT	8	0
49	Augustine et al. 2007	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	100	21	Up to 12 months post-KT	21	0
50	Kim et al. 2007	South Korea	Yes	No	IFN-γ	12 pc per $2x10^5$ PBMCs	45	11	N/A	N/A	N/A
51	Augustine et al. 2008	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	130	24	N/A	24	0
52	Bestard et al. 2008	Germany	No	Randomly after 24 months post-KT	IFN-γ	20 pc per $3x10^5$ PBMCs	34	17	N/A	N/A	N/A
53	Reinsmoen et al. 2008	Germany	Yes	No	IFN-γ	N/A (per $2x10^5$ PBMCs)	30	22	N/A	18	4
54	Kim et al. 2009	South Korea	No	Protocol: 3 times in 2 months post-KT	IL-10	N/A (per $2x10^5$ PBMCs)	42	11	Up to 2 weeks post-KT	N/A	N/A
55	Koscielska-Kasprzak et al. 2009	Poland	Yes	No	IFN-γ	N/A	53	14	Up to 12 months post-KT	N/A	N/A
56	Cherkassky et al. 2011	USA	Yes	Protocol: 3 times in 6 months post-KT	IFN-γ	N/A (per $2x10^5$ PBMCs)	31	1	Up to 5 months post-KT	1	0
57	Kim et al. 2012	South Korea	Yes	No	IFN-γ	12 pc per $2x10^5$ PBMCs	154	18	Up to 12 months post-KT	15	5
31	Bestard et al. 2013	Spain	Yes	Protocol: at 6 months post-KT	IFN-γ	25 pc per $3x10^5$ PBMCs	60	14	Up to 12 months post-KT	13	1
32	Hricik et al. 2013	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	118	25	Up to 12 months post-KT	25	0
58	Nazari et al. 2013	Iran	No	Protocol: at 3 months post-KT	IFN-γ	N/A (per $1x10^5$ PBMCs)	30	10	N/A	N/A	N/A
33	Crespo et al. 2015	Spain	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	157	42	N/A	37	5
59	Hricik et al. 2015	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	176	15	Up to 6 months post-KT	15	0
60	Hricik et al. 2015	USA	Yes	No	IFN-γ	25 pc per $3x10^5$ PBMCs	15	6	Up to 16 months post-KT	6	0
61	Slavcev et al. 2015	Czech	Yes	No	IFN-γ	N/A (per 5x104 PBMCs)	47	22	Up to 12 months post-KT	17	5
62	Crespo et al. 2017	Spain	No	Protocol: at 3 and 6 months post-KT	IFN-γ	19 pc per 3x105 PBMCs	161	31	Up to 6 months post-KT	24	7
63	Crespo et al. 2017	Spain	No	Protocol: at 6 months post-KT	IFN-γ	25 pc per 3x105 PBMCs	75	22	Up to 6 months post-KT	17	5
64	Mohammadi et al. 2017	Iran	Yes	Protocol: 3 times in 3 months post-KT	"IFN-γ, IL-10, IL-17"	N/A (per 1x105 PBMCs)	57	12	Up to 12 months post-KT	N/A	N/A
65	Schachtner et al. 2017	Germany	Yes	No	IFN-γ	25 pc per 3x105 PBMCs	15	7	N/A	7	0
66	Gandolfini et al. 2018	Spain	Yes	No	IFN-γ	25 pc per 3x105 PBMCs	168	15	N/A	14	1
67	Van Besouw et al. 2019	Netherlands	Yes	Protocol: at 6 months post-KT	IL-21	18 pc pre-KT and 62 pc post-KT per 3x105 PBMCs	81	28	Pre-KT: up to 6 months Post-KT: up to 60 months	26	10
68	Schachtner et al. 2020	Germany	Yes	No	IFN-γ	25 pc per 3x105 PBMCs	150	36	Up to 12 months post-KT	33	3

ABMR, antibody-mediated rejection; aTCMR, acute T cell-mediated rejection; N/A, not available; pc, producing cells; PBMCs, peripheral blood mononuclear cells

Fourteen studied did not report the timing of acute rejection. The other 18 studies reported that acute rejection occurred from week 2 to month 60 post-transplantation. For the calculation of the OR, 26 studies provided sufficient information on the patients with positive and negative ELISPOT results and the incidence of rejection.[31-34, 42-46, 48-53, 56, 58, 60, 61, 63-69] Eighteen studies presented the actual values of cytokine producing-cells to allow the calculation of the SMD.[31, 34, 42-45, 47-49, 51, 53, 54, 58, 59, 62, 63, 65, 68] Finally, 10 studies were examined for the association between ELISPOT results and allograft function. Six studies used the 6-variable MDRD equation,[33, 44, 46, 51, 52, 58] 1 study used the simplified 4-variable MDRD equation,[60] and 3 studies did not specify which MDRD equation was used.[32, 53, 63] The assessment of the risk of bias and applicability concerns, evaluated by QUAD-2, is shown in Supplementary Table S1.

Diagnostic performance of donor-specific ELISPOT assay for predicting acute rejection

The pooled diagnostic OR is illustrated in Figure 3. A positive pre-transplantation IFN-γ ELISPOT predicted acute rejection with an OR of 3.29 (95%-CI 2.11 to 5.13; p-value < 0.001, I^2 47.9%, Q-test p-value = 0.009), and a positive post-transplantation IFN-γ ELISPOT was associated with acute rejection with an OR of 6.84 (95%-CI 2.48 to 18.89; p-value < 0.001, I^2 64.6%, Q-test p-value = 0.013). There was only one study that associated rejection with a pre-transplantation and post-transplantation IL-21 ELISPOT assay (OR 11.0, 95%-CI 2.26 to 53.64; p = 0.003, and OR 8.46, 95%-CI 1.87 to 38.31; p = 0.006), respectively. After combining the OR of all cytokines, patients with a positive pre-transplant donor-specific assay were at a 3.50-fold higher risk for acute rejection (95%-CI 2.25 to 5.45; p -value < 0.001, I^2 49.6%, Q-test p-value = 0.007) compared with patients with negative ELISPOT. In the same way, patients with a post-transplant donor-specific ELISPOT had a 9.20-fold higher risk for acute rejection (95%-CI 3.47 to 24.41; p-value < 0.001, I^2 67.3%, Q-test p-value = 0.004). When sub-grouped into studies that reported aTCMR only (*i.e.,* excluding mixed-type rejection and ABMR), the pooled OR of patients with a positive ELISPOT was 2.81 (95%-CI 1.90 to 4.16; p-value < 0.001, I^2 25.2%, Q-test p-value = 0.045) for the pre-transplant ELISPOT assay, and 12.65 (95%-CI 6.59 to 24.28; p-value < 0.001, I^2 0%, Q-test p-value = 0.930) for the post-transplant ELISPOT (Supplementary Figure S1).

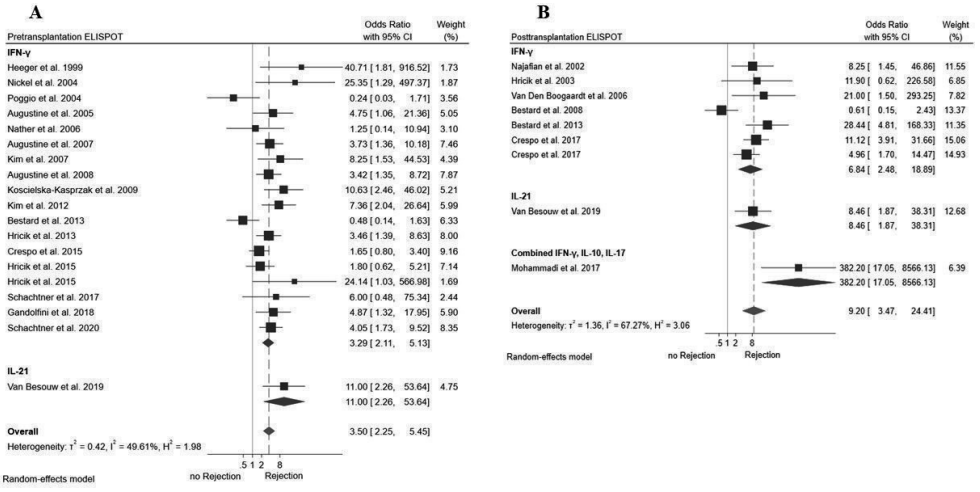

Figure 3: Forest plot of OR for acute rejection in patients with positive pre-transplantation ELISPOT assay (A) and post-transplantation ELISPOT assay (B).

The donor-specific ELISPOT assay was unable to differentiate patients with and without ABMR (pooled OR 0.79, 95%-CI 0.27 to 2.28; p-value = 0.665, I^2 0%, Q-test p-value = 0.532, Supplementary Figure S2), or patients with and without *de novo* DSA (pooled OR 1.56, 95%-CI 0.09 to 26.64; p-value = 0.757, I^2 82.5%, Q-test p-value = 0.017, Supplementary Figure S3).

Studies of donor-specific IFN-γ ELISPOT contained sufficient information to be combined into a pooled sensitivity and specificity analysis as shown in Table 2. The sensitivity and specificity of the ELISPOT test was at its highest when used in the post-transplantation period for aTCMR (sensitivity 0.81, 95%-CI 0.69 to 0.89; specificity 0.74, 95%-CI 0.59 to 0.85). The timing of post-transplantation ELISPOT in each study varied but was most frequently tested somewhere in the period between 3 and 6 months after transplantation, and associated with acute rejection up to 12 months post-transplantation (Table 1). In contrast, the pre-transplantation or post-transplantation IFN-γ ELISPOT assay had poor diagnostic performance for ABMR. However, only 379 patients were included in the analysis of the association between ELISPOT and ABMR, compared with 1,599 patients in the studies of ELISPOT and aTCMR (Table 2). The PPV and NPV of the IFN-γ ELISPOT assay depend on the prevalence of acute rejection, which was 32% (95%-CI 25% to 39%) in this meta-analysis. The pooled PPV and NPV of the pre-transplantation IFN-γ ELISPOT was 43% (95%-CI 36% to 50%) and 81% (95%-CI 74% to 88%), respectively. For the post-transplantation

IFN-γ ELISPOT, the pooled PPV was 54% (95%-CI 45% to 63%) and the pooled NPV was 79% (95%-CI 70% to 87%). The nomograms for the PPV and NPV of the pre-transplantation and post-transplantation IFN-γ ELISPOT for acute rejection are plotted in Supplementary Figure S4.

Table 2: Sensitivity and specificity of IFN-γ ELISPOT assay for acute rejection

Total rejection	Number of patients reported	Pooled sensitivity	95%-CI	Pooled specificity	95%-CI
Pre-KT ELISPOT	1,485	0.63	0.54-0.71	0.65	0.55-0.75
Post-KT ELISPOT	414	0.73	0.62-0.82	0.69	0.51-0.83
aTCMR	**Number of patients reported**	**Pooled sensitivity**	**95%-CI**	**Pooled specificity**	**95%-CI**
Pre-KT ELISPOT	1,219	0.60	0.51-0.68	0.65	0.52-0.77
Post-KT ELISPOT	380	0.81	0.69-0.89	0.74	0.59-0.85
ABMR	**Number of patients reported**	**Pooled sensitivity**	**95%-CI**	**Pooled specificity**	**95%-CI**
Pre-KT ELISPOT	304	0.11	0.01-0.63	0.50	0.40-0.60
Post-KT ELISPOT	75	0.20	0.01-0.70	0.57	0.45-0.69

ABMR, antibody-mediated rejection; aTCMR, acute T cell-mediated rejection

The funnel plot of the log OR for acute rejection is depicted in Supplementary Figure S5, and demonstrates incomplete symmetry. This indicates the possibility of some publication bias due to under-reporting of negative studies. The p-value from Egger's test was 0.085 for pre-transplantation ELISPOT and 0.117 for post-transplantation ELISPOT.

Difference of actual ELISPOT frequencies in patients with and without rejection

Figure 4 displays the SMD between patients with and without rejection. Patients with rejection had higher donor-specific ELISPOT frequencies compared with patients without rejection (SMD 1.71, 95%-CI 0.57 to 2.86; p-value = 0.003, I^2 98.0%, Q-test p-value < 0.001). This analysis was only possible for the total rejection incidence due to the lack of ELISPOT frequencies reported for the subgroups of aTCMR and ABMR. To illustrate the actual frequencies of the ELISPOT assay for each type of cytokine, the frequencies of cytokine-producing cells were pooled (Figure 5). The mean ± SD of IFN-γ-producing cell frequencies in patients with and without acute rejection was 176 ± 287 *versus* 86 ± 172 producing cells per 3 x 10^5 PBMCs pre-transplantation (p-value = 0.033), and 246 ± 256 *versus* 77 ± 87 producing-cells per 3 x 10^5 PBMCs post-transplantation (p-value = 0.015). IL-17-producing-cells from patients with and without rejection were significantly different in the post-transplantation period (161 ± 7 *versus* 51 ± 18 producing-cells per 3 x 10^5 PBMCs;

p-value < 0.001). The frequencies of IL-21-producing cells in the pre-transplantation and post-transplantation ELISPOT were significantly different in patients with and without acute rejection (35 ± 38 *versus* 12 ± 10 producing-cells per 3 x 10^5 PBMCs; p-value = 0.011 in pre-transplantation, and 60 ± 69 *versus*. 25 ± 33 producing-cells per 3 x 10^5 PBMCs; p-value = 0.02 in the post-transplantation phase). IL-10 was not included in the analysis of SMD due to its distinctive feature as an anti-inflammatory and regulatory cytokine[27, 70]. However, the actual frequencies of pre-transplantation and post-transplantation donor-specific IL-10-producing cells were significantly different between patients with and without rejection (1,246 ± 51 *versus* 1,161 ± 34 producing cells per 3 x 10^5 PBMCs pre-transplantation; p-value < 0.001, and 501 ± 517 *versus* 863 ± 593 producing cells per 3 x 10^5 PBMCs post-transplantation; p-value = 0.025).

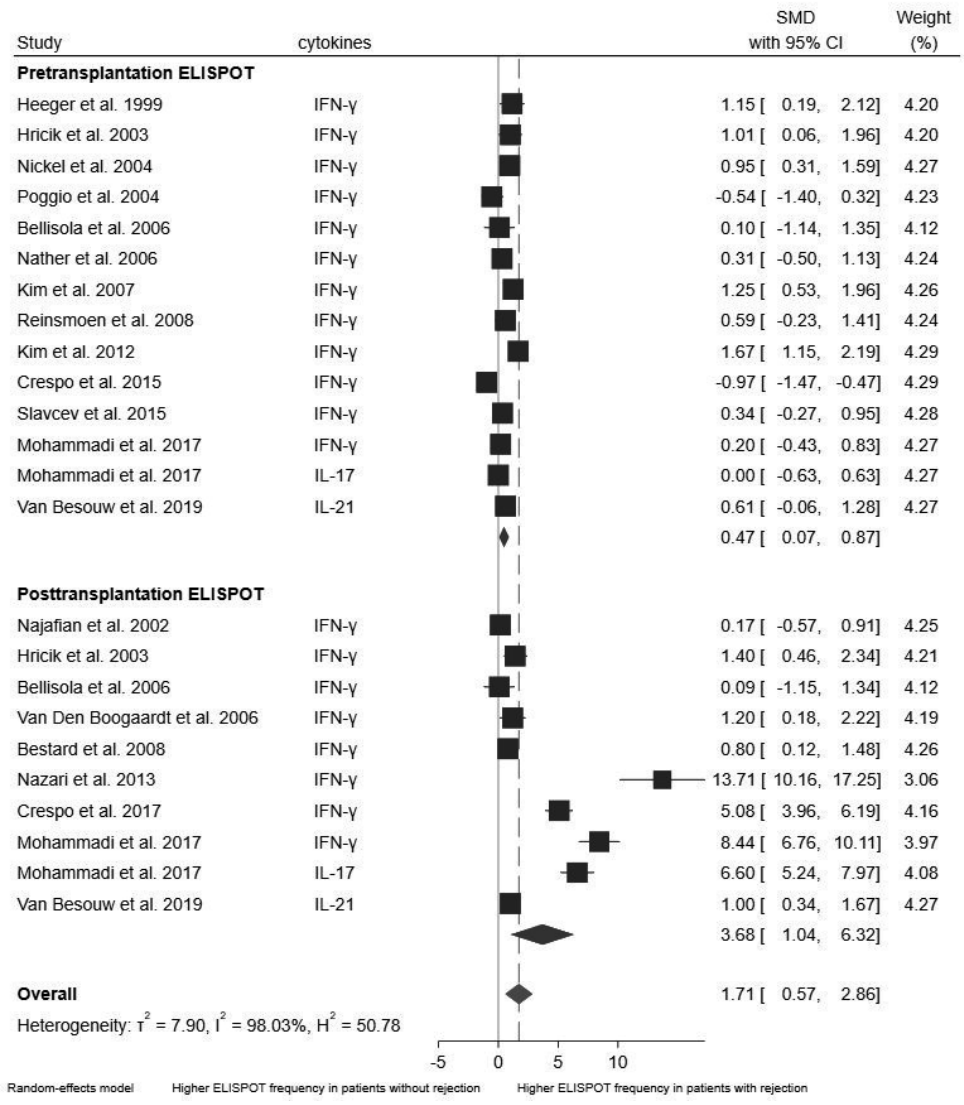

Figure 4: Forest plot of SMD showing the difference of ELISPOT frequencies between patients with and without rejection.

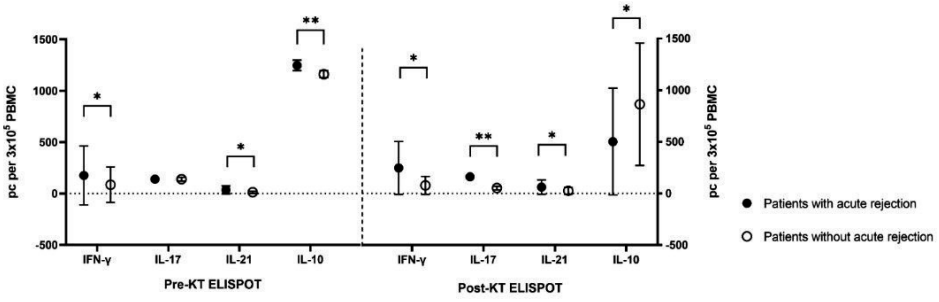

Figure 5: Pooled actual frequencies of cytokine producing-cells (with 95%-CI) for patients with and without acute rejection, categorized by the timing of ELISPOT assay (*, p-value < 0.05; **, p-value < 0.001; pc, producing-cells).

The association between kidney allograft function and donor-specific ELISPOT

The SMD of eGFR between patients with a positive and negative ELISPOT is shown in Figure 6. Patients with a positive donor-specific ELISPOT had a significantly lower eGFR both at month 6 and month 12 after transplantation (pooled SMD -0.59, 95%-CI -0.83 to -0.34; p-value < 0.001, I^2 73.3%, Q-test p-value < 0.001). The actual eGFR values in patients with a positive ELISPOT were significantly lower compared with patients with negative ELISPOT, regardless of the timing of the ELISPOT measurement or the timing of the eGFR measurement (Figure 7). There was not enough data to perform a meta-analysis of the association between serum creatinine and ELISPOT result, since only few studies reported serum creatinine as an endpoint.

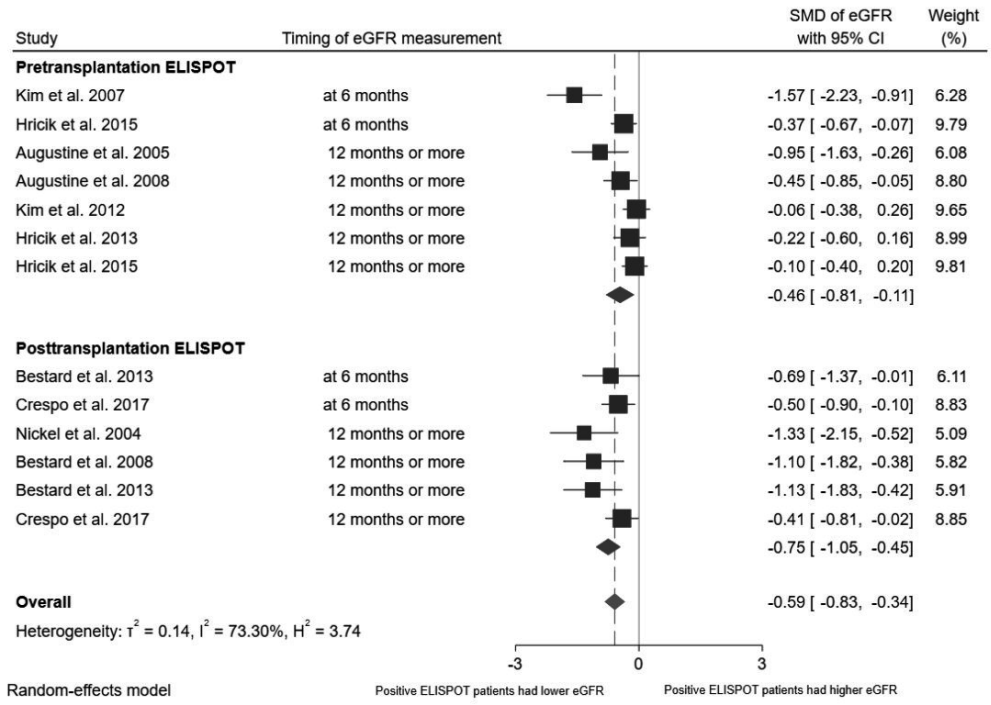

Figure 6: Forest plot of SMD showing the difference of eGFR between patients with positive and negative ELISPOT results.

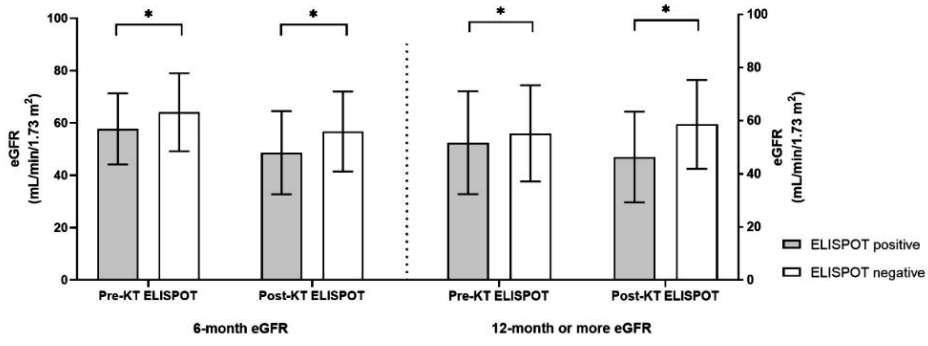

Figure 7: Pooled eGFR (with 95%-CI) of patients with positive and negative ELISPOT results, categorized by the timing of ELISPOT assay and timing of eGFR measurement (*, p-value < 0.05).

DISCUSSION

The results of this systematic review and meta-analysis demonstrate that ELISPOT is a useful immune-monitoring tool that can assist clinicians in stratifying the risk for acute rejection. Moreover, patients with a positive ELISPOT result were at higher risk for inferior kidney allograft function at 6 and 12 months after transplantation. Risk stratification using the donor-reactive ELISPOT assay can therefore guide personalization of an individual patient's immunosuppressive therapy.

The immune-monitoring tools currently in routine use for identifying rejection risk in kidney transplant recipients have poor predictive values. Only surrogate biomarkers such as immunosuppressive drug concentrations and the formation and titer of DSA have been implemented in routine diagnostics. None of these biomarkers evaluate the actual ongoing *in vivo* (or *in vitro*) interaction between the recipient's immune system and donor antigens, which should be the phenomenon of interest when assessing and monitoring rejection risk.[30, 71, 72] As a test that evaluates donor-reactive T lymphocyte-mediated immune responses (via the direct antigen presentation pathway), the ELISPOT assay determines the frequency of cytokine-producing cells and has been extensively investigated in relation to acute rejection.[73] In addition, evidence from previous studies showed that the results of the donor-reactive ELISPOT assay are dynamic and relate to the state of immunosuppression of an individual patient, and may assist with the adjustment of immunosuppressive medication doses.[32, 34, 52, 57, 58]

Our meta-analysis reported several cytokines used in the ELISPOT assay, including IFN-γ, IL-17, IL-21, and IL-10. Most studies reported results for IFN-γ, and demonstrated that this cytokine is useful as a predictor of acute rejection. We cannot draw conclusions about the diagnostic performance of IL-17 and IL-21, because only one study investigated each cytokine.[65, 68] Interestingly, the frequency of IL-10-producing cells was higher in patients with acute rejection compared to patients without acute rejection.[74] As IL-10 is an anti-inflammatory and regulatory cytokine, a high number of IL-10-producing cells in patients with acute rejection likely reflects the response to inflammation and rejection in the allograft, rather than being the cause.[75-78] In contrast, the post-transplantation IL-10 ELISPOT results showed lower levels of IL-10-producing cells in patients with acute rejection compared with patients without acute rejection. However, as the 95%-CI of the post-transplantation IL-10 ELISPOT frequency analysis was exceptionally wide, this indicates a high degree of heterogeneity. Moreover, IL-10 can be secreted from many cells that are included in recipient PBMCs fractions during the ELISPOT procedure.[26, 79] There might thus be significant variation in the source of IL-10, that at least partially contributes to the inconsistency of ELISPOT frequencies observed. Future studies could examine this possibility by purifying PBMCs cell

subtypes to investigate ELISPOT responses in distinct T lymphocyte subsets.

For the type of acute rejection, sensitivity and specificity were best when the IFN-γ ELISPOT assay was used for the diagnosis of aTCMR, reflecting that alloreactive T lymphocytes mostly contribute to aTCMR rather than ABMR.[80] However, the number of patients included in the studies of ELISPOT dedicated to ABMR were limited and therefore the performance characteristics in predicting this outcome could not be fully evaluated. Interestingly, other studies that were not included in this meta-analysis, showed the potential of the ELISPOT assay in detecting donor-specific antibody-secreting cells, which might be a more relevant predictive biomarker for ABMR.[81-83] A next step in ELISPOT research could be the development of an assay that simultaneously measures the effector cytokines IFN-γ, IL-17, and IL-21. This might increase the sensitivity and specificity of the ELISPOT assay for predicting acute rejection after transplantation.

Both the pre-transplantation and the post-transplantation ELISPOT assay had a good NPV (81% and 79%, respectively) but a poor to moderate PPV (43% and 54%, respectively) for acute rejection. This indicates that the pre-transplantation donor-specific ELISPOT assay is an immune stratification tool that reliably predicts the absence of acute rejection. The high NPV of the ELISPOT assay suggests that the assay may best be used to identify patients with a low risk of rejection and in whom immunosuppression can thus be safely minimized, rather than use the test to identify high-risk patients who may require more intensive immunosuppression. However, the PPV and NPV are not intrinsic properties of the test and depend on the prevalence of acute rejection. The PPV and NPV of the ELISPOT assay are thus subject to change when applied in different populations.

As one might expect, kidney allograft function, represented by eGFR, was inferior in patients with positive ELISPOT compared with the negative ELISPOT patients. This finding supports the association of an alloimmune response that contributes to acute rejection, and may result in poor allograft function.[1, 2, 84] However, the inferior allograft function observed in this meta-analysis is not necessarily the result of acute rejection only. Patients with a positive donor-reactive ELISPOT can also experience a more subtle alloimmune response, so called chronic ABMR or chronic TCMR, which causes a slow decline of kidney allograft function.[85-87] Moreover, other post-transplant complications can contribute to renal allograft dysfunction such as calcineurin inhibitor-mediated nephrotoxicity, BK virus nephropathy, or recurrent glomerular disease.[88, 89] Apart from acute rejection, clinicians should be aware of all possible causes of allograft dysfunction, even in the presence of a positive ELISPOT result.

With regard to personalized immunosuppressive therapy, we propose that the ELISPOT assay can inform treatment decisions by 3 distinct approaches. The first approach regards the pre-transplantation donor-reactive ELISPOT. It may inform the clinician about

the recipient's immune status, and guide initial immunosuppression and choice of induction therapy. Patients with high levels of pre-transplant ELISPOT frequencies might need more potent immunosuppressive therapy such as T lymphocyte-depleting agents, while patients with a low level of donor-reactivity may only require an IL-2 receptor antagonist with standard low-exposure maintenance immunosuppression. Second, ELISPOT assay results could assist clinicians in appropriately adjusting immunosuppressive regimens. A standardized protocol for repeat ELISPOT testing, *e.g.,* every 3-6 months in the first year after transplantation, might aid in discriminating patients in whom immunosuppression can be safely reduced *versus* those in whom tapering of immunosuppression should be avoided. Third, the ELISPOT results could potentially guide the treatment of patients who are diagnosed histologically with borderline acute cellular rejection. There is ongoing uncertainty whether borderline acute rejection represents true rejection (and should thus be treated) or that it merely reflects harmless infiltration of the graft, or even immunoregulation, and is therefore best left untreated.[87, 90] The role of ELISPOT in individualizing immunosuppression is being studied in an ongoing randomized, controlled trial (ClinicalTrials.gov identifier: NCT03465397).

Our meta-analysis has strengths and limitations. This is the first meta-analysis that includes both pre- and post-transplantation donor-specific ELISPOT studies and investigates several cytokines, other than IFN-γ. We demonstrated a consistent association between ELISPOT results and allograft function. However, some included studies did not clearly mention the type of rejection nor the timing of acute rejection, complicating interpretation of the temporal relationship between the ELISPOT diagnostic performance and acute rejection. This issue is particularly important for the post-transplantation ELISPOT. Not every study clearly described the exact timing of the ELISPOT measurement in relation to the timing of acute rejection (see Table 1). While the pre-transplantation ELISPOT appears to predict acute rejection, the findings from post-transplantation ELISPOT should be more cautiously interpreted as they often represent an association rather than a true prediction. Moreover, we found significant variation in ELISPOT cutoff frequencies that were used to identify positive and negative ELISPOT results in each study. This variation could be due in part to differences in the source of the donor antigens, which included both donor PBMCs and spleen cells. In studies investigating living donor kidney transplantation, PBMCs were used for the ELISPOT assay, whereas spleen cells of the donor were used in case of deceased donor kidney transplantation. In almost every study, both types of donor (and thus both PBMCs and splenocytes) were used, and no study reported the ELISPOT results based on the donor source. To the best of our knowledge, no studies have systematically investigated the effect of the type of stimulator cell on ELISPOT results. In addition, differences in the responder cell fractions (*e.g.* PBMCs *versus* purified T-lymphocytes), and the sensitivity of the ELISPOT reader might also contribute to the ELISPOT results. Future studies should consider these factors in

the design and analysis of ELISPOT assay studies.

In summary, donor-specific ELISPOT assays for cytokine-producing cells are useful tools to identify patients at risk for acute rejection, and may allow stratification of patients into high and low immunological risk kidney transplant recipients and guide immunosuppressive therapy. Further optimization of the ELISPOT technique and a standardization of the timing and use in clinical practice may lead to an improvement in its diagnostic performance.

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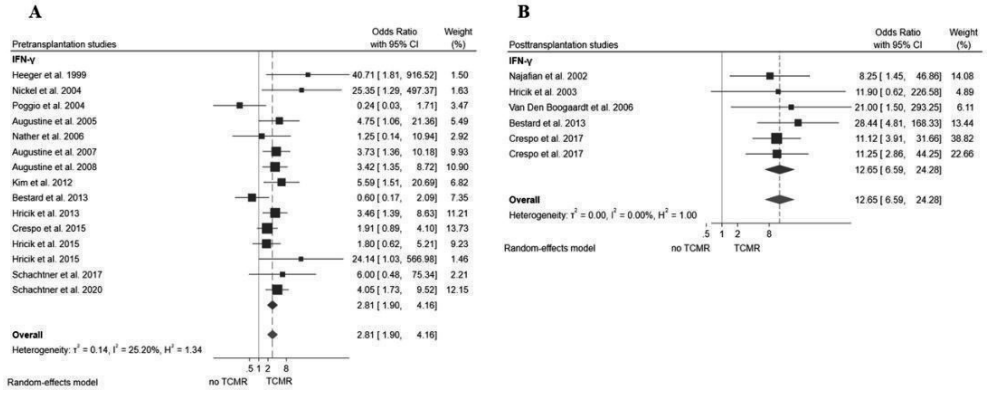
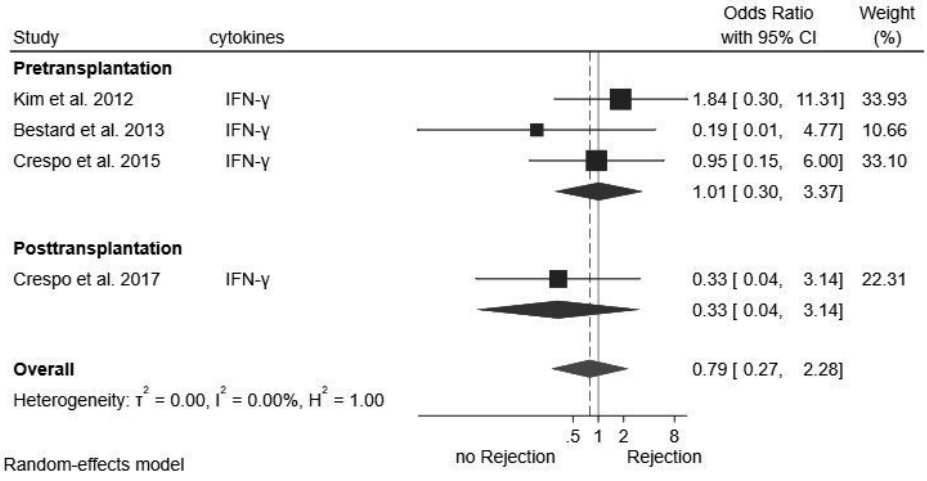
SUPPLEMENTARY DATA

Supplementary Table S1: QUADS-2 for risk of bias assessment

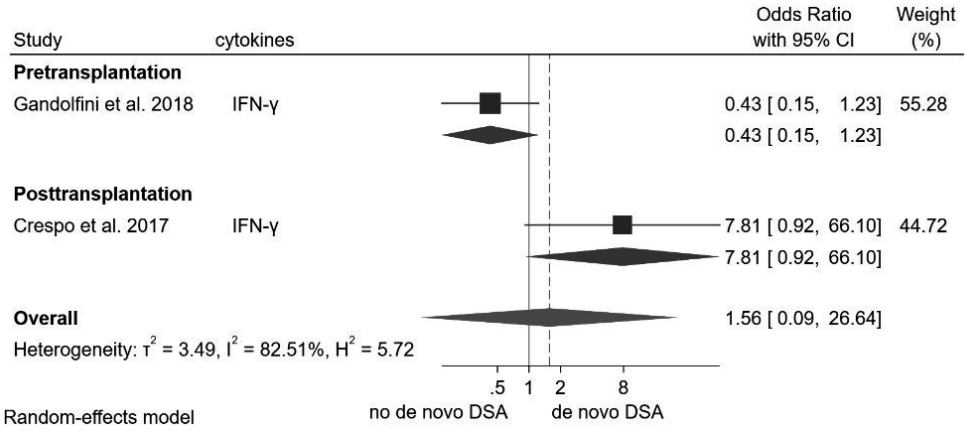
Authors and year of publication	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Heeger et al. 1999	Low	Low	Low	Low	Low	Low	Low
Najafian et al. 2002	Low	Low	Low	High	Low	Low	Low
Hricik et al. 2003	Low	Low	Low	High	Low	Low	Low
Nickel et al. 2004	Low	Low	Low	High	Low	Low	Low
Poggio et al. 2004	Low	Low	Low	High	Low	Low	Low
Augustine et al. 2005	Low	Low	Low	Low	Low	Low	Low
Bellisola et al. 2006	Low	Low	Low	Low	Low	Low	Low
Nather et al. 2006	Low	Low	Low	Low	Low	Low	Low
Van Den Boogaardt et al. 2006	Low	Low	Low	High	Low	Low	Low
Augustine et al. 2007	Low	Low	Low	Low	Low	Low	Low
Kim et al. 2007	Low	Low	High	Low	Low	Low	High
Augustine et al. 2008	Low	Low	Low	Low	Low	Low	Low
Bestard et al. 2008	Low	Low	Low	High	Low	Low	Low
Reinsmoen et al. 2008	Low	Low	Low	Low	Low	Low	Low
Kim et al. 2009	Low	Low	Low	Low	Low	Low	Low
Koscielska-Kasprzak et al. 2009	Low	Low	Low	Low	Low	Low	Low
Nickel et al. 2009	Low	Low	Low	Low	Low	Low	Low
Cherkassky et al. 2011	Low	Low	Low	Low	Low	Low	Low
Kim et al. 2012	Low	Low	Low	Low	Low	Low	Low
Bestard et al. 2013	Low	Low	Low	Low	Low	Low	Low
Hricik et al. 2013	Low	Low	Low	Low	Low	Low	Low
Nazari et al. 2013	Low	Low	Low	Low	Low	Low	Low
Crespo et al. 2015	Low	Low	Low	Low	Low	Low	Low
Hricik et al. 2015	Low	Low	Low	Low	Low	Low	Low
Hricik et al. 2015	Low	Low	Low	Low	Low	Low	Low
Slavcev et al. 2015	Low	Low	Low	Low	Low	Low	Low
Crespo et al. 2017	Low	Low	Low	Low	Low	Low	Low
Crespo et al. 2017	Low	Low	Low	Low	Low	Low	Low
Mohammadi et al. 2017	Low	Low	High	Low	Low	Low	High
Schachtner et al. 2017	Low	Low	Low	Low	Low	Low	Low
Gandolfini et al. 2018	Low	Low	Low	Low	Low	Low	Low
Van Besouw et al. 2019	Low	Low	Low	Low	Low	Low	Low
Schachtner et al. 2020	Low	Low	Low	Low	Low	Low	Low



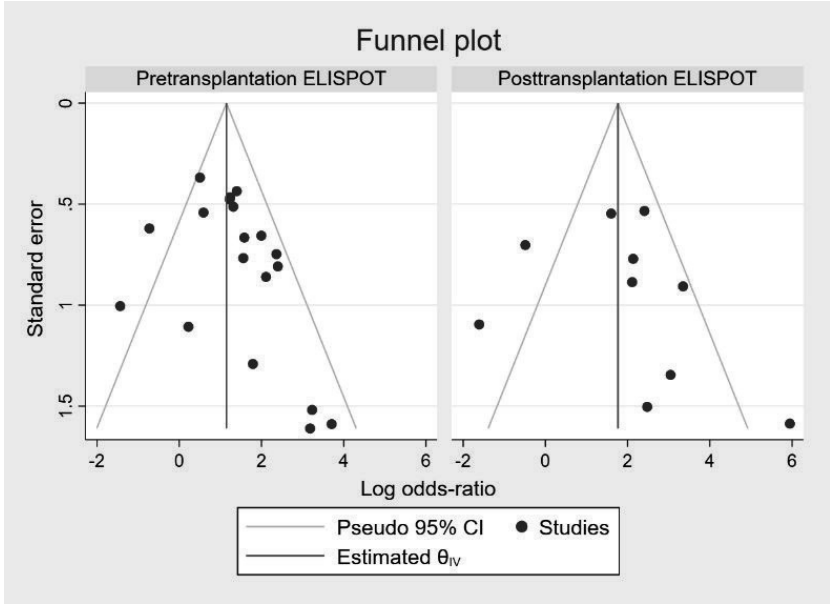
Supplementary Figure S1: Forest plot of OR for aTCMR in patients with positive pretransplantation ELISPOT assay results (A) and posttransplantation ELISPOT assay results (B).



Supplementary Figure S2: Forest plot of OR for ABMR in patients with positive pretransplantation and post-transplantation ELISPOT assay results.



Supplementary Figure S3: Forest plot of OR for *de novo* DSA in patients with positive pretransplantation and posttransplantation ELISPOT assay results.



Supplementary Figure S4: Funnel plot of log OR and standard error for acute rejection.

Chapter 5

A Systematic Review and Meta-Analysis of Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay for BK Polyomavirus Immune Response Monitoring After Kidney Transplantation

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ABSTRACT

BK virus (BKV) infection after kidney transplantation can cause BKV nephropathy (BKVAN) resulting in graft dysfunction and allograft loss. The treatment for BKVAN is reduction of the immunosuppressive load which increases the risk of kidney transplant rejection. There is no biomarker to monitor BKV activity besides BK viral load. The value of the Enzyme-Linked Immunosorbent Spot (ELISPOT) assay as a tool to monitor the recipient's anti-BKV immune response after transplantation was investigated systematically. Electronic databases, including MEDLINE, Scopus, and the Cochrane Central Register of Controlled Trials were searched for studies of ELISPOT evaluating the immune response against BKV. BKV status was categorized as "active BKV infection" and as "resolving BKV infection". Random-effects model meta-analysis was performed to determine the diagnostic performance of the ELISPOT assay, after stratifying patients into groups based on positive and negative ELISPOT results. One-hundred twenty-seven articles were identified of which nine were included. Patients with negative ELISPOT had an increased risk of having active BKV replication (odds ratio of 71.9 (95%-CI 31.0-167.1). Pooled sensitivity was 0.95 (95%-CI 0.89-0.98) and specificity was 0.88 (95%-CI 0.78-0.94). The standardized mean difference of the number of IFN-γ producing cells between patients with active BKV infection compared with patients who had resolving BKV infection was -2.09 (95%-CI -2.50, -1.68). The ELISPOT assay is a useful tool for BKV risk assessment and in combination with BKV load may support clinicians in guiding immunosuppressive therapy in patients with BKV replication.

INTRODUCTION

Patient and kidney transplant survival have much improved compared with the previous era due to better immunosuppressive drug therapy and a significant increase in the knowledge of transplantation immunology.[1-3] However, infectious complications, particularly BK virus (BKV) infection, is an important obstacle to allograft longevity.[4-8]

BKV replication is the consequence of both the reactivation, which is more common, or *a primo* infection.[9] After kidney transplantation, 30-40% of patients develop early BKV replication which manifests as BK viruria, whereas 10-20% will progress to BK viremia if the immune response fails to contain BKV replication. Eventually, BKV-associated nephropathy (BKVAN) occurs in 1-10% of kidney transplant recipients.[10] The incidence of BKVAN is higher among patients who receive more potent immunosuppression, which is more common these days as more patients with higher immunological risk receive transplants.[7, 11-13] Currently, there is no established treatment for BKVAN.[5, 7, 8, 14] The general recommendation is to lower the patient's net immunosuppressive state,[15] however, this may increase the donor-specific immune response and result in rejection.

Tools to monitor a patient's immune response against BKV during immunosuppressive therapy are lacking. Previous studies showed that interferon-γ (IFN-γ) is a cytokine with broad antiviral activities, including BKV, and that its expression is increased in response to BKV infection.[16, 17] The enzyme-linked immunosorbent spot (ELISPOT) assay has been developed to evaluate T lymphocyte function (*i.e*. the frequency of IFN-γ producing responsive cells) against BKV.[18-20] Patients with BKV infection can then be stratified into risk groups. Those with the negative BKV ELISPOT, *i.e.* the high-risk group, who have an insufficient immune response against BKV and are therefore more likely to have persistent BK viremia and develop BKVAN.[21] On the contrary, patients with positive BKV ELISPOT have an adequate immune response against BKV, and are considered to be at low-risk for BKV-related complications.[20, 22]

The objective of the present study was to systematically analyze publications describing the clinical use of IFN-γ ELISPOT in kidney transplant recipients who experienced BKV replication, and provide an evidence-based assessment on whether this ELISPOT assay can be applied in clinical practice.

MATERIALS AND METHODS

Data sources and searches

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.[23] The literature search was conducted in MEDLINE, Scopus, and the Cochrane Central Register of Controlled Trials to identify eligible studies on 21 August 2020. We also manually reviewed the references listed in the retrieved articles.

For MEDLINE, we used the following Medical Subject Heading terms (MeSH): ("Enzyme-Linked Immunospot Assay"[Mesh]) AND "Kidney Transplantation"[Mesh]. For Scopus, the following search terms were applied: TITLE-ABS-KEY (ELISPOT AND Transplantation). For the Cochrane Central Register of Controlled Trials, we use the MeSH descriptor which exploded all trees of [Enzyme-Linked Immunospot Assay] and [Kidney Transplantation].

Study selection

Retrospective and prospective studies that investigated the use of ELISPOT in kidney transplantation were included. Only studies that applied the ELISPOT assay for the monitoring of immune responses against BKV were included in the meta-analysis. Studies that did not correlate ELISPOT to BKV-related clinical endpoints were not included. The ELISPOT assay could be used in both pre-transplantation and post-transplantation studies. In order to analyze the sensitivity and specificity, included studies had to report cutoff values of their ELISPOT assays. The total number of patients with and without BKV replication, and the number of patients with positive or negative ELISPOT assay results had to be reported or had to be calculated from the information provided in the manuscripts. Studies that reported the actual number of the IFN-γ producing cells in the ELISPOT assay, either at the individual patient level or reporting the mean of the study population, were also included in the analysis of the standardized mean difference (SMD). Only studies with adequate information, in accordance with the Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015[24] were included in the review. Two authors (S.U. and S.K.) independently screened the titles and abstracts of the electronic citations, and full-text articles were retrieved for comprehensive review, and were independently rescreened. Disagreements were resolved through consensus and arbitration by D.H. and C.B.

Data extraction and quality assessment

The following data were extracted from each study: author's name, year of publication, country of origin, type of study, timing of ELISPOT testing, the total number of patients

with positive and negative ELISPOT tests, and the number of patients with BKV replication in each group. The cutoff for a positive ELISPOT assay in each study and the patients' actual number of IFN-γ producing cells were included for the analyses. In the post-transplantation ELISPOT studies, the ELISPOT results were retrieved from 2 time points. The first measurements were at the time that BKV replication was diagnosed or when viral load was actively increasing, which in our review we defined as having the "active BKV infection". The second ELISPOT results were collected when the infection was resolving or closest to the time of BK viral clearance, which we defined as "resolving BKV infection". If the ELISPOT values at these time points were not available, the actual values in these studies were not included in the analyses. Every type of BKV antigen used for stimulating the recipient's peripheral blood mononuclear cells (PBMC) was covered, including large T, small t, virion protein 1 (VP1), VP2, VP3, and mixed BK antigen. Patient characteristics, including immunosuppressive regimen and allograft function, were obtained from each study if available.

The Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2)[25] was used to evaluate the risk of bias. This tool contains 4 domains, which are patient selection, index test, reference standard, and flow and timing.

Data synthesis and analysis

As global measures of accuracy across all test threshold, we calculated the pooled diagnostic odds ratio (OR) for active BKV infection in patients with positive IFN-γ ELISPOT compared with patients who had negative IFN-γ ELISPOT, and the area under the summary receiver operating characteristic (SROC) curve. In calculating the OR, a continuity correction was applied to all cells in studies with any zero-cell count. The standardized mean difference (SMD) of the IFN-γ producing cells from patients with active and resolving BKV infection were calculated after normalizing the actual value of the ELISPOT assay to the number of IFN-γ producing cells per $3x10^5$ PBMC. For studies not providing mean and standard deviation (SD), the estimation method by Wan *et al*. was applied.[26] All pooled estimates were calculated using random effects models. A funnel plot was used to demonstrate possible publication bias, and Egger's method was used to test for asymmetry of the funnel plot. The existence of heterogeneity among study effect sizes was examined using the I^2 index and the Q-test p-value. An I^2 index higher than 75% reflects medium to high heterogeneity. The analyses were performed using Stata Statistical Software Release 15.1 with the user written commands midas, metandi and metadta (StataCorp LLC, College Station, TX) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA).

Ethical considerations

This meta-analysis and systematic review did not directly obtain data from human or animal subjects. All of the included studies' information was published in the scientific journals without the possibility to identify the individual patients.

RESULTS

Characteristics of the studies

A total of 651 articles were identified. The flow diagram of the included and excluded studies is shown in Figure 1. After exclusion of irrelevant and duplicated studies, 127 articles underwent full-text review. Nine articles fulfilled the inclusion criteria and were included in the meta-analysis.[27-35] The summary of study characteristics is shown in Table 1. All studies evaluated the relationship between the IFN-γ ELISPOT assay and BKV infection in the post-transplantation period. Two of these nine studies also assessed pre-transplantation measurements.[33, 34] The nine studies varied in study design and technical approach. First, the BK viral antigens used for each study varied, including large T antigen in 7 studies, small t antigen in 4 studies, VP1 antigen in 6 studies, VP2 antigen in 4 studies, VP3 antigen in 4 studies, and mixed antigen in 3 studies. All studies measured IFN-γ as the cytokine for the T lymphocyte-specific immune response against BK viral antigens. Second, the definitions of BKV infection among these studies were different, and ranged from positive decoy cells in urine, viruria, viremia, to full-blown BKVAN. Third, the number of PBMCs used in the IFN-γ ELISPOT assay varied substantially. To compare the outcomes of the different studies, we normalized the actual value of the ELISPOT assay to the number of IFN-γ producing cells per $3x10^5$ PBMC. The QUADS-2 risk of bias assessment for each study is shown in Table 2.

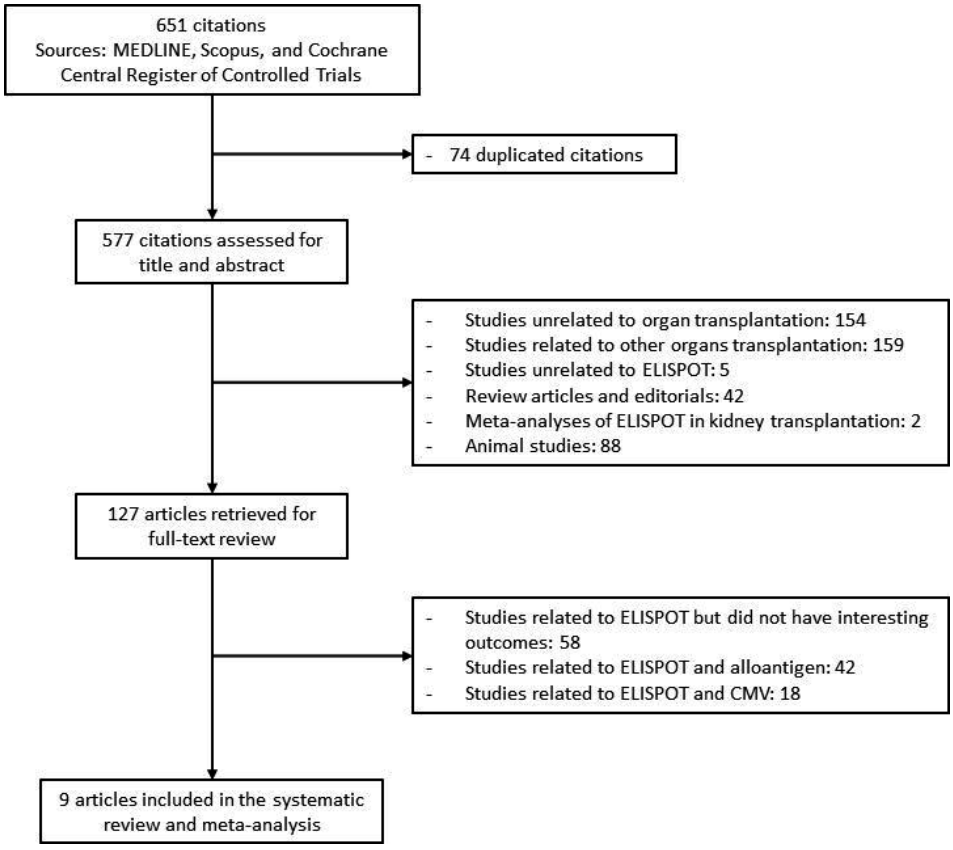

Figure 1: Flow diagram of study selection

Table 1: Summary of studies reporting data of IFN-γ ELISPOT assays in BKV-infected kidney transplant recipients.

Reference	Authors and year of publication	Country of origin	Timing of ELISPOT	Cutoff value after subtraction of negative control	BK antigen used for ELISPOT assay	Patient with active BKV infection (n)	Patients with resolving BKV infection (n)	Onset of BK viremia (months after transplantation)	Patients who never had BKV infection (n)	Definition of BKV infection
28	Binggeli et al. 2007	Switzerland	Post-transplantation	69 IFN-ɤ pc per 10^6 PBMC	Large T, VP1	22	20	N/A	-	Viremia
29	Prosser et al. 2008	USA	Post-transplantation	not mentioned (IFN-ɤ pc per 10^4 PBMC)	Large T	8	8	16 ± 11	-	BKV-associate nephropathy
30	Chakera et al. 2011	UK	Post-transplantation	50 IFN-ɤ pc per 10^6 PBMC	Large T, small t, VP1, VP2, VP3	9	9	N/A	8	Urine decoy cell and viremia
31	Schachtner et al. 2011	Germany	Post-transplantation	10 IFN-ɤ pc per 10^6 PBMC	Large T, small t, VP1, VP2, VP3	18	17	14 ± 18	-	Viremia
32	Costa et al. 2014	Italy	Post-transplantation	5 IFN-ɤ pc per $2x10^5$ PBMC	Mixed antigen	12	-	N/A	137	Viruria or viremia
33	Schachtner et al. 2014	Germany	Post-transplantation	10 IFN-ɤ pc per $2.5x10^5$ PBMC	Large T, small t, VP1, VP2, VP3	12	12	2 ± 1	17	Viruria or viremia
34	Mutlu et al. 2015	Turkey	Pre- and post-transplantation	10 IFN-ɤ pc per $2x10^5$ PBMC	Mixed antigen	12	6	5 ± 2	26	Viremia
35	Schachtner et al. 2015	Germany	Pre- and post-transplantation	25 IFN-ɤ pc per $3x10^5$ PBMC	Mixed antigen, large T, VP1	16	-	3 ± 4	92	Viremia
36	Bae et al. 2020	South Korea	Post-transplantation	not mentioned (IFN-ɤ pc per $3x10^5$ PBMC)	Large T, small t, VP1, VP2, VP3	17	34	13 ± 14	17	Viremia

n: number

pc: producing cells

N/A: not available

Table 2: QUADS-2 for risk of bias assessment

Authors and year of publication	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Binggeli et al. 2007	Low	High	Low	Low	Low	Low	Low
Prosser et al. 2008	Unclear	Low	Low	Low	Low	Low	Low
Chakera et al. 2011	High	Low	Low	Low	Low	Low	Low
Schachtner et al. 2011	Low	Low	Low	Low	Low	Low	Low
Costa et al. 2014	High	Low	Low	High	High	Low	Low
Schachtner et al. 2014	Low	Low	Low	Low	High	Low	Low
Mutlu et al. 2015	High	Low	Low	High	High	Low	Low
Schachtner et al. 2015	Low	Low	Low	Low	Low	Low	Low
Bae et al. 2020	Low	Low	Low	Low	Low	Low	Low

Considering that only 2 studies[33, 34] evaluated the BKV ELISPOT assay before kidney transplantation, we decided to focus our analyses on publications reporting post-transplantation studies. The included studies used varying definitions of BKV infection as shown in Table 1, including decoy cell-positive, viruria, viremia, or BKVAN. Each study measured IFN-γ ELISPOT response to BK viral antigen and correlated this with the clinical course of the patients, which was classified as "active BKV infection" and "resolving BKV infection". Four studies[27, 29, 30, 33] reported sufficient information on the ELISPOT cutoff values, the number of patients with positive and negative ELISPOT test results, and the BKV infection status at the time that ELISPOT was tested, which allowed us to perform meta-analysis for the diagnostic efficacy. The pooled ELISPOT values in patients with active BKV infection compared with patients who had resolving BKV infection was formulated from 7 studies,[27-30, 32, 33, 35] which could also be used for the calculation of the SMD. One study[31] did not report adequate information on the timing of the ELISPOT assay and patients' BKV status, and therefore was excluded from the final analysis.

Post-transplantation ELISPOT assay for the monitoring of BKV infection

We examined the patients who had BKV infection, including patients with active BKV or resolving BKV infection, and compared the IFN-γ ELISPOT results between these groups. Patients who had IFN-γ ELISPOT values less than the cutoff were regarded as having a "negative ELISPOT", whereas patients with an IFN-γ ELISPOT test result higher than the cutoff were considered as having a "positive ELISPOT". The cutoff values used in each study

were different as shown in Table 1. Patients with negative ELISPOT were at higher risk for active BKV infection compared to patients with positive ELISPOT who were more likely to have resolving BKV infection (diagnostic OR 71.91, 95%-CI 30.96-167.05, p-value < 0.001, I^2 = 0%, Q-test p-value = 0.80; Figure 2). The results were consistent in every subgroup of BK viral antigen studied. Figure 3 displays the sensitivity and specificity of the ELISPOT assay for detecting patients with active BKV infection. Overall, the ELISPOT assay performed with pooled sensitivity of 0.95 (95%-CI 0.89-0.98) and specificity of 0.88 (95%-CI 0.78-0.94). When a negative ELISPOT was considered to indicate a positive result for the risk of active BKV infection, the pooled positive likelihood ratio (PLR) was 6.3 (95%-CI 3.8-10.4) and the pooled negative likelihood ratio (NLR) was 0.09 (95%-CI 0.05-0.15; Figure 4). The hierarchical summary receiver operating characteristic (HSROC) curve was analyzed (Figure 5), which had an area under the HSROC curve of 0.97 (95%-CI 0.95-0.98). Finally, the funnel plot of the log of OR was not completely symmetrical, although the observed studies were within the 95%-CI (Supplementary Figure S1). Egger's test also suggested there was evidence of plot asymmetry with p-value of 0.01.

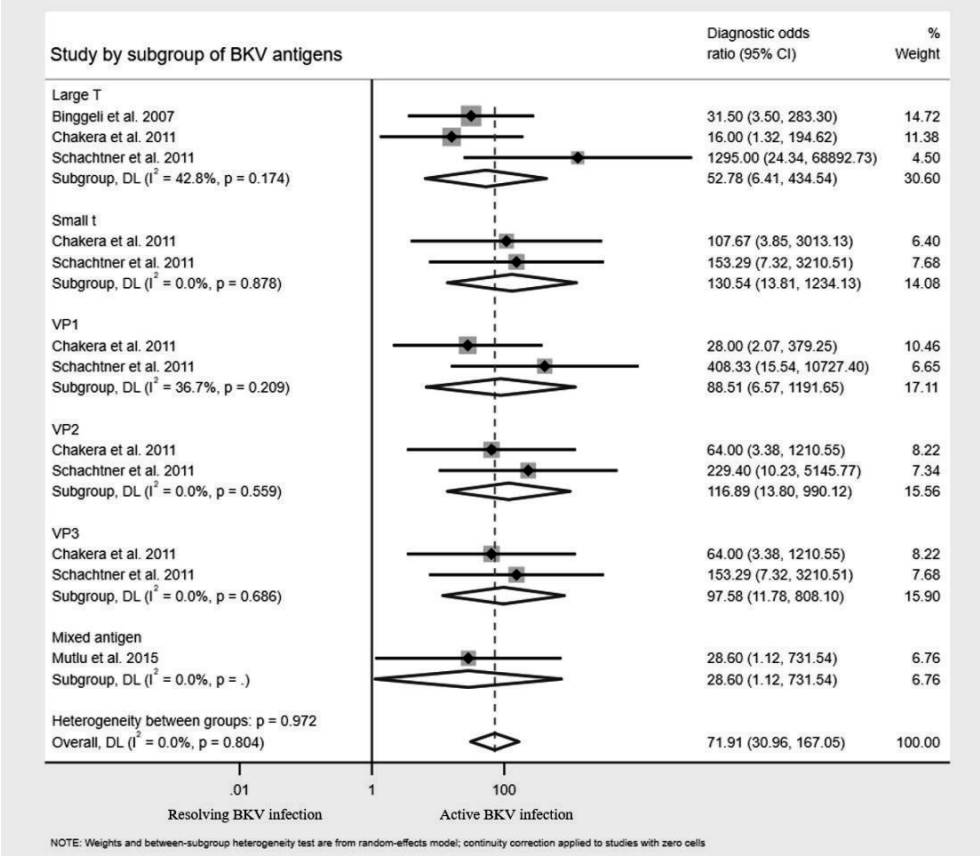

Figure 2: Diagnostic odds ratio of patients with negative compared to positive BKV-specific IFN-γ for having active BKV infection.

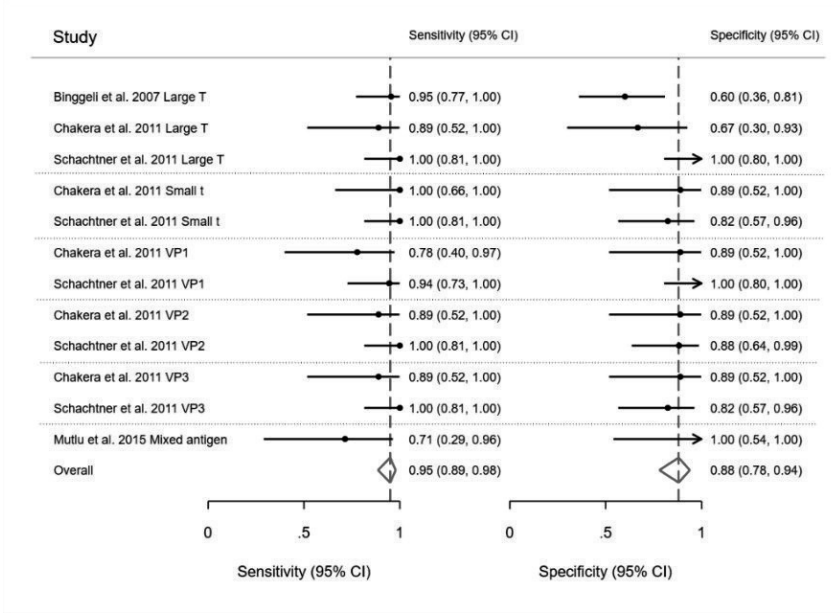

Figure 3: Sensitivity and specificity of the BKV-specific IFN-γ ELISPOT assay for the diagnosis of active BKV replication.

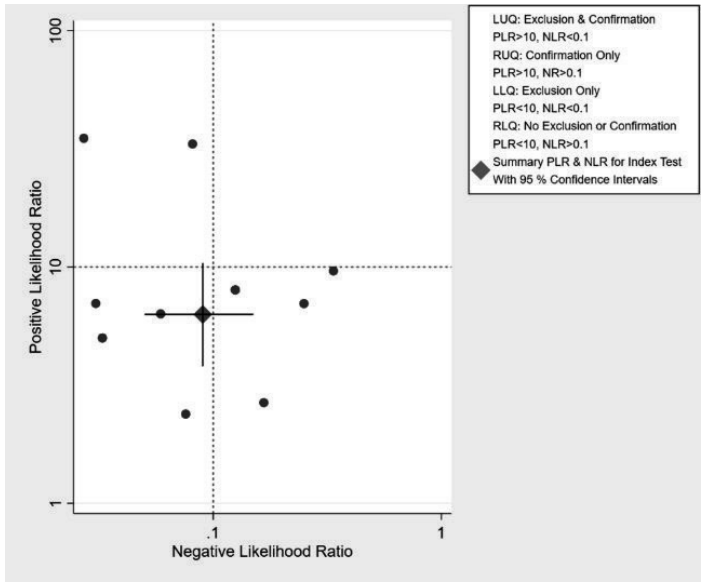

Figure 4: Scatter plot of the positive likelihood ratio (PLR) and the negative likelihood ratio (NLR) of having active BKV infection, when patients with negative BKV-specific IFN-γ ELISPOT were considered at risk of active BKV infection.

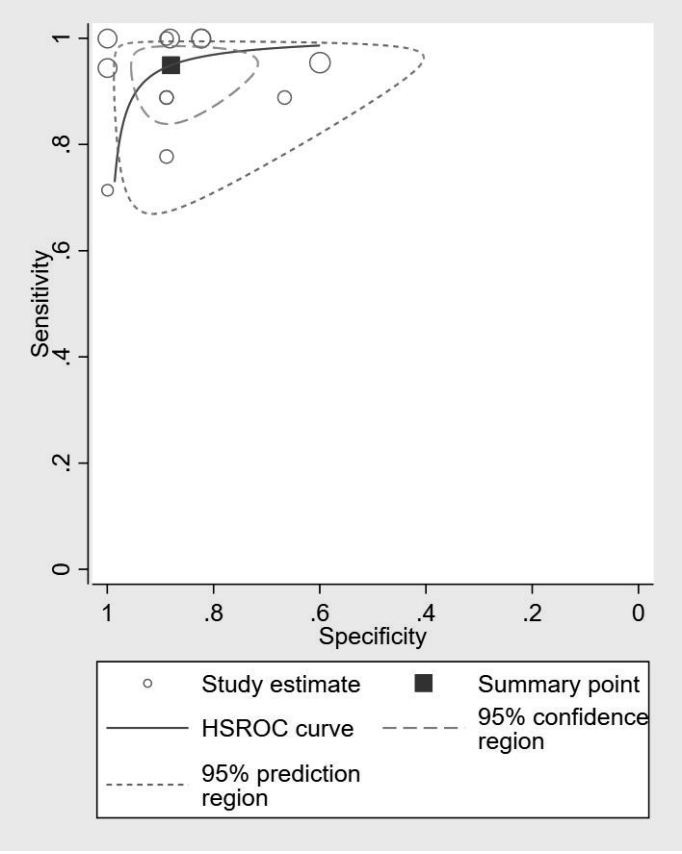

Figure 5: The hierarchical summary receiver operating characteristic (HSROC) curve of BKV-specific IFN-γ ELISPOT assay against BK viral antigens, with area under the curve of 0.97 (95%-CI 0.95-0.98).

Differences in IFN-γ ELISPOT test results between patients with active and resolving BKV infection

We calculated the SMD of the IFN- γ ELISPOT value between the patients with active BKV infection as compared with patients who had resolving BK infection. Figure 6 summarizes the SMD from each study and shows the pooled SMD of -2.09 (95%-CI -2.50, -1.68, p-value < 0.001, I^2 = 79.4%, Q-test p-value < 0.001). To illustrate the difference between patients with active and resolving BKV infection, mean and SD of the number of IFN-γ producing cells (pc) was plotted for each BK viral antigen used. Supplementary Figure S2 shows mean ± SD of the number of IFN-γ pc from the large T and small t antigen, in the patients with active BKV infection compared with the patients with resolving BKV infection

(8.3 ± 7.8 *vs*. 34.4 ± 25.7 IFN-γ pc/3x10^5 PBMC, and 6.6 ± 8.6 *vs*. 34.9 ± 20.7 IFN-γ pc/3x10^5 PBMC, respectively). The number of IFN-γ pc that responded to VP1, VP2, and VP3 antigen are described in Supplementary Figure S3 (18.2 ± 22.6 *vs*. 56.8 ± 56.0 IFN-γ pc/3x10^5 PBMC, 7.1 ± 7.7 *vs*. 33.2 ± 16.5 IFN-γ pc/3x10^5 PBMC, and 7.0 ± 8.1 *vs*. 36.0 ± 23.3 IFN-γ pc/3x10^5 PBMC, respectively).

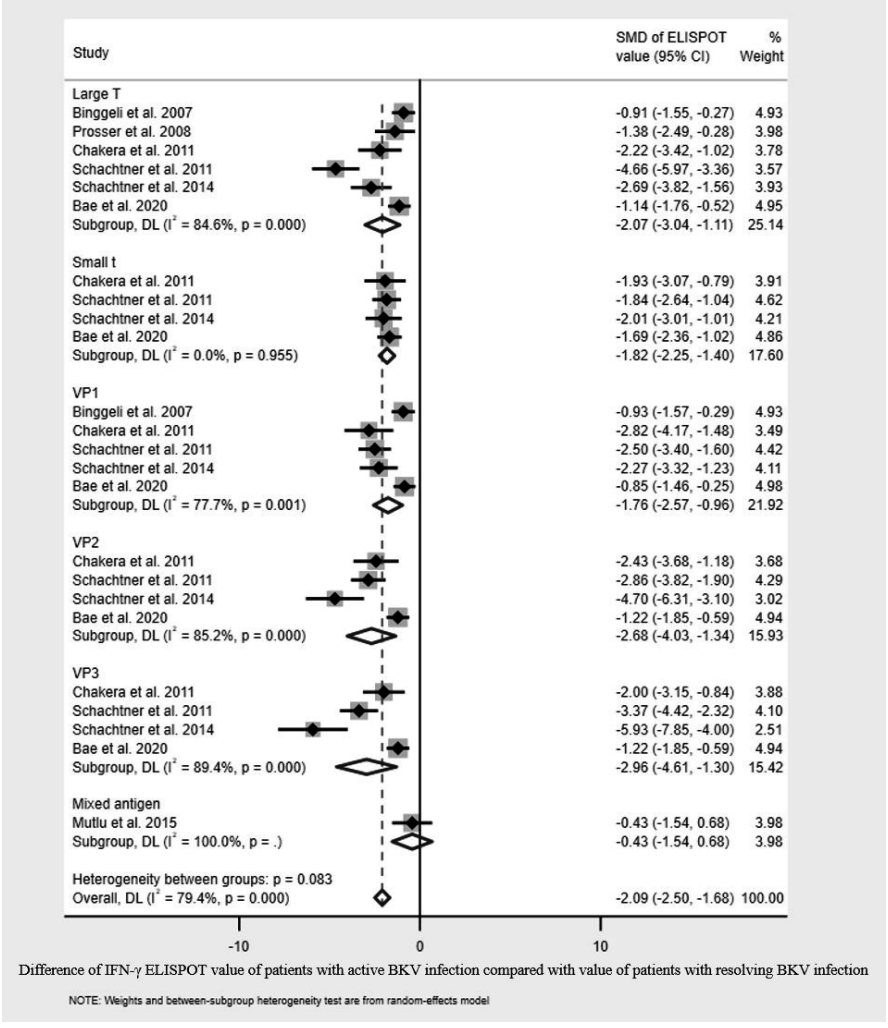

Figure 6: The standardized mean difference (SMD) of the IFN-γ ELISPOT value between patients with active BKV infection and patients with resolving BKV infection.

DISCUSSION

This study is the first systematic review with meta-analysis of the IFN-γ ELISPOT assay that correlated T cell BKV responsiveness with clinical BKV infection in kidney transplant recipients. Overall, the IFN-γ ELISPOT assay has a high capability to discriminate patients with active BKV infection from patients with resolving BKV infection. Patients with negative IFN-γ ELISPOT were at a 71.9-fold higher risk to have active BKV infection compared to the patients with positive IFN-γ ELISPOT. However, the test has NLR of 0.09 and PLR of 6.3, which signifies that the assay is suitable for exclusion, but not for confirmation of active BKV infection.

BKV is an important causes of kidney allograft loss due to the lack of effective treatment.[21, 36, 37] The current practice is to prevent significant BKV replication, including the surveillance for early BK viremia or viruria and minimizing risk factors that are known for BKV reactivation.[10, 15, 38] Besides the well-recognized risks such as intensified immunosuppression, the degree of human leukocyte antigen (HLA) mismatches, and kidney allograft ischemic reperfusion injury,[7, 10, 12, 13, 36] more recent evidence suggests that the mismatch between high donor BKV IgG sero-reactivity and low recipient sero-reactivity significantly increases the risk of BK viremia.[39] Moreover, the lack of donor-specific BKV genotype neutralizing antibody in the recipient also significantly associated with BKV infection after transplantation.[40] To control BKV replication, cells of both innate and adaptive immunity are involved. BKV-specific CD4+ T lymphocytes suppress BKV by the production and secretion of proinflammatory cytokines, particularly IFN-γ, tumor necrosis factor (TNF), and the serine protease granzyme B.[41] Different BKV antigens also activate different subsets of T lymphocytes, for example; VP1 mainly activated CD4+ T lymphocytes, while large T antigen stimulates CD8+ T lymphocytes.[27] In our view, the results of the BKV-specific ELISPOT could assist the clinician in monitoring and treating of BKV by two approaches.

First, by performing an anti-BKV IFN-γ ELISPOT assay at the time that BK viruria or viremia is first detected. By this method one will be able to classify patients as having a positive or negative ELISPOT result. The former will be more likely to have self-limited BKV replication, transient BK viremia, and a good prognosis without substantial changes to their immunosuppressive treatment. On the contrary, patients with negative ELISPOT will possibly progress to persistent BK viremia or BKVAN and may therefore need more aggressive interventions.[21] Second, another suitable period is when immunosuppressive reduction has been implemented for the treatment of BKV infection. The IFN-γ ELISPOT assay could then serve as to guide the clinician whether or not to lower the immunosuppressive medications. Patients who have increasing numbers of IFN-γ pc to BKV continuously since the beginning of the intervention are more likely to eventually clear the virus. The number of

BKV-specific IFN-γ pc that a clinician should target are depicted in Supplementary Figures S2 and S3 which should be accompanied by the patients' clinical course.

This ELISPOT technique is a highly sensitive method for the quantification of cytokine-producing cells after stimulation with a stimulus which in this case is a BKV antigen.[42] All included studies standardized their ELISPOT assay by including positive controls, either the superantigen *Staphylococcus enterotoxin* B or phytohemagglutinin, and negative controls. A few studies reported that the BKV antigens can cross-react with JC virus[43-45] which is an extremely rare cause of allograft nephropathy.[46] Other limitations of the ELISPOT assay are that it is time-consuming, relatively expensive, and that there is a need for well-trained personnel. Also, this assay does not provide information about the cellular source of the BKV-specific response. For this, flow cytometry is the better technique. Using intracellular cytokine staining by flow cytometry, Ahlenstiel-Grunow T. e*t al*. showed that the high amount of CD4+ and CD8+ BKV-specific T lymphocytes are of importance to control the virus and prevent BKVAN.[47] Moreover, the poly-functional CD8+ T lymphocytes which secrete IFN-γ, interleukin 2 (IL-2), and TNF-α are needed for BKV clearance, while the mono-functional CD8+ T lymphocytes are only effective for suppression of low-level BK viremia.[48] Altogether, the information from the ELISPOT assay and the amount of BKV-specific T lymphocytes and their functions, should be included in future trials exploring the value of these techniques in management strategy of BKV infection.

BKV-specific IFN-γ ELISPOT should be interpreted together with the BK viral load results. In current clinical practice, it is difficult to differentiate BK viremic patients who will achieve BK viral clearance from those who will have progressive BK viremia leading to BKVAN. Knowing the ELISPOT result at the time of viral load testing, would allow clinicians to make informed decisions based on the patient's immune response against BKV. Patients could then be stratified as high or low risk for developing BKVAN, and immunosuppression adjusted accordingly. This add-on value of the ELISPOT to supplement BK viral load testing would help prevent unnecessary aggressive immunosuppression reduction that leads to concurrent or superimposed acute rejection in patients with BKV infection, a scenario which remains problematic in kidney transplantation. However, since there are variations of BKV-specific IFN-γ ELISPOT protocol among laboratories, including the type of BKV antigens used, the amount of recipient's PBMC used in the assay, and the cutoff values. Development of a standardized protocol with evidence-based threshold cutoffs to defined antigens and consistent PBMC concentrations is still needed before this method can be routinely applied in the clinics.

Our study is not without limitations. The funnel plot and Egger's p-value indicate that there may be publication bias in the meta-analysis. Further studies are needed to confirm

or reject our findings. Second, the studies of pre-transplantation number of BKV-specific IFN-γ pc were too few to include in the meta-analysis. Interestingly, Schachtner *et al*. showed that the risk of BKV reactivation could be predicted by the loss of pre-transplant BKV-specific immunity in the post-transplantation period.[34] These findings were in contrast with the results from Mutlu *et al*. which did not find an association between pre-transplantation BKV-specific immunity and the development of post-transplantation BKV reactivation.[33] Further studies are required regarding the evaluation of pre-transplantation BKV-specific immunity and choosing a particular immunosuppressive regimen. Third, we realize that the non-normal distribution of the number of BKV-specific IFN-γ pc might have caused bias in the estimation of the mean and SD. Nevertheless, we have used methods that provide the best estimate of the sample mean and SD from the skewed data.[26] In addition, to minimize to risk of bias from outlier values, the cutoff of the ELISPOT assay was used to classify patients as having positive and negative results, and was correlated with the clinical evidence of BKV reactivation.

In conclusion, this meta-analysis and systematic review demonstrates that the IFN-γ ELISPOT assay is a useful tool for assessing the post-kidney transplantation risk of BKV-associated complications. Patients with an adequate T lymphocyte BKV-specific immune response (positive ELISPOT) are more likely to achieve resolution of BKV infection.

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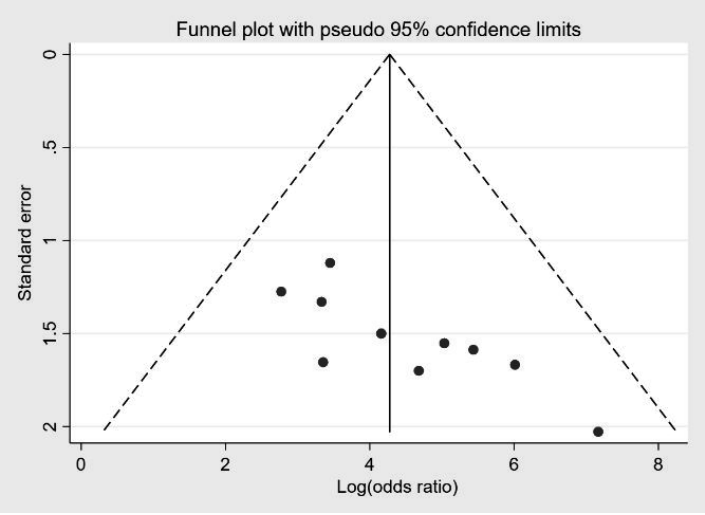
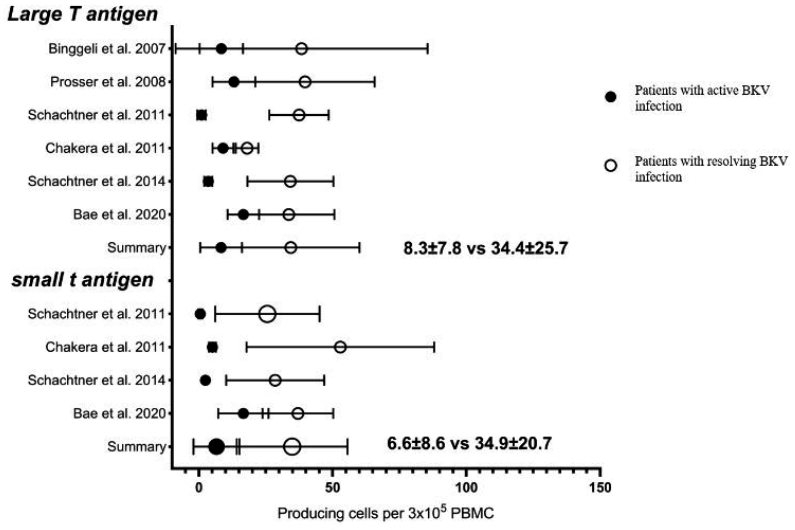
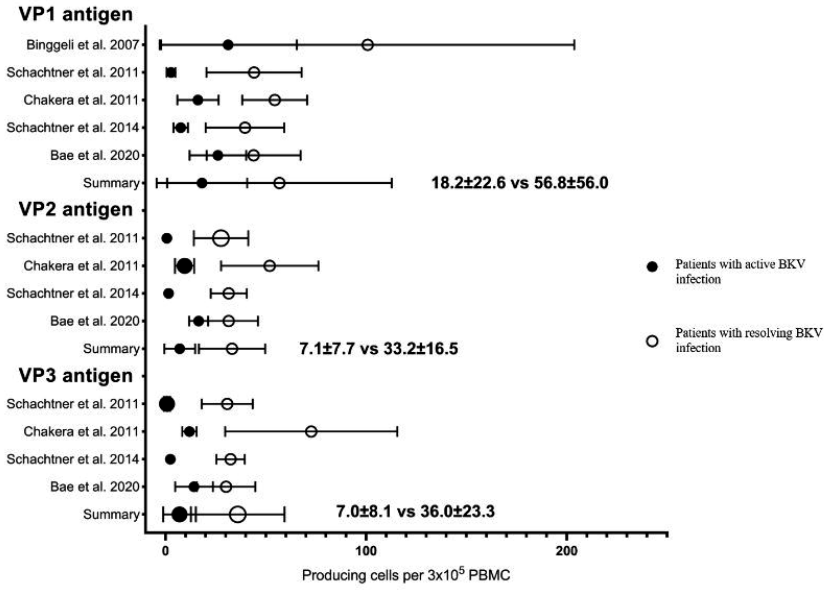
SUPPLEMENTARY DATA



Supplementary Figure S1: Funnel plot of the log of odds ratio and standard error



Supplementary Figure S2: IFN-γ ELISPOT values from the large T and small t antigens in patients with active BKV infection compared with patients with resolving BKV infection.



Supplementary Figure S3: IFN-γ ELISPOT values from the VP1, VP2, and VP3 antigens in patients with active BKV infection compared with patients with resolving BKV infection.

Chapter 6

The First Asian Kidney Transplantation Prediction Models for Long-Term Patient and Allograft Survival

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ABSTRACT

Introduction

Several kidney transplantation (KT) prediction models for patient and graft outcomes have been developed based on Caucasian populations. However, KT in Asian countries differs due to patient characteristics and practices. To date, there has been no equation developed for predicting outcomes amongst Asian KT recipients.

Methods

We developed equations for predicting 5- and 10-years patient survival (PS) and death-censored graft survival (DCGS) based on 6,662 patients in the Thai Transplant Registry. The cohort was divided into training and validation datasets. We identified factors significantly associated with outcomes by Cox regression. In the validation dataset, we also compared our models with another model based on KT in the United States (US).

Results

Variables included for developing the DCGS and PS models were recipient and donor age, background kidney disease, dialysis vintage, donor hepatitis C virus status, cardiovascular diseases, panel reactive antibody, donor types, donor creatinine, ischemic time, and immunosuppression regimens. The C-statistics of our model in the validation dataset were 0.69 (0.66-0.71) and 0.64 (0.59-0.68) for DCGS and PS. Our model performed better when compared with a model based on US patients. Compared with tacrolimus, KT recipients aged ≤ 44 years receiving cyclosporine A (CsA) had a higher risk of graft loss (adjusted HR 1.26, p=0.046). The risk of death was higher in recipients aged > 44 years and taking CsA (adjusted HR 1.44, p=0.011).

Conclusions

Our prediction model is the first based on an Asian population, can be used immediately after transplantation. The model can be accessed at www.nephrochula.com/ktmodels.

INTRODUCTION

Kidney transplantation (KT) is the most effective renal replacement therapy (RRT) in the current era. The benefit of KT over dialysis has been shown in many previous studies, in terms of both patient survival[1-3] and quality of life.[4] The success of KT is mainly due to improvements in tissue cross-matching and human leukocyte antigen (HLA) typing,[5] donor evaluations,[6, 7] and the evolving field of immunosuppressive medications.[8] With the introducing of mycophenolic acid (MPA)[9] and calcineurin inhibitors (CNIs)[10-12] as primary maintenance therapy, 5-year patient and graft survival has increased to 85-93% and 77-87% respectively in the Scientific Registry of Transplant Recipients (SRTR) in United States (US),[13] 92-95% and 81-87% in the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) Registry,[14] and 90-95% and 81-90% in the Australia and New Zealand Dialysis and Transplant Registry (ANZDATA).[15] In Asia, there are only few reports about long-term kidney transplant outcomes. The Japanese Renal Transplant Registry showed 5-years patient survival of 93-97% and 5-years graft survival of 87-94%.[16] These 5-year patient and graft survival rates are comparable with data from our Thai Transplant Registry (93-96% and 88-93%, respectively).[17]

Several kidney transplantation prediction models have been developed to inform both clinicians and patients, regarding patient and graft survival. However, all existing scoring tools were developed based on predominantly Caucasian populations, either from the United States or Europe.[18-25] A recent systematic review of these prediction models[26] showed the area under the curve (equivalent to the C statistic) between 0.60-0.80 as the discrimination scores for models validation. The most recent predictive score for post-transplantation outcomes developed by Molnar et al.[27] was also based on the SRTR in US, and included only 9% of Asians. To some extent, kidney transplant outcomes in Asian are different from other ethnicities,[13, 27-29] which could partly be explained by pharmacogenetic differences.[30, 31] Therefore, clinicians must be cautious when applying these models which were developed in a non-Asian, to an Asian population, and a model predicting kidney transplantation outcomes in an Asian population is needed.

In this present study, we developed a predictive model using data from the Thai Transplant Registry, and focused on death-censored graft survival and patient survival at 5 and 10 years post-transplant. We also compared our model with the latest model based on the SRTR in our validation dataset. To our knowledge, this is the first kidney transplantation prediction score derived from an entirely Asian population.

MATERIALS AND METHODS

Study Population

From a total of 7,107 kidney transplant recipients in the Thai Transplant Registry, 6,662 adults (age ≥ 18 years) patients were included into the study. The Thai Transplant Registry is organized by the Thai Transplant Society, the central organization for all transplantation in Thailand including kidney, liver, pancreas, lung, heart, and intestine transplantation. The transplant coordinators from 39 kidney transplant centers in Thailand submit electronic clinical information of transplant recipients to the central database annually. Although kidney transplantation in Thailand began in 1975, the information of cases transplanted earlier than 1987 were not complete enough to include in the analysis. In this study, we therefore included all patients transplanted from 1987 to April 2018. Most transplants in the registry were done in the period after January 1, 2001; only 8% of patients were transplanted between 1987-2000. This study was approved by the Thai Transplantation Society Committee, for using data for research purposes and improvement of Thai Kidney Transplantation (approval number 41/2561).

Data extraction

The variables available for kidney transplant recipients included age, gender, causes of end stage renal disease (ESRD), dialysis modalities, dialysis vintage, cardiovascular comorbidities (myocardial infarction/ischemia, peripheral vascular disease, cerebrovascular disease, and diabetes mellitus), panel reactive antibody (PRA), and hepatitis B and C virus status. Donor variables available were age, gender, type of donation, cause of brain death, hepatitis B virus (HBV) and hepatitis C virus (HCV) status, and best creatinine level. Individual data of recipient and donor HLA mismatches, and cold ischemic time were also included. Approximately 20% of Thai transplant recipients used de novo cyclosporine A (CsA) for primary immunosuppression, and about 45% received no induction[17]; therefore, we included the immunosuppressive regimens used for both induction and maintenance therapy in the analysis. The maintenance regimens in the registry were the regimens at discharge from hospital, which are usually continued during follow-up period unless medication-related adverse events occur.

Statistical Analysis

Continuous variables are shown as mean±standard deviation (SD), and categorical variables shown as percentages. The cohort was randomized into a training dataset (n=4,013) and validation dataset (n=2,649). Multiple imputation with 20 imputations were used for missing data in the training dataset. We imputed the cold ischemic time (8% missing), peak PRA (6% missing), dialysis vintage (25% missing), and donor serum creatinine (6% miss-

ing). For categorical variables, we imputed types of donor (12% missing), donor and recipient HBV and HCV status (11-13% missing), and recipient cardiovascular comorbidities (24%). The full method of multiple imputation can be found in Supplementary Table S1. The outcomes were overall patient survival, censored at the most recent follow-up visit, and death-censored graft survival. We used Cox proportional hazards regression to assess associations with our endpoints and available patient and donor variables using the training dataset. We assessed the linearity of continuous variables against the hazard function by initially modelling the covariate in quartiles and plotting the midpoint of the quartile against the log hazard. Variables with p-values <0.10 in univariate analysis were adjusted for in multivariable models. The final multivariate model was obtained using backward-selection based on Akaike's information criterion (AIC). The equations predicting 5- and 10-years outcomes were then developed using the coefficients from the multivariable Cox regression models based on the AIC. Applying Cox models in this way creates a risk equation which can be recalibrated to other cohorts, by replacing the mean value of predictors in our cohort with those of their own cohort, as well as replacing 5- and 10-year survival estimates with those from their own cohort. The equations were subsequently applied to the validation dataset, and the power of the model to discriminate outcomes was tested using Harrell's C statistic.[32] We calibrated the DCGS and PS models by assessing agreement between observed and predicted events within 10 years using a modified Hosmer-Lemeshow χ^2 statistic. The Kaplan-Meier estimator was used to obtain the observed incidence of events, and the predicted incidence of events after risk was categorized into deciles. Calibration plots of the observed and predicted survival probabilities for those with risk scores dichotomized at 50% were plotted and examined. We then compared the discriminative power of our survival models with the most updated SRTR-based models[27] using the C statistic. The difference between models was analyzed using linear combinations of parameters. Because the combination of CNIs (tacrolimus or CsA) and mycophenolate (MPA) has become the mainstay of maintenance regimens in the current era, but without definite evidence of superiority between different CNIs used in long-term follow up,[33-38] we also compared the survival function to assess if differences existed between tacrolimus and CsA, when combined with mycophenolate-based regimens. We assessed the proportional hazards assumption in our Cox models by formally testing whether the log hazard ratio function was constant over time.[39] All statistical analysis was conducted using Stata Statistical Software Release 15.1 (StataCorp LLC, College Station, TX, USA).

RESULTS

Characteristics of Thai Transplant Registry

Baseline characteristics of the training and validation cohort are shown in Table 1. Recipient age at transplantation was 44.2±11.9 years, and recipients were predominantly male (63.1%). Glomerular disease was the most common known cause of ESRD (24.9%). Mean dialysis vintage was 3.8±3.4 years, and hemodialysis was the most utilized modality (86.7%). Eighteen percent of patients had cardiovascular comorbidities, including myocardial ischemia or infarction, peripheral vascular diseases, ischemic or hemorrhagic strokes, and diabetes mellitus. Most patients were considered as having low level of sensitization with approximately 88% having 0% reactivity of panel reactive antibody (PRA). The donors were mostly male (63.4%), and 49.9% were living donations. For induction immunosuppression, most transplant recipients received either IL-2 receptor antagonists (IL2RA) (44.2%) or no induction (46.1%). Basiliximab was the most used IL2RA (35.0% of patients). Lymphocyte depleting agents were used as induction therapy in 9.7% of patients, 8.6% of the total cohort were given thymoglobulin. CNIs were prescribed in 95.5% of patients, and tacrolimus was more common than CsA. In only 2.3% of patients was a de novo mammalian target of Rapamycin inhibitor (mTORi) used as a part of the maintenance regimen. Median follow-up was 4.8 (interquartile range 2.0-9.1) years. Patient survival at 5 years was 92.2% and at 10 years was 86.4%. Death-censored graft survival at 5 years was 87.9% and at 10 years was 75.8%.

Table 1: Baseline characteristics in training and validation cohort

Variables	Total cohort (n=6,662)	Training cohort (n=4,013)	Validation cohort (n=2,649)	P-value for difference between Training and Validation cohort*
Recipient characteristics				
Recipient age[a], years (mean±SD)	44.2±11.9	44.1±11.8	44.3±12.1	0.393
Recipient male gender, percent	63.1	63.2	62.9	0.810
Causes of kidney diseases, percent				
Glomerular diseases	24.9	25.0	24.9	
Diabetic nephropathy	9.7	10.0	9.3	0.660
Others[b]	17.6	17.2	18.0	
Undefined etiology[c]	47.8	47.8	47.8	
Mode of dialysis, percent				
Hemodialysis	86.7	86.6	86.8	0.399
Peritoneal dialysis	9.2	9.6	8.9	
Preemptive transplantation	4.1	3.8	4.3	
Dialysis vintage, years (median, IQR)	2.9 (1.2-5.5)	2.9 (1.2-5.5)	2.8 (1.2-5.6)	0.703
Previous transplantation, percent	3.3	2.9	3.9	0.110
Recipient HBsAg positive, percent	3.8	3.7	3.9	0.772
Recipient Anti-HCV positive, percent	2.9	2.5	3.4	0.044
Recipients with cardiovascular comorbidities[d]	17.8	17.4	18.3	0.637
Panel reactive antibody, percent				
0%	88.2	88.3	88.1	
1-20%	5.3	5.2	5.3	0.920
21-80%	4.4	4.3	4.4	
>80%	2.2	2.2	2.2	
Donor characteristics				
Donor age, years (mean±SD)	37.4±12.8	37.5±12.8	37.4±12.8	0.661
Donor male gender, percent	63.4	64.1	62.3	0.112
Types of donor, percent				
Living donor	49.9	48.7	51.6	0.085
Traumatic deceased donor	36.6	37.5	35.2	
Cerebrovascular deceased donor	13.5	13.8	13.2	
Donor HBsAg positive, percent	2.3	2.3	2.2	0.969
Donor Anti-HCV positive, percent	1.3	1.2	1.4	0.365
Donor best creatinine, mg/dl (mean±SD)	0.95±0.47	0.95±0.48	0.95±0.46	0.589
Transplant characteristics				
Human leukocyte antigen mismatch (HLA MM), percent				
0 MM	21.5	20.9	22.3	0.398
1-3 MM	55.8	56.3	55.2	
4-6 MM	22.7	22.8	22.5	
Transplantation year, percent				
1987-2000	8.2	8.3	8.0	0.847
2001-2010	37.0	37.0	37.0	
2011-2018	54.8	54.7	55.0	
Cold ischemic time, hours (median, IQR)	11.1 (0.7-20)	11.7 (0.7-20.2)	9.7 (0.7-19.8)	0.046

Table 1: Baseline characteristics in training and validation cohort (continued)

Variables	Total cohort (n=6,662)	Training cohort (n=4,013)	Validation cohort (n=2,649)	P-value for difference between Training and Validation cohort*
Induction therapy, percent				
No induction	46.1	45.6	46.8	0.573
Lymphocyte depleting agents[e]	9.7	9.9	9.3	
IL-2 receptor antagonists[f]	44.2	44.5	43.9	
Calcineurin inhibitors (CNIs), percent				
No CNIs	4.5	4.8	4.0	0.283
Tacrolimus[g]	56.4	56.3	56.7	
Cyclosporine A	39.1	38.9	39.3	
Mycophenolate[h], percent	75.3	75.3	75.4	0.968
Mammalian target of Rapamycin inhibitor (mTORi)[i], percent	2.3	2.4	2.0	0.341
Azathioprine, percent	7.3	7.6	6.9	0.272
Prednisolone, percent	95.8	95.8	95.7	0.721
Follow-up duration, years (median, IQR)	4.8 (2.0-9.1)	4.8 (2.0-9.0)	4.9 (2.1-9.2)	0.683

[a]Age at transplantation

[b]Other causes of kidney diseases included cystic kidney diseases, tubulointerstitial nephropathy, obstructive uropathy, and ischemic nephropathy.

[c]Undefined etiology is diagnosed as a cause of ESRD when kidney biopsy was not done and there are no other clues of systemic diseases.

[d]Cardiovascular comorbidities included myocardial ischemia/infarction, peripheral vascular diseases, cerebrovascular diseases (hemorrhagic/ischemic stroke), and diabetes mellitus.

[e]Thymoglobulin, anti-lymphocyte globulin, OKT3, and alemtuzumab

[f]Basiliximab and Daclizumab

[g]Twice daily and once daily tacrolimus

[h]Mycophenolate mofetil and mycophenolate sodium

[i]Sirolimus and everolimus

*P-value calculated from t-test or Mann-Whitney test for normally distributed and skewed continuous variables respectively, and the chi-square test for categorial variable

Cox regression analysis and prediction equation development

The coefficients of every variable in the multivariate analysis were used to create the prediction equation. Univariate and multivariate models for predicting death-censored graft failure and patient death and shown in tables 2 and 3, respectively. Variables that significantly contributed to death-censored graft failure in univariate analysis with p-value<0.10 were recipient and donor age, ESRD cause, dialysis vintage, recipient and donor anti-HCV status, peak PRA, type of donor, donor sex, donor best creatinine, cold ischemic time, CNIs, MPA, and prednisolone use. Recipient and donor age, donor gender, causes of ESRD, recipient

cardiovascular morbidities, type of donors, donor best creatinine, cold ischemic time, HLA mismatch, CNIs, MPA, and prednisolone use were significantly associated with patient death in univariate models. The final multivariate equations chosen after applying backward-selection based on Akaike's information criterion (AIC) are shown in Supplementary Table S2. These models can be accessed as web-based calculator at www.nephrochula.com/ktmodels.

Table 2: Univariate and multivariate Cox regression models for predicting death-censored graft failure

Variables	Univariate analysis		Multivariate analysis		
	Unadjusted hazard ratio (95% CI)	p-value	Adjusted hazard ratio (95% CI)	Coefficient	p-value
Recipient characteristics					
Recipient age (per 1 year increase)	0.98 (0.98-0.99)	<0.001	0.98 (0.97-0.99)	-.0205659	<0.001
Recipient male gender (vs female)	0.95 (0.81-1.11)	0.502	-	-	-
Cause of ESRD		0.042			0.125
Glomerular diseases (ref.)	1		1		
Diabetic nephropathy	0.76 (0.55-1.05)	0.094	1.01 (0.71-1.42)	.0049955	0.977
Miscellaneous	0.74 (0.58-0.94)	0.014	0.82 (0.64-1.05)	-.1990233	0.122
Undefined etiology	0.82 (0.69-0.98)	0.029	0.81 (0.67-0.97)	-.2095562	0.026
Dialysis vintage (per 1 year increase)	1.03 (1.00-1.05)	0.064	1.00 (0.97-1.04)	.0036739	0.815
Previous kidney transplantation (vs no)	0.91 (0.59-1.40)	0.664	-	-	-
Recipient HBsAg (positive vs negative)	0.91 (0.56-1.47)	0.700	-	-	-
Recipient Anti-HCV (positive vs negative)	1.50 (1.05-2.15)	0.027	1.34 (0.87-2.04)	.289465	0.181
Recipient cardiovascular comorbidities (presence vs absence)	1.15 (0.88-1.51)	0.789	-	-	-
Peak PRA (per 1 percent increase)	1.01 (1.00-1.01)	0.033	1.00 (1.00-1.01)	.0044695	0.071
Donor characteristics					
Donor age (per year increase)	1.02 (1.01-1.02)	<0.001	1.02 (1.01-1.02)	.0158413	<0.001
Donor male gender (vs female)	1.16 (0.98-1.36)	0.077	1.05 (0.88-1.26)	.0493865	0.590
Type of donors		<0.001			0.003
Living donor (ref.)	1		1		
Traumatic deceased donor	1.30 (1.10-1.56)	0.003	1.01 (0.71-1.42)	.0059296	0.973
Cerebrovascular deceased donor	2.49 (1.94-3.19)	<0.001	1.64 (1.11-2.41)	.4930883	0.012
Donor HBsAg (positive vs negative)	1.40 (0.81-2.40)	0.226	-	-	-
Donor Anti-HCV (positive vs negative)	1.83 (1.10-3.04)	0.021	1.45 (0.80-2.65)	.3719404	0.224
Donor best serum creatinine (per 1 mg/dl increase)	1.15 (1.01-1.31)	0.042	1.08 (0.93-1.25)	.0745416	0.334
Transplantation characteristics					
HLA mismatch (per 1 mismatch increase)	1.01 (0.96-1.05)	0.770	-	-	-
Cold ischemic time (per 1 hour increase)	1.02 (1.01-1.03)	<0.001	1.02 (1.01-1.04)	.0210662	0.005
Induction therapy (vs no induction)	1.01 (0.86-1.19)	0.873	-	-	-
Calcineurin inhibitors		<0.001			<0.001
No CNIs (ref.)	1		1		
Tacrolimus	0.34 (0.26-0.46)	<0.001	0.50 (0.36-0.70)	-.6921427	<0.001
Cyclosporine A	0.41 (0.31-0.54)	<0.001	0.60 (0.44-0.83)	-.5048233	0.002
Mycophenolate (vs no mycophenolate)	0.75 (0.63-0.88)	<0.001	0.80 (0.67-0.95)	-.2234541	0.013
mTORi (vs no mTORi)	0.89 (0.55-1.44)	0.627	-	-	-
Prednisolone (vs no prednisolone)	0.29 (0.22-0.39)	<0.001	0.38 (0.28-0.52)	-.9624822	<0.001

Table 3: Univariate and multivariate Cox regression models for predicting patient death

Variables	Univariate analysis		Multivariate analysis		
	Unadjusted hazard ratio (95% CI)	p-value	Adjusted hazard ratio (95% CI)	Coefficient	p-value
Recipient characteristics					
Recipient age (per 1 year increase)	1.02 (1.02-1.03)	<0.001	1.02 (1.01-1.03)	.018879	<0.001
Recipient male gender (vs female)	1.15 (0.93-1.42)	0.195	-	-	-
Cause of ESRD		<0.001			0.060
Glomerular diseases (ref.)	1		1		
Diabetic nephropathy	2.43 (1.69-3.48)	<0.001	1.60 (1.05-2.43)	.4682175	0.028
Miscellaneous	1.54 (1.10-2.14)	0.011	1.38 (0.97-1.94)	.3188803	0.069
Undefined etiology	1.58 (1.21-2.08)	0.001	1.39 (1.05-1.83)	.3277265	0.021
Dialysis vintage (per 1 year increase)	1.02 (0.98-1.06)	0.306	-	-	-
Previous kidney transplantation (vs no)	0.63 (0.32-1.25)	0.189	-	-	-
Recipient HBsAg (positive vs negative)	1.04 (0.55-1.96)	0.907	-	-	-
Recipient Anti-HCV (positive vs negative)	1.37 (0.84-2.25)	0.211	-	-	-
Recipient cardiovascular comorbidities (presence vs absence)	1.61 (1.20-2.17)	0.002	1.55 (1.09-2.21)	.4391922	0.016
Peak PRA (per 1 percent increase)	1.00 (0.99-1.00)	0.375	-	-	-
Donor characteristics					
Donor age (years)	1.01 (1.00-1.02)	0.041	1.01 (1.01-1.02)	.0138043	0.002
Donor male gender (vs female)	1.27 (1.03-1.57)	0.028	1.00 (0.79-1.27)	.0024487	0.984
Type of donor		<0.001			0.032
Living donor (ref.)	1		1		
Traumatic deceased donor	1.78 (1.42-2.23)	<0.001	1.38 (0.90-2.12)	.3225336	0.140
Cerebrovascular deceased donor	1.63 (1.12-2.39)	0.011	1.08 (0.64-1.82)	.0735826	0.783
Donor HBsAg (positive vs negative)	0.96 (0.43-2.16)	0.923	-	-	-
Donor Anti-HCV (positive vs negative)	1.62 (0.82-3.18)	0.162	-	-	-
Donor best serum creatinine (per 1 mg/dl increase)	1.25 (1.09-1.43)	0.001	1.17 (0.98-1.40)	.1548611	0.089
Transplantation characteristics					
HLA mismatch (per 1 mismatch increase)	0.95 (0.89-1.00)	0.059	0.95 (0.90-1.01)	-.0491318	0.119
Cold ischemic time (per 1 hour increase)	1.03 (1.02-1.04)	<0.001	1.01 (1.00-1.03)	.0138675	0.146
Induction therapy (vs no induction)	0.99 (0.80-1.22)	0.894	-	-	-
Calcineurin inhibitors		<0.001			0.001
No CNIs (ref.)	1		1		
Tacrolimus	0.27 (0.19-0.39)	<0.001	0.45 (0.29-0.68)	-.8020715	<0.001
Cyclosporine A	0.38 (0.27-0.53)	<0.001	0.57 (0.38-0.86)	-.5560733	0.007
Mycophenolate (vs no mycophenolate)	0.60 (0.48-0.74)	<0.001	0.62 (0.49-0.78)	-.4730699	<0.001
mTORi (vs no mTORi)	0.93 (0.50-1.75)	0.825	-	-	-
Prednisolone (vs no prednisolone)	0.28 (0.20-0.39)	<0.001	0.44 (0.29-0.66)	-.8273759	<0.001

Validation of prediction equations and comparison with external model

We tested our equation in the validation dataset. Harrell's C statistic for DCGS was 0.69 (95%CI 0.66-0.71), and 0.64 (95%CI 0.59-0.68) for PS. The C statistic of DCGS from our model performed significantly better than a recently published model based on SRTR in US[27] (0.55, 95%CI 0.52-0.58) (p-value for difference<0.001). For PS, our equation had improved discriminating properties compared to the US-based model with C-statistic and 95%CI shifting by 5% to the right (0.58, 95%CI 0.54-0.63), although the difference was not

statistically significant (p-value for difference=0.068) (Table 4). Model calibration for DCGS and PS are shown in Supplementary Table S3-S6 and Supplementary Figure S1-S2.

Table 4: Harrell's C-statistics of the Thai Registry model in the validation dataset, compared with the United States' Scientific Registry of Transplant Recipients (SRTR)-based equation.

Outcomes	C-statistics for predicting outcomes (95% CI)		
	US SRTR model	Thai Registry model	p-value for difference
Death-censored graft survival	0.55 (0.52-0.58)	0.69 (0.66-0.71)	<0.001
Patient survival	0.58 (0.54-0.63)	0.64 (0.59-0.68)	0.068

Maintenance immunosuppression regimen and survival outcomes in multivariate analysis

The adjusted DCGS function, categorized by maintenance immunosuppression regimen and recipient age is shown in Figure 1. We dichotomized recipient age at 44 years, which was the mean and median value in this registry. DCGS of older recipients (aged >44 years) was not significantly affected by the maintenance regimen used (adjusted hazard ratio 1.17, 95% CI 0.90-1.52, for CsA, compared with tacrolimus in an MPA-based regimen). Conversely, recipients aged ≤44 years had a significantly higher risk of graft failure when treated with a CsA+MPA, versus a tacrolimus+MPA regimen (aHR 1.26, 95%CI 1.01-1.57).

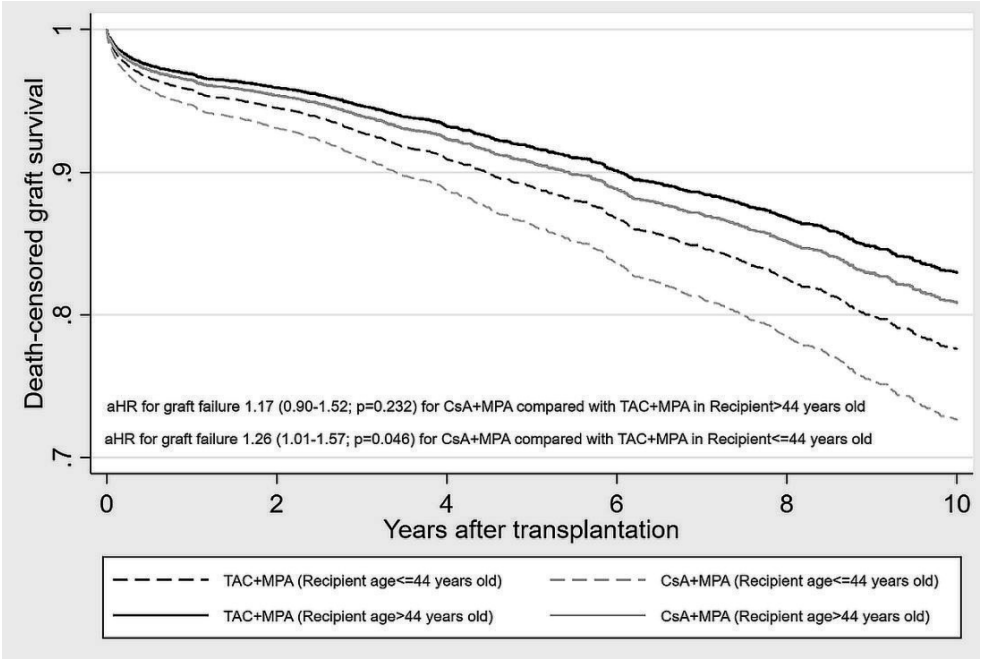

Figure 1: Adjusted death-censored graft survival function by Cox regression (adjustment made for recipient and donor age, donor anti-HCV status, peak PRA, type of donors, cold ischemic time, and prednisolone use). aHR; adjusted hazard ratio, TAC; tacrolimus, CsA; cyclosporine A, MPA; mycophenolate

Survival of recipients younger than 44 years was not affected by CNI used (aHR 1.20, 95%CI 0.82-1.77, for CsA compared with tacrolimus in an MPA-based regimen) (Figure 2). However, survival of older recipients (>44 years) who received CsA with MPA was significantly inferior to those received tacrolimus with MPA (aHR 1.44, 95%CI 1.09-1.91).

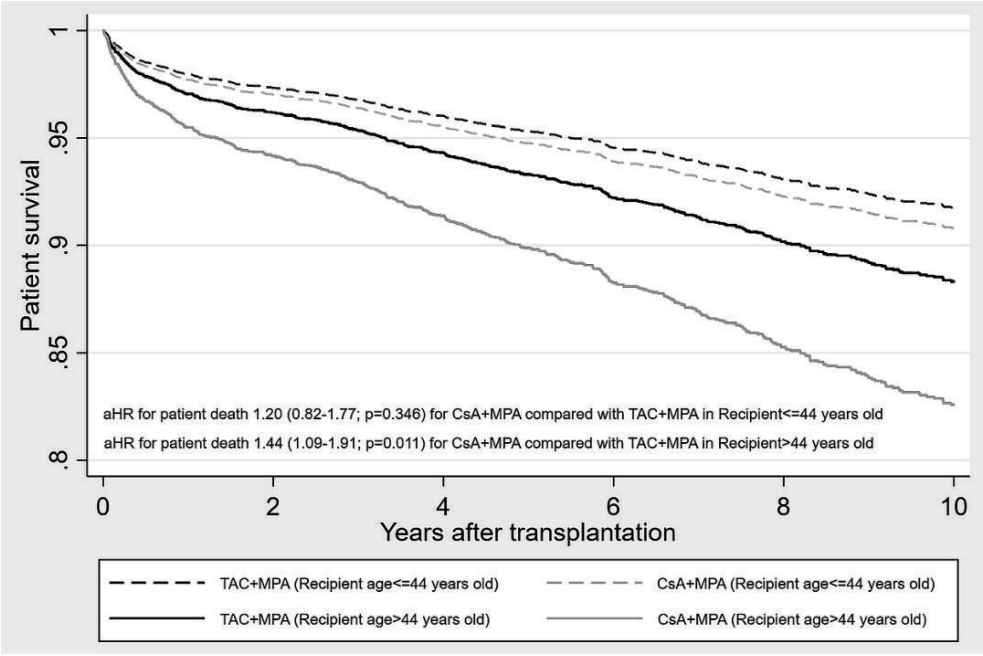

Figure 2: Adjusted patient survival function by Cox regression (adjustment made for recipient and donor age, causes of ESRD, recipient cardiovascular morbidities, type of donors, donor best creatinine, and prednisolone use). aHR; adjusted hazard ratio, TAC; tacrolimus, CsA; cyclosporine A, MPA; mycophenolate

DISCUSSIONS

Our study provides the first prediction score for long-term graft and patient survival after kidney transplants in an Asian population. Our analysis identified factors that significantly contributed to graft and patient survival, and these were used to create the models (Table 2 and 3). The predictive power of our models was better than the model from United States (Table 4), which emphasizes the importance of developing models to account for differences in patient characteristics in cohorts of different ethnicities.

The long-term outcomes of kidney transplantation in an Asian population show more favorable outcomes than those predominantly comprised of Caucasians.[28, 29] The 5-year death-censored graft survival and patient survival in our registry were 87.9% and 92.2%, respectively, which were comparable with estimates from the Japanese registry.[16] Living donor kidney transplantation was performed in approximately 50% of transplants in Thailand, which was higher than the rate of living donation in US (around 25%)[13] and Europe (around 30%),[14] although much lower than living donation rates in Japan (around 90%).[16] Our data

are representative of kidney transplantation in Thailand with more than 90% of transplants performed after 2000, the year which all maintenance immunosuppressive medications were already available, including tacrolimus and mycophenolate. These medications remain the backbone immunosuppressants of the current kidney transplantation era. Our maintenance immunosuppression regimen resembles that used in other parts of the world, with tacrolimus together with mycophenolate as the most used combination. However, induction therapy in Thailand is different from other countries, since very few patients received lymphocyte depleting agents (9.7%) (Table 1), compared with 70% in US.[13] The low rate of lymphocyte depleting agents used is likely due to the relatively lower immunologic risks of Thai recipients. In support of this, up to the time this registry was analyzed (April 2018), Thailand has not implemented calculated PRA (cPRA) into the organ allocation system, and our result showed that 88% of patients had 0% of PRA. In contrast, 0% CPRA was only found in about 60% of US patients.[13] Moreover, around 50-70% of US patients had 4-6 HLA mismatches,[13] but only 23% of Thai patients had the same degree of HLA mismatches (Table 1). With a higher rate of low immunologic risk transplantation in Thai recipients compared with US recipients, lower numbers of Thai transplants received lymphocyte depleting agents.

Our model provides estimates of graft and patient survival in the long-term period immediately post-transplant. We included maintenance immunosuppressive medications into the models, since these medications are usually decided preoperatively and started before or at the time of transplantation.[40] After transplantation, these maintenance medications are usually continued throughout follow up. Tacrolimus is the main CNI used in Thailand (Table 1). However, CsA is also prescribed de novo, and has a role in some patients, especially those with poorly controlled diabetes. In addition, 1 mg of tacrolimus costs around 4.1 US dollars, while 1.3 US dollars for 25 mg of CsA might be considered a more economical choice for Thai diabetes patients. Although some studies have demonstrated that tacrolimus is superior in terms of graft function and graft survival compared to CsA,[41, 42] heterogeneity of effect sizes was found in a Cochrane Collaboration meta-analysis comparing these outcomes between tacrolimus and CsA transplant recipients.[10] Tacrolimus showed less graft loss at 6 months post-transplant, but the same rate of graft loss at 1 and 5 years, compared with CsA. In many recent studies, CsA had been shown to increase the acute rejection rate compared with tacrolimus,[35, 37, 38, 43] but with the same patient and graft survival.[35-37, 43, 44] Differences in these outcomes could be due to differences in target CsA concentrations, timing of therapeutic drug monitoring, formulations of CsA used, concurrent immunosuppressive medications, and the studied population.[37, 45, 46] In our study, the effects of CsA and tacrolimus when combined in a MPA-based regimen were not consistently different in all subgroups. Younger patients maintained with CsA+MPA were at a significantly higher risk for death-censored graft failure (aHR 1.26, p=0.046), but in older recipients this risk was slightly reduced and not significant-

ly different (aHR 1.17, p=0.232) (Figure 1). This might be partially explained by a study that showed higher prevalence of biopsy-proven CNI nephrotoxicity from CsA compared with tacrolimus.[34] Therefore, older recipients would have a relatively shorter period of allograft and lower cumulative CNI exposure, but the younger recipients would be more likely to have higher cumulative CNI exposure thus resulting in a higher incidence of CNI nephrotoxicity amongst CsA users. Moreover, young recipients are known to be at risk for acute rejection,[40] and the relatively lower immunosuppression by CsA might increase the risk of rejection and shorten graft survival.

Patient survival in our study was not different in recipients younger than 44 years old who received CsA versus tacrolimus in MPA-based regimen (aHR 1.20, p=0.346) (Figure 2). On the contrary, recipients older than 44 years old who received CsA+MPA were at higher risk for death (aHR 1.44, p=0.011). The different medication-related adverse effects between CsA and tacrolimus might contribute to the death rates in older patients, who are less tolerant to drug toxicities. Although some CNI-associated systemic toxicities, such as bone pain syndrome,[47] posterior reversible encephalopathy syndrome (PRES),[48] and thrombotic microangiopathy,[49] can occur with both type of CNIs, the incidence of hyperlipidemia, bone fractures, hypertension, and cardiovascular events is higher amongst those receiving CsA.[35, 44, 50] This must be considered along with the higher incidence of post-transplant diabetes mellitus (PTDM) in recipients receiving tacrolimus.[51] However, previous studies have shown CsA is associated with higher cardiovascular risk profile,[52] major cardiovascular events,[35, 53] and cardiotoxicities[54] compared with tacrolimus.

Our study has several strengths. This is the first predictive scoring system for long-term outcomes of kidney transplantation in Asians, which has fair to good predictive power for patient and graft survival. Our models in general should not be directly applied by using the coefficients from Cox regression models based on different datasets. We have compared our model with another recent model developed from transplant recipients of different ethnicities and our model was better at discriminating outcomes, underlining the clinical importance of developing clinical tools based on racially distinct groups. Differences in patient characteristics, type of kidney donors, and immunosuppressive regimens between Thailand and other countries are factors which likely contribute to transplantation outcomes. Nevertheless, our study also has some limitations. First, we have not tested our models in an external Asian cohort, which should ideally be performed in another Indo-Asian or East-Asian ethnicity group comparable to Thailand. Second, type of insurance is used for prediction in the US equation, but was not available in our dataset, which may have contributed to the lower discriminative of these equations in our cohort. However, most transplant related expenses in Thailand are now covered by the Universal Healthcare System, mitigating the impact of this limitation. Third, the adjusted HR for patient death and graft failure according

to the immunosuppression regimens should be interpreted cautiously (Figure 1 and Figure 2), since the aHRs for different immunosuppressive regimens are very similar. We applied the adjusted Cox regression model to reduce potential bias and confounding, and clinician should weigh medications adverse effect profile and long-term outcomes in each individual patient. Further research to improve the predictive power of the models must include all related factors that contribute to graft and patient outcomes. These factors, including changes in immunosuppressants and socioeconomic status, should be included dynamically by using artificial intelligence or machine learning, and should be generally accessible. More importantly, pharmacogenomics would most likely improve the precision of the models. By including CYP3A5 genotype which affects CNIs metabolism[55] and is expressed differently in different ethnic groups,[56] we might able to predict nephrotoxicity and rejection episodes.

The model calibration for patient survival showed the expected deaths from our equation tended to be higher than the actual events at 5 years, particularly in patients with risk scores higher than the 50th percentile, which is around 8% at 5 years (Supplementary Table S5 and Supplementary Figure S2). This model overestimation needs to be carefully interpreted when discussing implications in patients with these elevated risks. However, patient survival still fitted with the actual events at 10 years (Supplementary Figure S2). Moreover, these high-risk patients might receive benefit from more intensive care and surveillance to help modify this predicted risk.

In conclusion, the first Asian-based kidney transplantation model for long-term outcomes was developed and can be accessed at www.nephrochula.com/ktmodels. This model will be a useful tool for physicians to evaluate 5- and 10-years PS and DCGS, and provide more intensive follow-up to the high-risk patients. Further external validation on the applicability of this model to other Asian populations is required.

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SUPPLEMENTARY DATA

Supplementary Table S1: Missing values and multiple imputation method.

Variables	Missing data (%)
Cold ischemic time	8.1
Recipient HBsAg	11.1
Recipient Anti-HCV	13.3
Dialysis vintage	25.0
Peak PRA	6.4
Recipient cardiovascular comorbidities	24.4
Type of donor	12.4
Donor HBsAg	11.2
Donor Anti-HCV	12.7
Donor best serum creatinine	6.1
Multiple imputation was done separately in the training and validation dataset. We assumed that data was missing at random. We used sequential imputation by chained equations with 20 imputations added. Continuous covariates were imputed using linear models and logistic models were used for categorical predictors. Imputation integrity was tested by examining the imputed value distributions and patterns. The independent variables consisted of recipient and donor age, recipient and donor sex, causes of kidney disease, graft and patient survival outcomes, censoring indicator, and survival time.	

Supplementary Table S2: Predictive equations derived from Thai Transplant Registry.

Outcomes	Equations
Death-censored graft survival	((-.6657178*Tacrolimus "YES" 1 "NO" 0)+(-.4895407*CsA "YES" 1 "NO" 0)+(-.2467738*MPA "YES" 1 "NO" 0)+(-.9526094*Prednisolone "YES" 1 "NO" 0)+(.0208071*Cold ischemic time (hours))+(-.0204749*Recipient age (years))+(.0043734* Peak Panel Reactive Antibody (percent))+(.0157974*Donor age (years))+(.0373527*Traumatic head injury donor "YES" 1 "NO" 0)+(.519852*CVA donor "YES" 1 "NO" 0)+(.5717543*Donor Anti-HCV positive "YES" 1 "NO" 0))+1.6174455
5-year graft loss = 1 - (0.8786^exp (Death-censored graft survival)) 10-year graft loss = 1 - (0.7551^exp (Death-censored graft survival))	
Patient survival	((-.8033568*Tacrolimus "YES" 1 "NO" 0)+(-.5426932*CsA "YES" 1 "NO" 0)+(-.4974057*MPA "YES" 1 "NO" 0)+(-.8264266*Prednisolone "YES" 1 "NO" 0)+(.0186543*Recipient age (years))+(.4308922*Diabetic kidney disease "YES" 1 "NO" 0)+(.2998744*Other causes of ESRD "YES" 1 "NO" 0)+(.3542467*Undefined cause of ESRD "YES" 1 "NO" 0)+(.4287579* Recipient CVD "YES" 1 "NO" 0)+(.5565913*Traumatic head injury donor "YES" 1 "NO" 0)+(.3047587*CVA donor "YES" 1 "NO" 0)+(.1698932*Donor best serum creatinine (mg/dl)))+.22735532
5-year patient death = 1 - (0.9210^exp (Patient survival) 10-year patient death = 1 - (0.8609^exp (Patient survival)	

Supplementary Table S3: Model calibration for 5-year death-censored graft survival.

Decile risk groups	N	Observed event	Expected event	Observed/Expected event ratio
1 (lowest risk)	245	5	13.7	0.36
2	252	24	18.7	1.28
3	227	15	19.4	0.77
4	254	15	24.3	0.62
5	229	25	24.8	1.01
6	227	22	27.8	0.79
7	226	27	31.2	0.87
8	229	44	37.4	1.18
9	206	36	41.4	0.87
10 (highest risk)	237	64	84.0	0.76
Total	**2,332**	**277**	**322.7**	**0.86**
p-value=0.466				

Supplementary Table S4: Model calibration for 10-year death-censored graft survival.

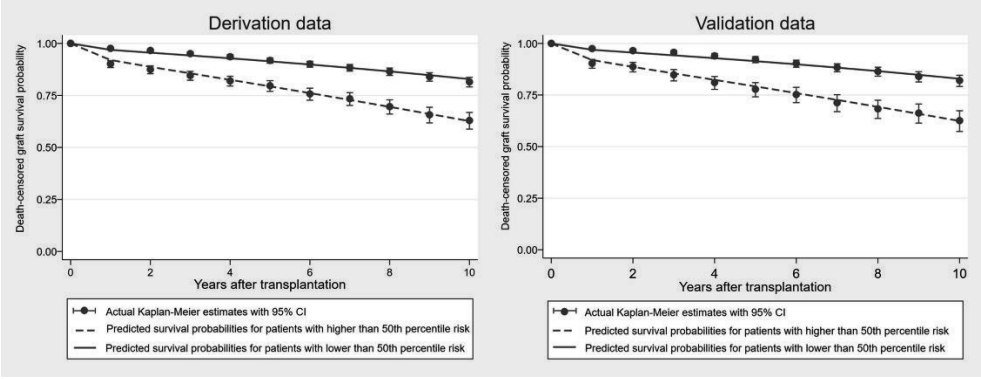
Decile risk groups	N	Observed event	Expected event	Observed/Expected event ratio
1 (lowest risk)	245	13	29.6	0.44
2	252	42	39.9	1.05
3	227	39	41.0	0.95
4	254	45	51.0	0.88
5	229	57	51.7	1.10
6	227	45	57.3	0.79
7	226	50	63.7	0.78
8	229	72	75.1	0.96
9	206	82	81.1	1.01
10 (highest risk)	237	96	142.8	0.67
Total	**2,332**	**541**	**633.1**	**0.85**
p-value=0.182				

Supplementary Table S5: Model calibration for 5-year patient survival.

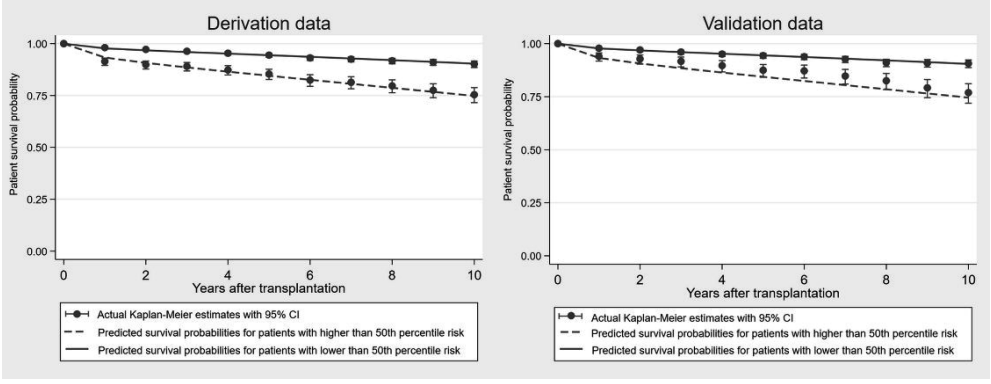
Decile risk groups	N	Observed event	Expected event	Observed/Expected event ratio
1 (lowest risk)	241	6	8.0	0.75
2	235	5	10.6	0.47
3	240	14	13.2	1.06
4	217	19	14.1	1.35
5	218	22	16.4	1.34
6	244	13	21.1	0.62
7	237	18	23.5	0.77
8	229	15	26.9	0.56
9	247	22	36.8	0.60
10 (highest risk)	224	33	62.2	0.53
Total	**2,332**	**166**	**232.7**	**0.71**
p-value=0.252				

Supplementary Table S6: Model calibration for 10-year patient survival.

Decile risk groups	N	Observed event	Expected event	Observed/Expected event ratio
1 (lowest risk)	241	15	14.4	1.04
2	235	10	19.0	0.53
3	240	18	23.5	0.77
4	217	24	25.0	0.96
5	218	24	28.9	0.83
6	244	25	37.0	0.68
7	237	33	41.1	0.80
8	229	28	46.6	0.60
9	247	49	62.9	0.78
10 (highest risk)	224	62	97.9	0.63
Total	**2,332**	**288**	**396.5**	**0.73**
p-value=0.851				



Supplementary Figure S1: Calibration of Cox model for death-censored graft survival in the derivation and validation datasets. The survival probabilities are categorized into the higher than 50th percentile risk group, and the lower than 50th percentile risk group.



Supplementary Figure S2: Calibration of Cox model for patient survival in the derivation and validation datasets. The survival probabilities are categorized into the higher than 50th percentile risk group, and the lower than 50th percentile risk group.

Chapter 7

Immune Subsets from Ficoll Density Gradient Separation in Kidney Transplant Recipients

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Many studies in transplantation have used peripheral blood mononuclear cells (PBMCs) to assess immunological risk and to unravel the mechanisms of rejection and tolerance.[1-3] Examples include functional T and B lymphocyte studies by ELISPOT,[1, 4, 5] T lymphocyte proliferation studies,[2] gene expression profiling,[3] and intracellular tacrolimus concentration measurements.[6] However, the PBMC fraction consists of various leukocyte subpopulations, including T-, B-, and NK lymphocytes, and monocytes, with potential contamination by granulocytes. By using Ficoll density gradient separation to isolate the PBMCs, the proportion of low-density granulocytes is increased in inflammatory conditions such as sepsis,[7] burn wounds,[8] or in autoimmune disease.[9] The biological validity of these studies could therefore be affected if based on faulty methodological assumptions.[7, 10]

Surprisingly, no studies report on whether the proportion of PBMC subpopulations from transplant recipients differ from healthy controls, or if rejection affects cellular PBMC composition. We compared the proportions of PBMC subsets in kidney transplant recipients during a biopsy-proven acute T cell-mediated rejection (TCMR) episode (n=11) with those from kidney transplant recipients without acute rejection (n=12) and healthy controls (kidney transplant donors before transplantation) (n=10). All kidney transplant recipients received tacrolimus (targeted pre-dose concentration 7-14 ng/mL), mycophenolate mofetil (fixed-dose 2 g/day), and prednisolone (total daily dose 5-20 mg/day). PBMCs were obtained from heparinized blood using the standard Ficoll density gradient procedure (Ficoll-Paque, GE Healthcare, Uppsala, Sweden), and were frozen in RPMI-1640 with glutamax (Life Technologies/Gibco BRL, Paisley, Scotland) with 15% heat-inactivated human serum and 10% dimethyl sulfoxide (Merck, Darmstadt, Germany).[11] PBMCs were stored in liquid nitrogen at −190 °C until use.[1, 12] All participants gave written informed consent. The study was performed in accordance with the declaration of Helsinki (2013) and approved by the institutional review board of the Erasmus MC (No. 2018-035).[13]

PBMCs were thawed using RPMI medium with DNase (DNase I, Roche, Germany) and stained with anti-CD3-BV510, anti-CD19-PE-Cy7, anti-CD16-PE, anti-CD56-PerCP, anti-CD45-APC, anti-CD15-BV421, and anti-CD14-FITC. All antibodies were from Biolegend (San Diego, CA) except anti-CD14 (BD Bioscience, San Jose, CA). PBMC subsets were measured on a FACS Canto II flow cytometer (BD Biosciences). Flow cytometric data was analyzed by Kaluza Analysis Software version 2.1 (Beckman Coulter Life Sciences, Indianapolis, IN).

Figure 1 illustrates the gating strategy and Table 1 summarizes the composition of the PBMCs from the patients and healthy controls. Overall, T lymphocytes predominated in the PBMC fraction (mean: 52.4±12.9%), followed by monocytes (28.3±12.3%) and B lymphocytes (9.1±5.2%). There was no significant difference between kidney transplant re-

cipients (with or without acute TCMR) and healthy controls regarding the proportion of T-, B-, and NK lymphocytes, monocytes, and granulocytes in PBMCs after the Ficoll procedure.

In contrast to previous studies that showed a significant increase in the proportion of granulocytes in patients suffering from inflammation compared with controls,[7, 10] there was no statistically significant difference in the cellular make-up of the PBMC fraction of kidney transplant recipients with acute TCMR compared to non-rejecting kidney transplant recipients. The results in our study show that acute rejection might therefore be considered as a relatively "milder" state of inflammation in the peripheral blood compartment compared with septicemia or other systemic inflammatory diseases.[8-10]

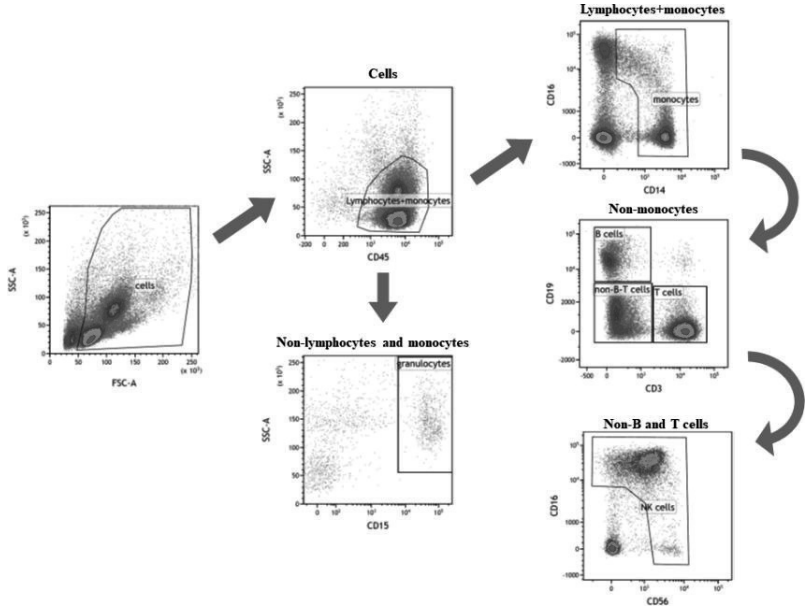

Figure 1: Representative data of the flowcytometric analysis.

Table 1: PBMC subsets in healthy controls and kidney transplant recipients with and without acute T cell-mediated rejection.

Variable	All subjects	Healthy controls	Recipients without rejection	Recipients with acute TCMR	P-value for non-rejection recipients vs. acute TCMR recipients	P-value for healthy controls vs. acute TCMR recipients	P-value across all 3 groups
Number of subjects	33	10	12	11	-	-	-
Time after transplantation, days (median, Q1-Q3)	-	-	19.5 (9.5-48.0)	12.5 (7.0-42.5)	0.38	-	-
Age, years (mean ± SD)	54.5 ± 11.3	53.4 ± 7.1	56.0 ± 13.4	54.0 ± 12.7	0.63	0.96	0.80
Male, n (%)	25 (76%)	7 (70%)	9 (75%)	9 (82%)	0.69	0.40	0.82
T lymphocytes, % (mean ± SD)	52.4 ± 12.9	55.4 ± 12.6	52.6 ± 13.3	49.4 ± 13.3	0.57	0.30	0.58
Monocytes, % (mean ± SD)	28.3 ± 12.3	24.1 ± 9.0	28.3 ± 11.4	32.0 ± 15.2	0.51	0.17	0.34
B lymphocytes, % (mean ± SD)	9.1 ± 5.2	7.0 ± 2.2	10.6 ± 6.0	9.3 ± 6.1	0.62	0.26	0.27
NK lymphocytes, % (mean ± SD)	7.7 ± 5.3	10.8 ± 5.5	7.2 ± 4.4	5.5 ± 5.3	0.42	0.09	0.06
Granulocytes, % (mean ± SD)	2.6 ± 4.4	2.7 ± 5.0	1.4 ± 2.0	3.8 ± 5.8	0.20	0.67	0.46

SD; standard deviation, TCMR; T cell-mediated rejection

Interestingly, a trend toward a lower proportion of NK lymphocytes in the PBMCs fraction of recipients with acute TCMR was observed. Previous studies have shown a significantly higher number of infiltrating NK lymphocytes in the kidney allografts with TCMR compared to kidneys affected by antibody-mediated rejection or kidneys without signs of rejection.[14-16] Moreover, the number of infiltrating NK lymphocytes was positively correlated with a higher Banff TCMR grade.[14] The present findings suggest that NK lymphocytes migrate from the periphery to the transplanted kidney. Since NK lymphocyte make up the smallest PBMC sub-fraction, the infiltration of the kidney transplant may have an observable effect in the peripheral compartment. However, further studies are needed to confirm this hypothesis.

Our study has limitations. First, the sample size was relatively small and might be underpowered to detect significant differences, although most of the measured immune cell subsets (except NK lymphocytes) show a large overlap in their proportion of PBMCs across all groups. Second, this study was conducted in kidney transplant recipients, and the results might be different in recipients of a non-renal organ transplant.

In conclusion, our study shows that PBMC fractions of immunosuppressed kidney transplant recipients, with or without rejection, are not noticeably influenced by the Ficoll separation procedure. These results may be useful for future immune monitoring studies of transplant recipients involving human PBMCs.

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Chapter 8

Association between the Intracellular Tacrolimus Concentration in $CD3^+$ T Lymphocytes and $CD14^+$ Monocytes and Acute Kidney Transplant Rejection

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Ther Drug Monit. 2022 March 21. In press

ABSTRACT

Background

Intracellular tacrolimus concentration in peripheral blood mononuclear cells (PBMCs) ($TAC_{[PBMC]}$) has been proposed to better represent its active concentration than its whole blood concentration. As tacrolimus acts on T lymphocytes and other white blood cells, including monocytes, we investigated the association of tacrolimus concentration in $CD3^+$ T lymphocytes ($TAC_{[CD3]}$) and $CD14^+$ monocytes ($TAC_{[CD14]}$) with acute rejection after kidney transplantation.

Methods

From a total of 61 samples in this case-control study, 28 samples were obtained during biopsy-proven acute rejection (rejection group), and 33 samples were obtained in the absence of rejection (control group). PBMCs were collected from both cryopreserved (retrospectively) and freshly obtained (prospectively) samples. $CD3^+$ T lymphocytes and $CD14^+$ monocytes were isolated from PBMCs, and their intracellular tacrolimus concentrations were measured.

Results

The correlation between tacrolimus whole-blood and intracellular concentrations was poor. $TAC_{[CD3]}$ was significantly lower than $TAC_{[CD14]}$ (median 12.8 *vs* 81.6 pg/million cells; $p<0.001$). No difference in $TAC_{[PBMC]}$ (48.5 *vs* 44.4 pg/million cells; $p=0.82$), $TAC_{[CD3]}$ (13.4 *vs* 12.5 pg/million cells; $p=0.28$), and $TAC_{[CD14]}$ (90.0 *vs* 72.8 pg/million cells; $p=0.27$) was found between the rejection and control groups. However, freshly isolated PBMCs showed significantly higher $TAC_{[PBMC]}$ than PBMCs from cryopreserved samples. Subgroup analysis of intracellular tacrolimus concentrations from freshly isolated cells did not show a difference between rejectors and nonrejectors.

Conclusions

Differences in $TAC_{[CD3]}$ and $TAC_{[CD14]}$ between patients with and without rejection could not be demonstrated. However, further optimization of the cell isolation process is required because a difference in $TAC_{[PBMC]}$ between fresh and cryopreserved cells was observed. These results need to be confirmed in a study with a larger number of patients.

INTRODUCTION

After kidney transplantation, tacrolimus treatment is routinely monitored by measuring whole blood pre-dose concentrations (C_0).[1-3] However, some kidney transplant recipients experience rejection despite adequate exposure to tacrolimus, suggesting that whole-blood C_0 does not fully reflect its immunosuppressive effect.[4]

Tacrolimus acts by inhibiting the enzyme calcineurin within peripheral blood mononuclear cells (PBMCs).[5] Recently, intracellular tacrolimus concentration in PBMCs has gained considerable interest, as it may correlate better with clinical outcomes after transplantation than whole blood tacrolimus concentration. Capron *et al*.[6] were the first to show that intracellular tacrolimus concentration in PBMCs was significantly associated with the risk of acute liver transplant rejection, whereas whole blood tacrolimus concentration was not. However, subsequent studies in both liver and kidney transplant recipients have not replicate these observations.[7-10] Apart from technical issues and differences in study design, this may be explained by the fact that the PBMC fraction consists of many different cell types, including $CD3^+$ T lymphocytes, $CD14^+$ monocytes, B lymphocytes, natural killer (NK) cells, and dendritic cells, all of which play different roles in allorecognition and rejection.[11-15]

The aim of the present study was to investigate the association between the occurrence of acute kidney transplant rejection and intracellular tacrolimus concentration in PBMCs, $CD3^+$ T lymphocytes, and $CD14^+$ monocytes. This is the first study in which intracellular tacrolimus concentration was measured in mononuclear cell subsets of kidney transplant recipients and correlated with acute rejection. In addition, some of the analytical and methodological limitations of previous studies were overcome, including the use of red blood cell (RBC) lysis buffer to eliminate contamination by erythrocytes, the effect of freezing on intracellular tacrolimus concentration in PBMCs was investigated, and a larger cohort of recipients was included and sampled on the day of a kidney transplant biopsy.

METHODS

Study population and endpoints

This was a case-control study. All patients participated in the LIBIDAR *(LIquid BIopsies for Minimally Invasive Detection of Acute Rejection*) study, which aimed to identify minimally invasive biomarkers for the diagnosis of acute kidney transplant rejection. In the LIBIDAR study, PBMCs and serum were collected from patients who underwent a for-cause kidney allograft biopsy. All patients in the present study received the same immunosuppressive therapy consisting of basiliximab induction therapy and maintenance treatment with

tacrolimus, mycophenolic acid (MPA), and prednisolone.

The primary aim of the present study was to examine the association between acute rejection and intracellular tacrolimus concentration in PBMCs ($TAC_{[PBMC]}$), $CD3^+$ T lymphocytes ($TAC_{[CD3]}$), and $CD14^+$ monocytes ($TAC_{[CD14]}$). Patients with biopsy-proven acute T-cell-mediated rejection (aTCMR; the "cases") were selected from the study database. Patients who underwent a for-cause biopsy and did not present rejection were selected as controls. Kidney transplant biopsies were classified by an experienced nephropathologist (M.C.C.-v.G.) according to the Banff 2019 classification.[16] Patients with biopsy findings showing borderline TCMR, antibody-mediated rejection (ABMR), or findings suggestive of ABMR (such as thrombotic microangiopathy or microvascular inflammation only [*i.e.,* without positive C4d staining and negative testing for donor-specific anti-HLA antibodies (DSA)]) were not included. An additional inclusion criterion was the availability of PBMCs to allow the measurement of intracellular tacrolimus. As the present study was initiated while the LIBIDAR study was ongoing, we included both cryopreserved PBMCs (from the patients already enrolled) and freshly isolated PBMCs (from the prospectively enrolled patients) from patients participating in the LIBIDAR study for the measurement of intracellular tacrolimus concentrations.

Cell isolation for intracellular tacrolimus concentration measurement

PBMCs were collected in the morning for a for-cause kidney transplant biopsy. PBMCs were isolated from heparinized blood (at the same time as whole blood C_0 sampling) using a standard Ficoll separation procedure at 25°Con the same day as the blood sampling. PBMCs were washed twice with 10 mL of phosphate-buffered saline (PBS) before 1 mL of RBC lysis buffer (eBioscience, Affymetrix, San Diego) was added, followed by incubation for 10 min at 25°C.[17] PBMCs were counted using a Sysmex XOP-300 cell counter (Sysmex, Lincolnshire, IL). The Sysmex XP-300 cell counter measures white blood cells in an analysis range of 1.0-99.9 × 10^3/μL with a variation coefficient of 3.5% or less.[18] Previously, we have determined the cell content and purity of Ficoll-separated PBMC isolates. Flow cytometric analysis of samples from healthy controls showed that the vast majority of cells consisted of mononuclear cells (88-100%), with lymphocytes accounting for >80%.[19] After PBMCs were isolated, 2 × 10^6 PBMCs were resuspended in 50 μL of PBS, snap-frozen as a cell pellet in liquid nitrogen, and stored at -80 °C until measurement of intracellular tacrolimus concentration.

For samples from patients who had already been enrolled in the LIBIDAR study before the beginning of the present study, cryopreserved PBMCs were thawed from -80 °C using DNase (DNase I; Roche, Germany) in Roswell Park Memorial Institute (RPMI) medium (RPMI 1640 medium without L-glutamine; Lonza, USA) and treated with RBC lysis

buffer at 25°C for 10 min. Two million PBMCs were then made into cell pellets and stored for measurement of intracellular tacrolimus concentration in the same way as fresh samples from prospectively enrolled patients. The only difference between cryopreserved and fresh PBMCs was the additional thawing and washing steps in the former.

For measurement of tacrolimus concentration in $CD3^+$ T lymphocytes and $CD14^+$ monocytes, negative selection using magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with MACS buffer was performed at 4 °C. The Pan T cell isolation kit (130-096-535; Miltenyi Biotec) and Pan monocyte isolation kit (130-096-537; Miltenyi Biotec) were used to isolate pure $CD3^+$ T lymphocytes and $CD14^+$ monocytes. The purity of the cells was checked after MACS separation by flow cytometry by staining with anti-human CD3-BV510 (317332; BioLegend, San Diego, CA), anti-human CD14-APC-H7 (560180; BD Biosciences, Franklin Lakes, NJ), anti-human CD45-APC (368512; BioLegend), and anti-human CD16-PE (302008; BioLegend). Cells were used for further analysis only if their purity was ≥90%. $CD3^+$ T lymphocytes and $CD14^+$ monocytes (2×10^6 cells each, counted with a Sysmex XOP-300 cell counter and then manually using a Neubauer counting chamber with trypan blue for confirmation) were resuspended in 50 µL of PBS and then sent for analysis of intracellular tacrolimus concentration.

Tacrolimus concentration measurement

A validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was used to evaluate whole blood and intracellular tacrolimus concentrations. Details regarding this assay have been previously published and discussed previously.[17] In brief, the MagSIMUS-TDM^{prep} kit from MagnaMedics containing MagSIMUS-TDM^{prep} Type I Particle Mix Beads and buffers was used (MagnaMedics Diagnosis B.V., Geleen, the Netherlands). The measured concentration was expressed as µg/L. The range of detection was 0.1–25 µg/L, with an inaccuracy of <5% and imprecision of <15%.[17] Intracellular tacrolimus concentration was then calculated and reported as pg/million cells (range: 5-1,250 pg/million cells).

Statistical analyses

Continuous variables were reported as mean ± standard deviation (SD) or median (first–third quartile) for normally and non-normally distributed data, respectively. Categorical variables were presented as numbers and percentages. The correlations between intracellular tacrolimus concentration in PBMCs, $CD3^+$, and $CD14^+$ cells, and whole blood C_0 concentration were analyzed using Spearman's rank correlation coefficient (r). Unpaired *t*-tests and Wilcoxon rank-sum tests were used to evaluate differences in continuous variables between the two groups. The Wilcoxon matched-pairs signed-rank test was used to analyze

the difference in intracellular tacrolimus concentration between $CD3^+$ T lymphocytes and $CD14^+$ monocytes. All statistical analyses were performed using the Stata Statistical Software (version 17.0; StataCorp LLC, College Station, TX, USA) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA).

Ethical considerations

This study was performed in accordance with the principles of the Declaration of Helsinki (seventh revision, October 2013, approved by the 64th World Medical Association General Assembly, Fortaleza, Brazil) and the Medical Research Involving Human Subjects Act (WMO). Study procedures were performed in accordance with the ethical standards of the institutional research committee that approved the present study (Erasmus MC Medical Ethical Review Board, number 2018-035). Written informed consent was obtained from all patients prior to inclusion.

RESULTS

Baseline characteristics

A total of 53 patients were included in this study. The patient characteristics are shown in Table 1. There was no significant difference in baseline characteristics between patients with and without rejection. A total of 61 biopsies were performed in the 53 patients. In total, 46 patients underwent 1 kidney transplant biopsy, 6 underwent 2 kidney transplant biopsies, and 1 underwent 3 kidney transplant biopsies. aTCMR was diagnosed in 28 biopsies, and an alternative diagnosis was made in 33 biopsies. The histological findings of the 61 biopsies are presented in Supplementary Table S1.

Table 1: Patient characteristics

Variable	Total patients = 53
Age, years (mean ± SD)	61.3 ± 12.4
Male, n (%)	32 (60%)
Caucasian, n (%)	34 (76%)
Cause of ESRD, n (%)	
Diabetic nephropathy	12 (23%)
Hypertensive nephropathy	10 (19%)
Glomerulonephritis	16 (30%)
Polycystic kidney disease	2 (4%)
Other	10 (19%)
Unknown	3 (5%)
Mode of renal replacement therapy, n (%)	
Hemodialysis	29 (54%)
Peritoneal dialysis	11 (21%)
Preemptive transplantation	13 (25%)
First kidney transplantation, n (%)	49 (92%)
Living-donor kidney transplantation, n (%)	22 (42%)
HLA mismatch (mean ± SD)	3.7 ± 1.5
PRA at the time of transplantation, % (median, Q1-Q3)	0 (0-4)
Time from transplantation to kidney biopsy, days (median, Q1-Q3)	9 (6-22)

ESRD, end-stage renal disease; HLA, human leukocyte antigen; PRA, panel reactive antibody

Correlation between tacrolimus concentration in whole blood, PBMCs, $CD3^+$, and $CD14^+$ cells

$TAC_{[PBMC]}$, $TAC_{[CD3]}$, and $TAC_{[CD14]}$ values are shown in Figure 1. $CD14^+$ monocytes had a significantly higher intracellular tacrolimus concentration than $CD3^+$ T lymphocytes (median 81.6 (60.3-120.8) *vs*. 12.8 (9.6-16.5) pg/million cells; $p < 0.001$). $TAC_{[CD3]}$ was significantly lower than $TAC_{[PBMC]}$ (12.8 (9.6-16.5) *vs*. 48.5 (32.6-86.1 pg/million cells; $p < 0.001$).

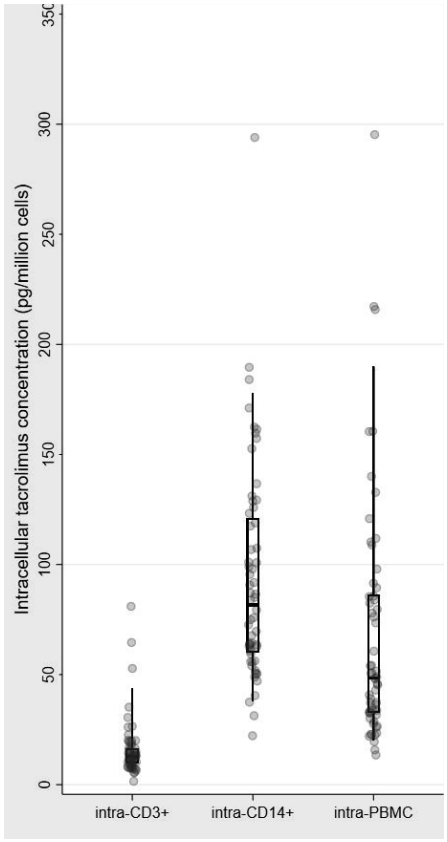

Figure 1: Comparison of intracellular tacrolimus concentration (n = 61) between $CD3^+$ T lymphocytes, $CD14^+$ monocytes, and PBMCs. The boxes describe the median with Q1and Q3, whereas the whiskers represent 95% CI.

Table 2 and Supplementary Figure S1 show the correlations between tacrolimus concentrations in different cell fractions. A poor correlation was found between whole blood tacrolimus concentration and intracellular tacrolimus concentration in PBMCs, $CD3^+$ T lymphocytes, and $CD14^+$ monocytes. $TAC_{[CD3]}$ and $TAC_{[CD14]}$ were moderately correlated with $TAC_{[PBMC]}$ ($r = 0.59$; $p < 0.001$ and $r = 0.67$; $p < 0.001$, respectively). The correlation between $TAC_{[CD3]}$ and $TAC_{[CD14]}$ was 0.64 ($p < 0.001$).

Table 2: Correlations between tacrolimus concentrations measured in different compartments.

Tacrolimus concentration	Whole blood	$TAC_{[PBMC]}$	$TAC_{[CD3]}$	$TAC_{[CD14]}$
Whole blood	1.00			
$TAC_{[PBMC]}$	0.27*	1.00		
$TAC_{[CD3]}$	0.43**	0.59**	1.00	
$TAC_{[CD14]}$	0.58**	0.67**	0.64**	1.00

Values represent Spearman's rank correlation coefficient.

$TAC_{[CD3]}$, intracellular tacrolimus concentration in $CD3^+$ T lymphocytes; $TAC_{[CD14]}$, intracellular tacrolimus concentration in $CD14^+$ monocytes; $TAC_{[PBMC]}$, intracellular tacrolimus concentration in peripheral blood mononuclear cells

* $p < 0.05$, ** $p < 0.001$

Association between intracellular tacrolimus concentration and acute rejection

Table 3 displays the comparison of tacrolimus concentrations in whole blood, PBMCs, $CD3^+$ T lymphocytes, and $CD14^+$ monocytes between patients with biopsy-proven acute rejection and those without. Whole blood tacrolimus concentration was not different between patients with and without rejection: 9.7 ± 3.5 *vs*. 9.2 ± 3.0 ng/mL; p = 0.51. In addition, $TAC_{[PBMC]}$ (48.5 (33.3-86.8) *vs*. 44.4 (32.3-83.1) pg/million cells; p = 0.78), $TAC_{[CD3]}$ (13.4 (10.0-18.2) *vs*. 12.5 (8.3-14.8) pg/million cells; p = 0.28), and $TAC_{[CD14]}$ (90.0 (62.5-136.3) *vs*. 72.8 (57.3-106.5) pg/million cells; p = 0.27) were not significantly different between these two groups. After normalization of intracellular tacrolimus concentration by the tacrolimus daily dose (for dose-corrected tacrolimus exposure) or the whole blood concentration, there were still no significant differences between patients with acute rejection and those with alternative biopsy findings (Table 3).

Table 3: Tacrolimus concentration from patients in the rejection and non-rejection groups.

Tacrolimus concentration	Non-rejection group (n = 33)	Rejection group (n = 28)	p-value
Whole blood pre-dose concentration, ng/mL (mean ± SD)	9.2±3.0	9.7±3.5	0.51
$TAC_{[PBMC]}$, pg/million cells (median, Q1-Q3)	44.4 (32.3-83.1)	48.5 (33.3-86.8)	0.78
$TAC_{[CD3]}$, pg/million cells (median, Q1-Q3)	12.5 (8.3-14.8)	13.4 (10.0-18.2)	0.28
$TAC_{[CD14]}$, pg/million cells (median, Q1-Q3)	72.8 (57.3-106.5)	90.0 (62.5-136.3)	0.27
$TAC_{[PBMC]}$ / whole blood concentration ratio (median, Q1-Q3)	5.7 (3.3-8.4)	6.2 (3.9-7.3)	0.96
$TAC_{[CD3]}$ / whole blood concentration ratio (median, Q1-Q3)	1.2 (1.0-1.8)	1.4 (1.1-1.8)	0.39
$TAC_{[CD14]}$ / whole blood concentration ratio (median, Q1-Q3)	9.3 (6.9-11.5)	8.7 (7.2-13.7)	0.62
$TAC_{[PBMC]}$ / tacrolimus daily dose ratio (median, Q1-Q3)	6.2 (3.2-10.4)	5.0 (2.5-9.7)	0.34
$TAC_{[CD3]}$ / tacrolimus daily dose ratio (median, Q1-Q3)	1.6 (0.9-2.5)	1.3 (0.8-1.9)	0.44
$TAC_{[CD14]}$ / tacrolimus daily dose ratio (median, Q1-Q3)	9.2 (6.5-15.3)	8.3 (6.4-15.0)	0.54
Plasma MPA concentration, mg/L (mean ± SD)	2.8 ± 1.9	2.5 ± 1.5	0.57

MPA, mycophenolate; $TAC_{[CD3]}$; intracellular tacrolimus concentration in $CD3^+$ T lymphocytes, $TAC_{[CD14]}$; intracellular tacrolimus concentration in $CD14^+$ monocytes, $TAC_{[PBMC]}$; intracellular tacrolimus concentration in peripheral blood mononuclear cells

Differences in $TAC_{[PBMC]}$, $TAC_{[CD3]}$, and $TAC_{[CD14]}$ between cryopreserved and fresh samples

We explored whether there was any difference in intracellular tacrolimus concentration between cryopreserved samples from retrospectively enrolled patients (*i.e.*, those who already had their samples frozen before the initiation of the present study, which needed additional thawing and washing steps before measurement of intracellular tacrolimus concentration) and freshly isolated samples from prospectively enrolled patients. In our study, 37 cryopreserved samples were thawed, and 24 samples were freshly isolated. Figure 2 displays the $TAC_{[PBMC]}$, $TAC_{[CD3]}$, and $TAC_{[CD14]}$ of the cryopreserved and freshly isolated samples. The median intracellular concentration from cryopreserved PBMCs was significantly lower than that of fresh PBMCs (34.5 (28.1-47.0) *vs*. 86.1 (56.8-136.4) pg/million cells; $p < 0.001$). $TAC_{[CD3]}$ and $TAC_{[CD14]}$ in cryopreserved samples were also significantly lower than those in fresh samples (12 (8.3-13.8) *vs*. 15.8 (9.8-28.5) pg/million cells; $p = 0.01$ for $TAC_{[CD3]}$ and 64.3 (52.5-99.0) *vs*. 117.1 (74.1-151.0) pg/million cells; $p < 0.001$ for $TAC_{[CD14]}$). In addition, after normalization of intracellular tacrolimus concentrations by the whole blood C_0, all values were significantly lower in cryopreserved samples than in fresh samples, including the $TAC_{[PBMC]}$ to whole blood concentration ratio (5.2 (2.8-7.3) *vs*. 9.8 (6.7-22.0); $p < 0.001$), $TAC_{[CD3]}$ to whole blood concentration ratio (1.3 (1.0-1.6) *vs*. 2.6 (1.6-4.3); $p < 0.001$), and $TAC_{[CD14]}$ to whole blood concentration ratio (8.3 (6.6-11.6) *vs*. 11.0 (9.3-12.7); $p = 0.031$).

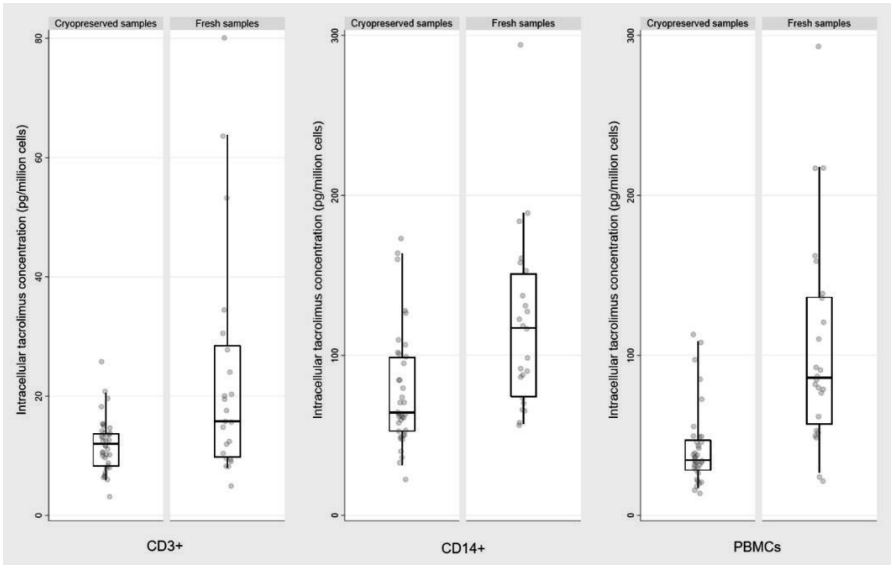

Figure 2: Intracellular tacrolimus concentration in PBMCs, $CD3^+$, and $CD14^+$ cells from cryopreserved samples (n = 37) and fresh samples (n = 24). The p-value from the Wilcoxon rank-sum test for the difference in concentration between cryopreserved and fresh samples was 0.014 for $CD3^+$ T lymphocytes and < 0.001 for both $CD14^+$ monocytes and PBMCs. The boxes describe the median with Q1and Q3, whereas the whiskers represent 95% CI.

Based on this finding, we separately analyzed the association between acute rejection and intracellular tacrolimus concentration in cryopreserved and freshly isolated samples, as shown in Supplementary Figure S2. There was no significant difference in intracellular tacrolimus concentration between the rejection (n = 17) and non-rejection (n = 20) groups when only cryopreserved samples were analyzed: $TAC_{[PBMC]}$, 34.0 (31.0-47.0) *vs*. 36.9 (25.0-47.0) pg/million cells, p = 0.97; $TAC_{[CD3]}$, 11.2 (8.3-13.4) *vs*. 12.8 (10.0-15.6) pg/million cells, p = 0.20; and $TAC_{[CD14]}$, 63.3 (51.8-87.6) *vs*. 65.0 (57.3-101.7) pg/million cells, p = 0.43. Similarly, there were no significant differences between the rejection (n = 11) and non-rejection (n = 13) groups in $TAC_{[PBMC]}$ [90 (53.0-160.5) *vs*. 85.5 (60.5-110.3) pg/million cells; p = 0.60], $TAC_{[CD3]}$ [15.6 (9.8-28.5) *vs*. 16.5 (11.7-23.5) pg/million cells; p = 0.88], and $TAC_{[CD14]}$ [98.8 (72.8-127.7) *vs*. 127.8 (90.0-151.0) pg/million cells; p = 0.37] in fresh samples.

DISCUSSION

To the best of our knowledge, this is the first study to measure intracellular tacrolimus concentrations in $CD3^+$ T lymphocytes and $CD14^+$ monocytes after solid organ trans-

plantation and to investigate their association with acute rejection. A poor correlation was observed between $TAC_{[CD3]}$ and whole blood C_0, and the same trend was observed for $TAC_{[CD14]}$. This is in line with previous studies that reported a poor correlation between $TAC_{[PBMC]}$ and whole blood C_0.[6-10, 20-22] The poor correlation between whole blood C_0 and intracellular tacrolimus concentrations emphasizes the importance of measuring tacrolimus concentration in its target compartment(s), as the latter cannot be extrapolated from whole blood concentration.

Several observations in this study warrant further discussion. $CD3^+$ T lymphocytes are the central effector cells in acute rejection and are important for humoral rejection by providing B cell help.[23-29] They are also the most abundant cells in the PBMC subfraction.[30, 31] Monocytes contribute to alloimmunity via diverse pathways, including antigen processing and presentation, co-stimulation, and cytokine production.[32] Interestingly, intracellular tacrolimus concentration was significantly lower in $CD3^+$ T lymphocytes than in $CD14^+$ monocytes (Figure 1). Moreover, the range of intracellular tacrolimus concentrations in $CD3^+$ T lymphocytes was narrower than that in $CD14^+$ monocytes.

The assay for the measurement of the intracellular tacrolimus concentration we used here has been validated,[9, 17, 22] and the $TAC_{[PBMC]}$ in our study lies within the same range and is comparable to that reported by others.[6, 8, 9, 20-22] Therefore, we suspect that the difference in tacrolimus concentration between $CD3^+$ T lymphocytes and $CD14^+$ monocytes may be related to a difference in the pharmacokinetics (distribution) of intracellular tacrolimus between these two cell types. Previous studies have shown that the activity of the efflux transporter P-glycoprotein (P-gp, assessed by the Rhodamine-123 assay) in $CD3^+$ T lymphocytes is higher than that in $CD14^+$ monocytes.[33-38] However, no study has shown that a difference in P-gp activity translates into a difference in intracellular tacrolimus concentration, which is dependent on this efflux transporter. We hypothesized that the higher activity of P-gp in $CD3^+$ T lymphocytes could be responsible for the lower intracellular tacrolimus concentration than that in $CD14^+$ monocytes. In our previous study, *ABCB1* (the gene encoding P-gp) expression was not significantly correlated with intracellular tacrolimus distribution in PBMCs.[9] Therefore, we chose not to genotype patients in this study for *ABCB1*.

Interestingly, P-gp can be upregulated during inflammatory states.[39, 40] One might speculate that intracellular tacrolimus concentration may be lower during an acute rejection (which is an inflammatory state and may be caused by mechanisms other than sub-therapeutic tacrolimus exposure) because of increased (inflammatory) expression of P-gp. Other inflammatory conditions, such as infections, which frequently complicate transplantation, may have the same effect. Prospective studies are needed to elucidate the causal relationship

between intracellular tacrolimus concentration, P-gp activity, and acute rejection.

Second, a difference in intracellular tacrolimus concentration was observed between cryopreserved and fresh samples. Intracellular tacrolimus concentration in cryopreserved samples was significantly lower than that in fresh samples. As the cell isolation process for $CD3^+$ T lymphocytes and $CD14^+$ monocytes and the tacrolimus concentration measurement method were identical for all samples, this finding suggests that intracellular tacrolimus was either lost during the freezing process or during the additional washing steps in the thawing process. This observation suggests that the use of fresh samples is preferred over frozen samples when measuring intracellular tacrolimus concentration.

Third, no difference in tacrolimus concentration in $CD3^+$ T lymphocytes, $CD14^+$ monocytes, or PBMCs was observed between patients with and without rejection. Only one study showed an association between acute rejection and lower $TAC_{[PBMC]}$ in liver transplant recipients.[6] Other studies on solid organ transplantation did not show this association.[7-9, 41] Although intracellular tacrolimus concentrations in PBMCs, $CD3^+$ lymphocytes, and $CD14^+$ monocytes were not different between patients with and without acute rejection, we believe that it is too early to abandon investigations into intracellular tacrolimus concentration because whole blood C_0, which is the standard in current clinical practice, cannot accurately predict acute rejection.[4] As intracellular tacrolimus concentrations in cryopreserved samples were significantly lower than those in fresh samples, more pharmacokinetic studies of intracellular tacrolimus concentration and optimization of the assay are needed.

Moreover, a study by Capron *et al*. revealed an association between $TAC_{[PBMC]}$ and acute rejection among liver transplant recipients treated with tacrolimus monotherapy.[6] It is possible that the intracellular concentrations of both tacrolimus and MPA are relevant to the net immunosuppressive status of patients receiving both drugs. The plasma MPA concentrations in our study did not differ between patients with and without rejection (Table 3). Only one study has measured intracellular MPA concentration in PBMCs and demonstrated that a lower intracellular MPA concentration poses a risk for rejection in kidney transplant receipients.[42] However, the sensitivity and specificity of intracellular MPA concentration in PBMCs for predicting rejection were only 70% and 67%, respectively. In addition, the activity of inosine monophosphate dehydrogenase, the target of MPA, is poorly correlated with intracellular MPA concentration.[42, 43] The combination of intracellular tacrolimus concentration with intracellular MPA concentration measurement may better reflect clinical outcomes and improve therapeutic drug monitoring.

Our study has several strengths. Compared with that in previous studies, the process of cell isolation and measurement of intracellular tacrolimus concentration in this study were improved by using RBC lysis buffer in all samples to eliminate RBC contamination. Second, sampling was performed on the same day as kidney allograft biopsy. In addition, the number of patients with acute rejection included in this study was larger than that of a previous study in which PBMCs were sampled on the same day as kidney transplant biopsy.[10] Importantly, because PBMCs are composed of different cell populations, more specific immune cell subsets have been investigated. Every patient received the same immunosuppressive regimen, enabling comparison of the intracellular tacrolimus concentration between patients with and without rejection. Despite these strengths and improvements, no differences in intracellular tacrolimus concentrations were observed between patients with and without rejection. However, the number of freshly isolated samples was relatively low. A prospective study that includes more patients and only freshly isolated samples is needed to establish the association between intracellular tacrolimus concentration and clinical outcomes. We believe that the present findings should not be considered final, or that intracellular tacrolimus is not clinically relevant. Rather, they should be regarded as a new path for future studies that should first focus on further optimizing the analytical process. Additionally, the use of a time-to-event analysis that includes all intracellular tacrolimus concentrations up until the diagnosis of acute rejection might better predict an acute rejection episode than values from a single time point.[44] As a T lymphocyte response usually takes several days to develop and peaks between 7-15 days after the initial stimulation (depending on the type of stimulation and the measurement method)[45, 46], such a time-to-event analysis should at least include all intracellular tacrolimus concentrations measured in the 2 weeks preceding the diagnosis of rejection. In the present study, kidney transplant biopsies were performed at a median of 9 days after transplantation. We suggest that, in any prospective study, intracellular tacrolimus concentration should be measured whenever a whole blood pre-dose concentration is measured for the duration of the first month after transplantation. Following this approach, most TCMRs are likely to be "captured" with ample samples to allow time-to-event analysis.

CONCLUSION

In conclusion, $TAC_{[CD3]}$ and $TAC_{[CD14]}$ were measured for the first time in kidney transplant recipients. $TAC_{[CD3]}$ was significantly lower than $TAC_{[CD14]}$, suggesting a difference between $CD3^+$ T lymphocytes and $CD14^+$ monocytes in terms of the pharmacokinetics of intracellular tacrolimus. $TAC_{[PBMC]}$, $TAC_{[CD3]}$, and $TAC_{[CD14]}$ were all poorly correlated with whole blood C_0, and were not significantly associated with acute rejection. However, the differences between $TAC_{[CD3]}$ and $TAC_{[CD14],}$ as well as the difference between cryopreserved and

fresh samples, shed some light on strategies to improve the pre-analytic process to preserve intracellular tacrolimus concentration. Our study emphasizes that this pre-analytical workup needs to be optimized before any large-scale clinical studies are initiated.

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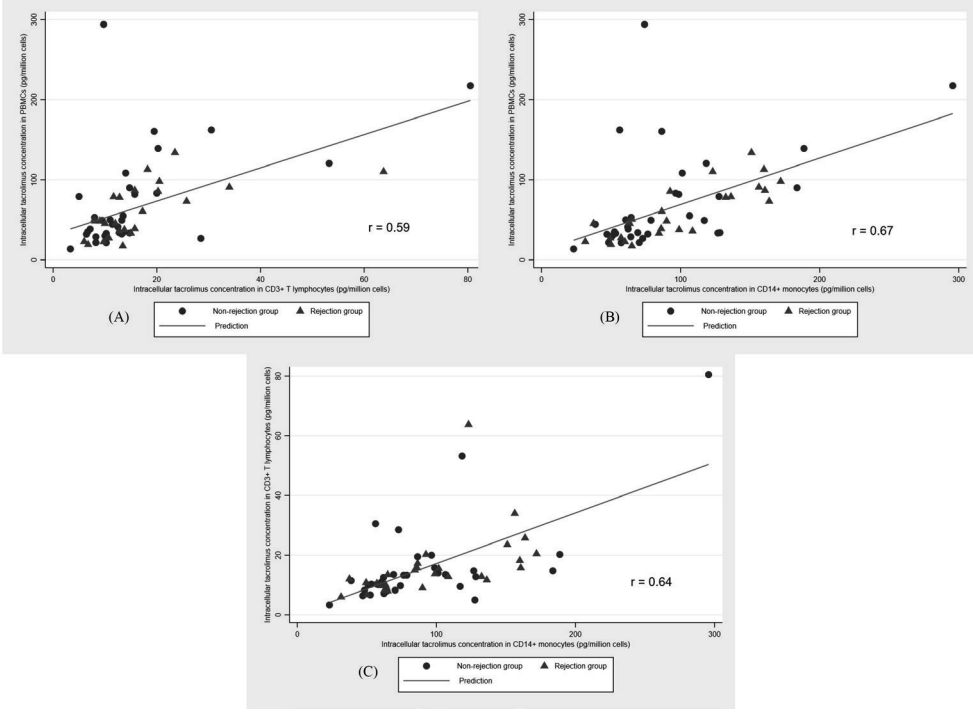
SUPPLEMENTARY DATA

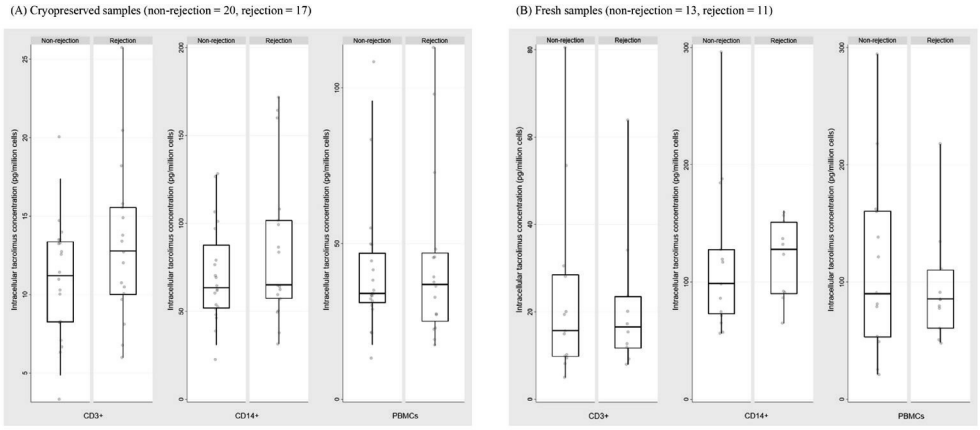
Supplementary Table S1: Kidney biopsy classifications in the control and rejection groups.

Non-rejection group (n = 33)		Rejection group (n = 28)	
• ATN	20 (61%)	• aTCMR 1A	4 (14%)
• Donor-derived arteriolosclerosis	10 (30%)	• aTCMR 2A	19 (68%)
• ATN with oxalate crystal deposition	1 (3%)	• aTCMR 2B	5 (18%)
• Diabetic nephropathy	1 (3%)		
• Renal infarction	1 (3%)		

ATN, acute tubular necrosis; aTCMR, acute T cell-mediated rejection



Supplementary Figure S1: Correlations between intracellular tacrolimus concentrations in different cell fractions.



Supplementary Figure S2: Intracellular tacrolimus concentration in PBMCs and $CD3^+$ and $CD14^+$ cells from cryo-preserved (A) and fresh samples (B). The non-rejection and rejection groups were compared. The boxes describe the median with Q1and Q3, whereas the whiskers represent the 95% CI.

Chapter 9

P-Glycoprotein, FK-Binding Protein-12, and the Intracellular Tacrolimus Concentration in T-Lymphocytes and Monocytes of Kidney Transplant Recipients

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ABSTRACT

Background

Transplant recipients may develop rejection despite having adequate tacrolimus whole blood pre-dose concentrations (C_0). The intra-immune cellular concentration is potentially a better target than C_0. However, little is known regarding intracellular tacrolimus concentration in T-lymphocytes and monocytes. We investigated the tacrolimus concentrations in both cell types and its relation with the expression and activity of FK-binding protein (FKBP)-12 and P-glycoprotein (P-gp).

Methods

T-lymphocytes and monocytes were isolated from kidney transplant recipients followed by intracellular tacrolimus concentration measurement. FKBP-12 and P-gp were quantified with Western blot, flow cytometry, and the Rhodamine-123 (Rh123) assay. Interleukin (IL)-2 and interferon-γ (IFN-γ) in T-lymphocytes were measured to quantify the effect of tacrolimus.

Results

Tacrolimus concentration in T-lymphocytes was lower than in monocytes (15.3(8.5-33.4) *vs*. 131.0(73.5-225.1) pg/million cells; $p<0.001$). The activity of P-gp (measured by Rh123 assay) was higher in T-lymphocytes than in monocytes. Flow cytometry demonstrated a higher expression of P-gp (normalized mean fluorescence intensity (MFI) 1.5(1.2-1.7) *vs.* 1.2(1.1-1.4); $p=0.012$) and a lower expression of FKBP-12 (normalized MFI 1.3(1.2-1.7) *vs.* 1.5(1.4-2.0); $p=0.011$) in T-lymphocytes than monocytes. Western blot confirmed these observations. The addition of verapamil, a P-gp inhibitor, resulted in a two-fold higher intra-T-cell tacrolimus concentration. This was accompanied by a significantly fewer cytokine-producing cells.

Conclusion

T-lymphocytes have a higher activity of P-gp and lower concentration of the FKBP-12 compared to monocytes. This explains the relatively lower tacrolimus concentration in T-lymphocytes. The addition of verapamil prevents loss of intracellular tacrolimus during the cell isolation process and is required to ensure adequate intracellular concentration measurement.

INTRODUCTION

Whole blood pre-dose concentration (C_0) monitoring is routinely performed to guide tacrolimus therapy after transplantation.[1, 2] However, therapeutic drug monitoring (TDM) using C_0 is not ideal as patients can still develop rejection or toxicity despite the tacrolimus C_0 being within the target concentration range.[3, 4] Although TDM with the use of an area-under the concentration *versus* time curve (AUC) may overcome some of these shortcomings, this strategy has important practical limitations including the need for multiple blood sampling and being time-consuming.[5, 6]

Tacrolimus' mechanism of action is to inhibit the enzyme calcineurin inside immune cells. Of the tacrolimus present in whole blood, more than 80% is located within erythrocytes which have a high concentration of the tacrolimus receptor FK binding protein (FKBP)-12.[7] The tacrolimus whole blood concentration is therefore largely determined by its concentration in cells that are not immunologically active.[5, 8-10] The measurement of the intracellular tacrolimus concentration in peripheral blood mononuclear cells (PBMCs) and in lymphocyte subsets has recently gained considerable interest.[4, 8, 11] Possibly, TDM based on the tacrolimus concentration measured in cells involved in allorecognition may be more relevant than conventional TDM based on whole blood concentrations.[12-16]

Previously, we developed a method to measure the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes and in $CD14^+$ monocytes.[17] $CD3^+$ T-lymphocytes isolated from kidney transplant recipients had a significantly lower intracellular tacrolimus concentration than $CD14^+$ monocytes.[17] We postulated that this may relate to differences in the activity of the efflux transporter P-glycoprotein (P-gp or ABCB1) or the expression of FKBP-12. Since there are only very limited data regarding the expression of P-gp and FKBP-12 in these cell types,[18, 19] a mechanistic study was performed with the aim to explain the causes of the differences in the intracellular tacrolimus concentration between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.

METHODS

Study overview

Experiments were performed using whole blood from healthy volunteers and kidney transplant recipients who received tacrolimus as part of routine clinical care. Kidney transplant recipients in this study participated in the LIBIDAR *(LIquid BIopsies for minimally invasive Detection of Acute Rejection*) study which aims to identify minimally invasive biomarkers for the diagnosis of acute kidney transplant rejection.[20] All patients gave written

informed consent. The study was performed in accordance with the declaration of Helsinki (2013) and was approved by the institutional review board of the Erasmus MC (Medical Ethical Review Board number 2018-035 for kidney transplant recipients and number 2018-1623 for healthy volunteers).

Study design and rationale

First, samples from kidney transplant recipients were analyzed to confirm the findings of our previous study that the intracellular tacrolimus concentration in T lymphocytes is lower than in monocytes.[17] Patient characteristics are described in Supplementary Table S1. After this had been confirmed, we performed multiple experiments, including the Rhodamine-123 (Rh123) assay, flow cytometric analysis, and Western blot, to investigate differences in P-gp and FKBP-12 expression between T lymphocytes and monocytes. These experiments were performed by using samples from healthy volunteers since only a limited volume of blood can be drawn from kidney transplant recipients. Finally, to test the hypothesis based on the outcomes of these experiments, we examined the effect of P-gp inhibition on the intracellular tacrolimus concentration in T lymphocytes from another cohort of kidney transplant recipients. These recipients formed a second group as described in Supplementary Table S2. We prospectively included these recipients to allow the addition of the P-gp inhibitor (verapamil) during the fresh cell isolation process.

The intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes

To confirm the findings of our previous study,[17] the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes was measured in samples of 16 kidney transplant recipients (details of these patients are provided in Supplementary Table S1). The timing of the intracellular sampling was the same as that of the whole blood tacrolimus C_0. Standard Ficoll separation was performed at room temperature (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) to isolate PBMCs from whole blood.[21] Red blood cell lysis buffer (eBioscience, San Diego, CA) was used to eliminate erythrocyte contamination. $CD3^+$ T-lymphocytes and $CD14^+$ monocytes were then isolated by use of negative selection Magnetic-Activated Cell Sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with the pan T cell isolation kit (Miltenyi Biotec) and pan monocyte isolation kit (Miltenyi Biotec). The purity of cells was checked after MACS separation by using flow cytometry and the cells were used for the intracellular tacrolimus measurement only if the purity was ≥90%. Cells were then counted with the Sysmex XP-300 cell counter and manually using a Neubauer counting chamber with trypan blue for confirmation. An overview of the cell isolation process is shown in Supplementary Figure S1. The details of the intracellular tacrolimus measurement assay were described previously.[22, 23] Briefly, a validated liquid chromatography-tandem mass spectrometry method (LC-MS/MS) was used. The concentration measured

is reported in µg/L and calculated and reported in pg/million cells (range 5-1,250 pg/million cells), and compared between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.

Determination of P-gp activity in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes

PBMCs were isolated from whole blood of 8 healthy volunteers (4 males and 4 females, mean age 29.4 ± 5.6 years) by use of standard Ficoll separation. Rh123 is a fluorescent dye that can be detected with flow cytometry under the FITC channel. Rh123, like tacrolimus, is a substrate of P-gp. The activity of P-gp was determined by using the flow cytometry to measure the percentage of Rh123-positive cells after Rh123 was loaded and allowed to efflux from the cells for 2 hours.[24] Verapamil is a potent P-gp inhibitor which was added to the medium to assess the activity of P-gp and its inhibition by verapamil. 1 x 10^6 PBMCs from each healthy volunteer were incubated with Rh123 (0.5 µg/mL) in 100 µL of RPMI 1640 medium with GlutaMAX (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) for 45 minutes (loading period). Thereafter, PBMCs were washed and incubated in Rh123-free medium for 2 hours at 4°C, 25°C, and 37°C to evaluate the effect of temperature on P-gp activity. These experiments were performed with or without the addition of verapamil hydrochloride (Centrafarm, the Netherlands) in a final concentration of 40 µmol/L to the medium and washing solutions. After 2 hours of incubation in the Rh123-free medium, PBMCs were stained with an anti-CD3-BV510 (Biolegend, San Diego, CA), an anti-CD14-PerCP (BD Biosciences, Franklin lakes, NJ), and an anti-CD45-APC (BD Biosciences) and then analyzed on a FACS Canto II flow cytometer (BD Biosciences). The percentage Rh123-positive cells in the $CD3^+$ T-lymphocyte and $CD14^+$ monocyte subsets were compared at these different conditions.

Expression of P-gp and FKBP12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes

PBMCs from healthy volunteers were used for the evaluation of P-gp and FKBP12 expression, and were stained for surface CD3, CD14, and CD45 as described above. Fc receptor blocker (BD Biosciences) was incubated for 10 minutes at room temperature before the staining of surface markers to minimize the non-specific binding of antibodies to the Fc receptor on monocytes.[25] For P-gp, an anti-CD243-PE monoclonal antibody (Invitrogen, Waltham, MA) was used and mouse anti-IgG2a-PE (Biolegend) was used as the isotype control. The anti-CD243 monoclonal antibody used was the UIC2 clone directed against the extracellular epitope of P-gp.[26] FKBP12 was stained intracellularly with an anti-FKBP12 monoclonal antibody (Invitrogen) conjugated with Alexa Fluor 488 (Abcam, Cambridge, UK). The isotype control for this FKBP12-antibody was mouse anti-IgG2b-Alexa Fluor 488 (Invitrogen). The antibodies used for surface staining (CD3, CD14, CD45, and CD243) were incubated for 30 minutes at 4°C in the dark. For the intracellular staining of FKBP12, cells were incubated with anti-FKBP12 for 30 minutes at room temperature in the dark, after

permeabilization with Permeabilizing solution 2 (BD Biosciences) for 10 minutes at room temperature. Samples were then analyzed with flow cytometry and the expression of P-gp and FKBP12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes was quantified by using the normalized mean fluorescence intensity (MFI) (target protein MFI: isotype MFI ratio).[27] The same analytic process was performed in the samples from kidney transplant recipients and results were compared with healthy volunteers.

Semi-quantification of P-gp and FKBP12

To further evaluate the expression of P-gp and FKBP12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes, Western blot analysis was performed. $CD3^+$ T-lymphocytes and $CD14^+$ monocytes were isolated from PBMCs of the healthy volunteers with Ficoll and MACS as described above. Cell lysates of $CD3^+$ T-lymphocytes and $CD14^+$ monocytes were made by adding RIPA buffer (ThermoFisher, Waltham, MA) with protease inhibitor (ThermoFisher), sonicated, and mixed at 4°C for 30 minutes.[28, 29]

Cell lysates (600 μg of protein in 40 μL of RIPA buffer) were loaded into the 4-20% Mini-PROTEAN TGX Precast gels (SDS-PAGE; Bio-Rad, Hercules, CA) and ran at 120 Volt. Polyvinylidene difluoride (PVDF) membranes were then blotted at 300 milliampere for 90 minutes and blocked by 5% non-fat milk in 0.1% Tween 20 in tris-buffered saline (TBS-T) for 60 minutes.

The primary antibodies, including mouse anti-human ABCB1 antibody (OriGene, Rockville, MD) and rabbit anti-human FKBP12 antibody (Cell Signaling Technology, Danvers, MA), were used to detect P-gp and FKBP12 bands in the PVDF membranes. Mouse anti-actin antibody (Invitrogen) was used as loading control. The primary antibodies were incubated overnight at 4°C. The secondary antibodies were incubated for 60 minutes at room temperature, including horse anti-mouse IgG (horseradish peroxidase-conjugated, Cell Signaling Technology) and goat anti-rabbit IgG (horseradish peroxidase-conjugated, Cell Signaling Technology). Membranes were then developed by the Clarity Western ECL Substrate (Bio-Rad) for chemiluminescence detection. P-gp and FKBP12 protein band densities were normalized by the band density of actin (the control protein) and compared between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.

The effect of P-gp inhibition by verapamil, intracellular tacrolimus concentration, and cytokine production in $CD3^+$ T-lymphocytes

For these experiments, blood was sampled from 8 kidney transplant recipients (details of these patient are shown in Supplementary Table S2). Next, Ficoll separation and MACS were performed to isolate pure $CD3^+$ T-lymphocytes for the measurement of the intracellular tacrolimus concentration as described above. Verapamil was added to the blood

samples immediately after sampling and in all solutions/buffers used for the cell isolation process, at a final concentration of 40 μmol/L. The intracellular tacrolimus concentrations in $CD3^+$ T-lymphocytes were then compared between samples with verapamil added and samples from the same patients without the addition of verapamil.

In addition to the intracellular tacrolimus concentration measurement, $CD3^+$ T-lymphocytes were stimulated overnight (16 hours) with CD3/CD28 Dynabeads Human T-Activator (Gibco) and protein transporter inhibitor cocktail (eBioscience). Verapamil was also added during the cell isolation and in the culture media. After the stimulation period, CD3/CD28 beads were removed and cells were stained with surface anti-CD3-BV510 and Via-Probe-PerCP (BD Biosciences) for viability. Interleukin (IL)-2 and interferon-γ (IFN-γ) were measured by intracellular staining with anti-IL-2-PE antibody (Invitrogen) and anti-IFN-γ-FITC antibody (BD Biosciences). The percentage of cytokine-positive cells was compared between the samples with and without verapamil by using the fluorescence-minus-one gating strategy.[30] Supplementary Figure S2 demonstrates the example of gating strategy.

To examine the effect of adding verapamil to different cell isolation steps, we performed a tacrolimus spiking experiment in blood from 8 healthy volunteers since only a limited amount of blood can be drawn from a kidney transplant recipient. Blood from each volunteer was spiked to achieve a whole blood tacrolimus concentration of 20 ng/mL and split into 3 groups based on the addition of verapamil. The first group underwent cell isolation without the addition of verapamil. In the second group, verapamil was added only after PBMCs were isolated. In the third group, verapamil was added to all steps of the cell isolation process. The intracellular tacrolimus concentrations were then compared between groups.

Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD) or median (quartile 1 – quartile 3) for normally and non-normally distributed data, respectively. Categorical data is shown as number with percentage. The Wilcoxon matched-pair signed-rank test was used to evaluate the difference of intracellular tacrolimus concentration between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes, P-gp and FKBP12 between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from flow-cytometric and western blot data, and the difference between samples with and without verapamil. Analysis of variance (ANOVA) was performed to test the difference of Rh123-positive $CD3^+$ T-lymphocytes and $CD14^+$ monocytes under different conditions.

Flow cytometric data was analyzed by Kaluza Analysis Software version 2.1 (Beckman Coulter Life Sciences, Indianapolis, IN). Image Lab Software version 6.1 (Bio-Rad Lab-

oratories, Hercules, CA) was used to analyze the protein band density from western blot. All statistical analyses were performed using Stata Statistical Software version 17.0 (StataCorp LLC, College Station, TX) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

The intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from kidney transplant recipients

Figure 1A displays the difference in the intracellular tacrolimus concentration between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from kidney transplant recipients. $CD3^+$ T-lymphocytes had a significantly lower intracellular tacrolimus concentration than $CD14^+$ monocytes: median (Q1-Q3) 15.3 (8.5-33.4) *vs*. 131.0 (73.5-225.1) pg/million cells; $p< 0.001$. The monocyte-to-T-lymphocytes intracellular tacrolimus concentration ratio was 7.09 (5.89-9.50). Whole blood tacrolimus concentration from the same samples was 9.5 (7.2-13.8) ng/mL (Supplementary Table S1). The correlation coefficient (*r*) between the whole blood C_0 and intra-T lymphocytes tacrolimus concentration was 0.77 ($p<0.001$), between the whole blood C_0 and intra-monocytes tacrolimus concentration it was 0.54 ($p=0.031$), and between the intra-T lymphocytes and intra-monocytes tacrolimus concentration the correlation was 0.60 ($p=0.014$). The scatter plots showing the correlation of tacrolimus concentration between different compartments are shown in Figure 1B. These findings confirm the results of our previous study.[17]

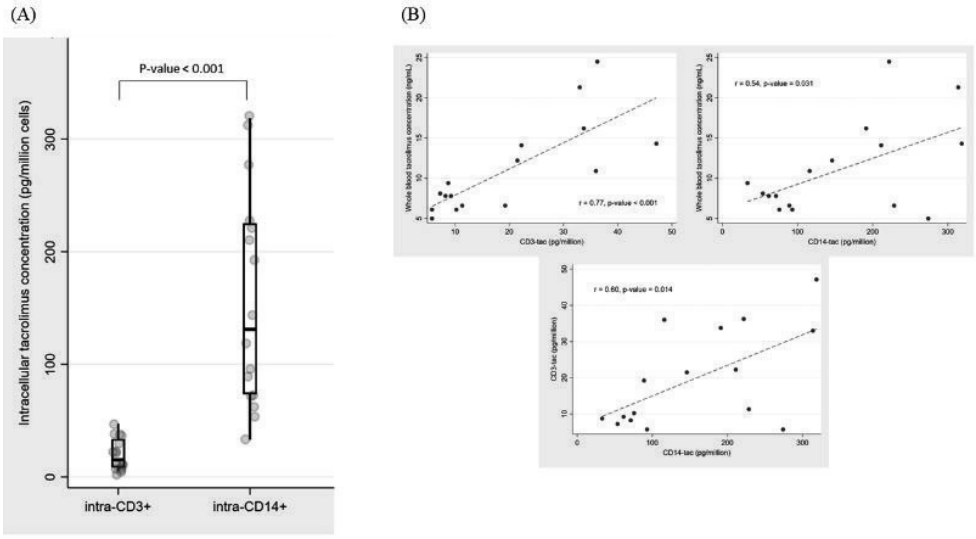

Figure 1: (A) The difference of the intracellular tacrolimus concentration between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes in kidney transplant recipients (n=16). (B) Correlations between tacrolimus concentration in $CD3^+$ T-lymphocyte, $CD14^+$ monocytes, and whole blood.

P-gp activity assessed with Rh123

Figure 2 illustrates representative data of P-gp activity assessed with Rh123 staining of $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from healthy volunteers under different conditions. The percentage of Rh123-postive $CD3^+$ T-lymphocytes decreased with increasing incubation temperatures demonstrating that the activity of P-gp becomes higher as the temperature increases. This finding suggests that at 4°C, the activity of P-gp is significantly decreased (hence the highest percentage of Rh123-positive cells) and becomes most active in the normal body temperature range (37°C; the lowest percentage of Rh123-positive cells). In contrast, $CD14^+$ monocytes did not show any difference in the percentage of Rh123-positive cells at the different incubation temperatures.

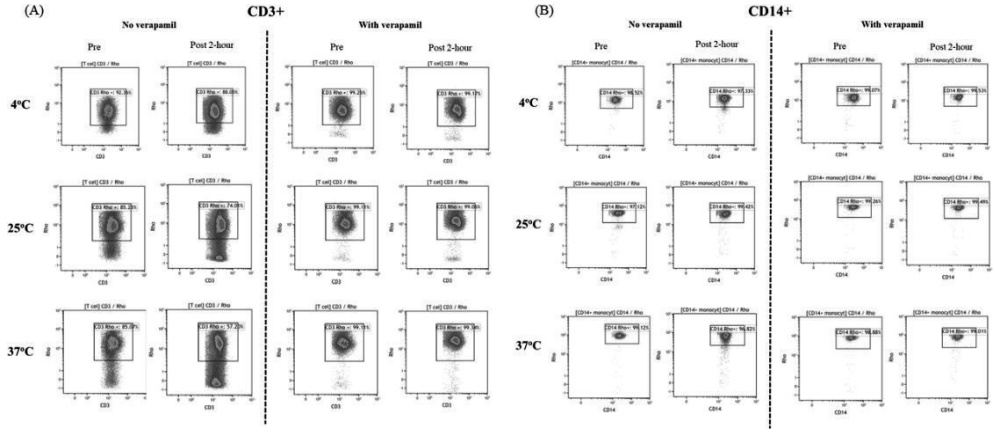

Figure 2: Representative data of the flow-cytometric analysis of the Rh123-positive $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from healthy volunteers. (A) Percentages of Rh123-postive $CD3^+$ T-lymphocytes in the different incubation temperatures and the effect of verapamil. (B) Percentages of Rh123-postive $CD14^+$ monocytes in the different incubation temperatures and the effect of verapamil.

The addition of verapamil to the culture medium could negate the effect of temperature on $CD3^+$ T-lymphocytes (Figure 2A). However, the addition of verapamil to $CD14^+$ monocytes did not alter the percentage of Rh123-positive cells, indicating lower baseline P-gp activity (Figure 2B). The summary of the percentages of Rh123-postive $CD3^+$ T-lymphocytes and $CD14^+$ monocytes is shown in Table 1, which demonstrates a significantly lower percentage of Rh123-postive $CD3^+$ T-lymphocytes with increasing temperature.

Table 1: Summary of the percentages of Rh123-postive $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.

Percentage of Rh123-positive cells	4ºC no verapamil	25ºC no verapamil	37ºC no verapamil	P-value	4ºC with verapamil	25ºC with verapamil	37ºC with verapamil	P-value
$CD3^+$ T-lymphocytes	93.7±4.4	80.3±8.9	60.6±14.4	<0.001	96.3±2.4	95.8±3.2	95.9±3.7	0.96
$CD14^+$ monocytes	98.9±1.1	99.4±0.9	98.6±1.1	0.45	99.5±0.4	99.4±0.9	98.5±2.5	0.50

Rh123; Rhodamine-123

P-gp and FKBP12 expression in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes

P-gp and FKBP-12 expression were analyzed by use of flow cytometry in samples of 8 healthy volunteers. In Figure 3A-C the gating strategy and a typical example illustrating P-gp and FKBP-12 expression is depicted. The expression of P-gp was significantly higher in $CD3^+$ T-lymphocytes than in $CD14^+$ monocytes: normalized MFI 1.5 (1.2-1.7) *vs.* 1.2 (1.1-

1.4); p=0.012 (Figure 3D). In contrast, the expression of FKBP12 was significantly higher in $CD14^+$ monocytes than $CD3^+$ T-lymphocytes: normalized MFI 1.3 (1.2-1.7) *vs.* 1.5 (1.4-2.0); p=0.011 (Figure 3D). We then compared P-gp and FKBP-12 expression between kidney transplant recipients and healthy volunteers in a new set of experiments. Both kidney transplant recipients and healthy controls showed that the P-gp expression was higher in $CD3^+$ T-lymphocytes than in $CD14^+$ monocytes, and the FKBP-12 expression was significantly higher in $CD14^+$ monocytes than in $CD3^+$ monocytes (Supplementary Figure S3).

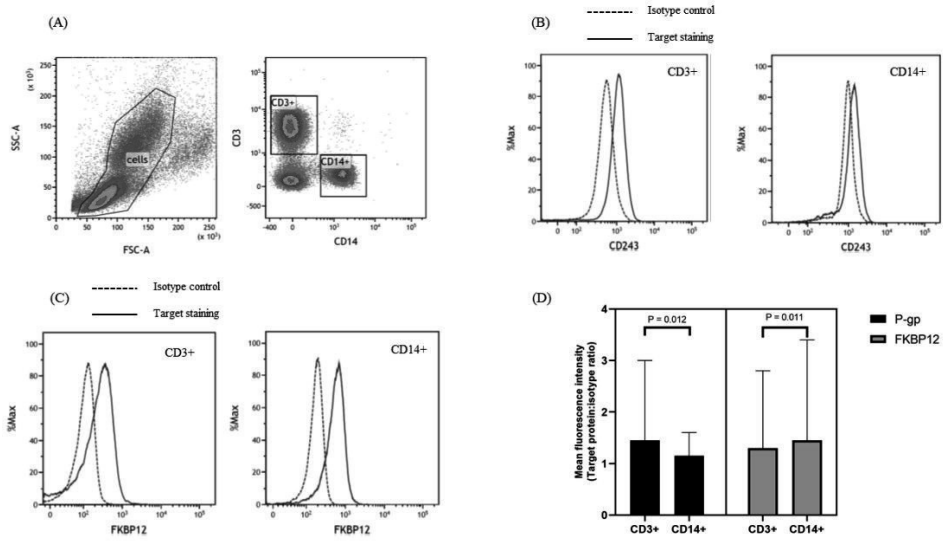

Figure 3: Flow-cytometric analysis of P-gp (CD243) and FKBP-12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from healthy volunteers. (A) Gating strategy of PBMCs. (B) MFI of P-gp in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes and their isotype controls. (C) MFI of FKBP-12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes and their isotype controls. (D) MFI (median with 95%-CI) of the staining of P-gp and FKBP-12 after subtraction of the isotype controls, comparing between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from 8 healthy volunteers.

Next, semi-quantitative analysis of P-gp and FKBP12 expression was performed. A representative result of the Western blot analysis is depicted in Figure 4A. In line with the flow-cytometric findings, $CD3^+$ T-lymphocytes had a significantly higher content of P-gp compared to $CD14^+$ monocytes: normalized band density: 65% (28-77) *vs*. 0.7% (0.1-3.0); p<0.001 (Figure 4B). The FKBP12 content of $CD14^+$ monocytes was significantly higher than that of $CD3^+$ T-lymphocytes: normalized band density: 55% (21-74) *vs*. 32% (19-39); p<0.001 (Figure 4B).

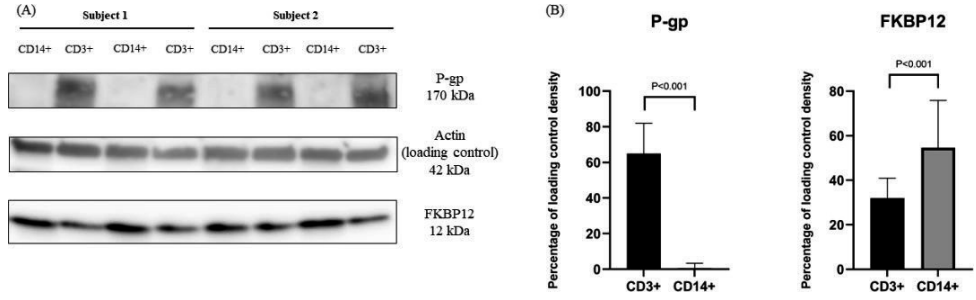

Figure 4: Western blot analysis of P-gp and FKBP-12 in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from healthy volunteers. (A) Representative protein band densities of P-gp, FKBP-12, and actin (the loading control protein) from 2 healthy volunteers. (B) Protein band density of P-gp and FKBP-12 normalized by the loading control density (median with 95%-CI), comparing $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from 8 healthy volunteers.

The effect of verapamil on the intracellular tacrolimus concentration and cytokines in $CD3^+$ T-lymphocytes

Blood samples of 8 kidney transplant recipients were obtained for the measurement of the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes. Samples from the same patients were split into 2 groups; the verapamil group and no verapamil group. Figure 5A displays the results of the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes of the kidney transplant recipients, comparing the samples with and without added verapamil. All verapamil-added samples had a higher intracellular tacrolimus concentration compared with samples without added verapamil. The median intracellular tacrolimus concentration increased 2.0-fold (1.8-3.6).

Representative data of the percentage of IL-2- and IFN-γ-positive cells are shown in Figure 5B and 5C, comparing $CD3^+$ T-lymphocytes with and without added verapamil. Figure 5D illustrates the summary data which shows a significantly higher percentage of IL-2-positive and IFN-γ-positive $CD3^+$ T-lymphocytes in samples without verapamil compared with the samples with added verapamil from the same patient (median IL-2-positive cells 20.0% (15.7-27.9) *vs*. 13.7% (7.5-20.4); p=0.012, median IFN-γ-positive cells 8.7% (6.0-10.8) *vs*. 5.5% (3.1-6.7); p=0.012). The samples from healthy volunteers (who did not receive tacrolimus) did not show any differences in both IL-2 (24.3% (21.9-28.4) *vs*. 25.0% (20.6-29.1); p=1.00) or IFN-γ-positive cells (14.0% (12.0-18.1) *vs*. 15.0% (11.6-19.6); p=0.40).

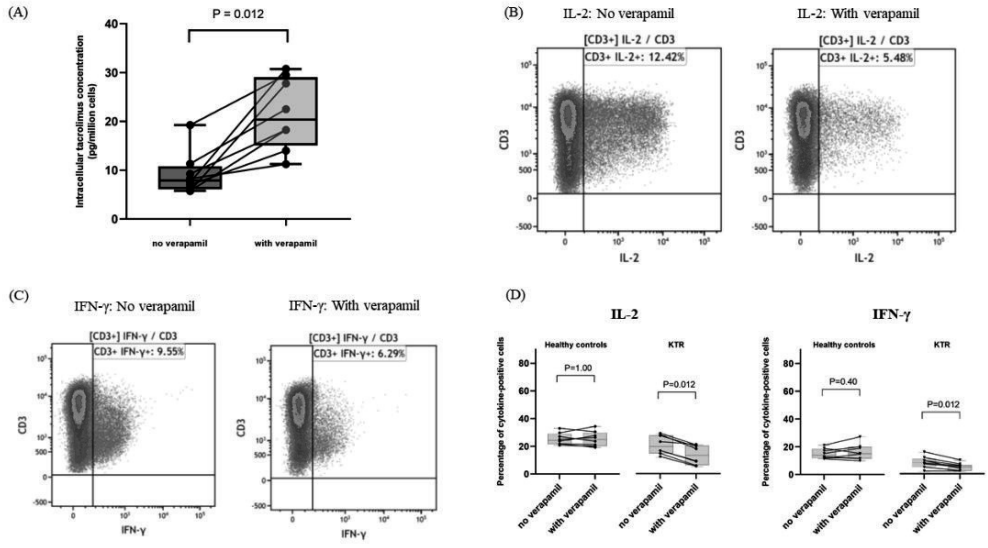

Figure 5: The effect of P-gp inhibitor on intracellular tacrolimus concentration and cytokines. (A) The effect of verapamil on the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes of kidney transplant recipients. Boxes represent the median with Q1 and Q3, and whiskers represent minimum-maximum. (B) Representative data of IL-2-positive cells from kidney transplant recipients (KTR) samples with and without verapamil. (C) Representative data of IFN-γ-positive cells from KTR samples with and without verapamil. (D) Summary data of the effect of verapamil on cytokines production in $CD3^+$ T-lymphocytes from healthy controls and KTR.

Since only a limited volume of blood can be drawn from kidney transplant recipients, blood from 8 healthy volunteers was obtained to demonstrate the effect of adding a P-gp inhibitor during different steps of the cell isolation process. Whole blood from each volunteer was spiked with tacrolimus to achieve the whole blood tacrolimus concentration of 20 ng/mL (mean ± SD 21.03 ± 2.69 ng/mL). Samples from each volunteer were divided into 3 groups according to the addition of verapamil. Supplementary Figure S4 shows that the intracellular tacrolimus concentrations in T-lymphocytes were significantly higher in group 2 (adding verapamil only after PBMCs were isolated) compared with group 1 (no verapamil added) (31.4 (25.5-37.1) vs. 21.3 (13.0-27.3) pg/million cells; p = 0.012). The intracellular tacrolimus concentrations in group 3 (adding verapamil in every step) were also higher than in group 2 (45.3 (106.5-39.3) vs 31.4 (25.5-37.1) pg/million cells; p = 0.012). These findings confirm that intracellular tacrolimus is lost during the cell isolation process and support the use of verapamil to inhibit P-gp activity throughout the whole isolation process.

DISCUSSION

Here, for the first time, it is demonstrated that $CD3^+$ T-lymphocytes have a higher expression of the tacrolimus efflux transporter P-gp and a lower concentration of the tacrolimus receptor FKBP-12 compared to $CD14^+$ monocytes. Furthermore, it was demonstrated that P-gp activity increases with higher temperature and that this temperature effect can be inhibited by adding verapamil. We believe these findings have important implications for the measurement of the intracellular tacrolimus concentration.

The intracellular tacrolimus concentration in PBMCs is a reasonable target for TDM of tacrolimus and may better correlate with clinical outcomes of tacrolimus therapy. In one study such a correlation was indeed observed.[31] A lower intra-PBMC tacrolimus concentration was associated with a higher risk of liver transplant rejection and higher rejection severity.[31] However, this finding was not confirmed in other studies in kidney and liver transplantation.[23, 32-35] One possible explanation for these conflicting findings may be that the PBMC fraction consists of many different immune cell subsets which have different roles in allorecognition and rejection. In a previous study, the intracellular concentration of tacrolimus was measured in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.[17] To our surprise, no association between the intracellular tacrolimus concentration in these cell types and acute T cell-mediated rejection was observed. A significantly lower intracellular tacrolimus concentration was however, measured in $CD3^+$ T-lymphocytes than in $CD14^+$ monocytes, a finding confirmed in the present study. It was postulated that this difference occurred because of differences in the activity and expression of P-gp and/or FKBP-12.

The concept of differential P-gp expression in human immune cells is not new. In 1992, Chaudhary *et al*. utilized Rh123 and 3,3′-Diethyloxacarbocyanine iodide ($DiOC_2(3)$; another fluorescent dye which is also a substrate of P-gp) and demonstrated that $CD56^+$ natural killer (NK) cells and $CD8^+$ T-lymphocytes had the highest expression of P-gp, whereas a lack of functionally active P-gp was found in $CD14^+$ monocytes.[18] A difference in P-gp expression between immune cells was also reported by Drach *et al*. In their study, the expression of messenger ribonucleic acid (mRNA) of the multidrug-resistance gene-1 (*MDR1*; which encodes P-gp) was measured in different human PBMC subsets by use of PCR. The highest expression of *MDR1* was observed in $CD56^+$ NK cells, followed by $CD3^+$ T-lymphocytes, and $CD19^+$ B lymphocytes, with a very low expression in $CD14^+$ monocytes and $CD15^+$ granulocytes.[19] Up until now, however, no study has demonstrated that this differential P-gp expression translates into a difference in the intracellular concentration of P-gp-dependent drugs such as tacrolimus. In our study, both flow cytometric and Western blot analysis showed results pointing in the same direction, with a higher expression of P-gp and a lower expression of FKBP-12 in $CD3^+$ T-lymphocytes compared with $CD14^+$ monocytes,

which is in line with previous studies.[18, 19] Some expression of P-gp on $CD14^+$ monocytes could be detected in our flow cytometric analysis but not in the Western blot. We suspect that this might relate to the non-specific binding of the monoclonal antibodies to the Fc receptor on monocytes which is a known limitation of flow cytometric analysis.[25]

FKBP-12 is the target of tacrolimus and is located within the cytoplasm of eukaryotic cells.[36] It is the smallest member of the FKBP family and is essential for mammalian life. Mutant mice deficient in FKBP-12 suffer from severe dilated cardiomyopathy and ventricular septal defects, or have defective neural tube closure, which is lethal in the embryonic period.[37] There is data to suggest that FKBP-12 binds to ryanodine receptors (RyRs), and that the FKBP-12-RyRs complex amplifies calcium-signaling in myocytes to evoke muscle contraction.[36] However, the exact physiologic function of FKBP-12 in humans has not been clearly identified and differences in the expression of FKBP-12 in human immune cells have never been reported. Our observations demonstrate that such differences exist and suggest that besides P-gp, the difference in FKBP-12 expression between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes may partly explain the difference in the intracellular tacrolimus concentration. The average mean cell volume of $CD14^+$ monocytes is larger than that of $CD3^+$ T-lymphocytes (413±21 *vs*. 176±6 μm^3)[38] and $CD14^+$ monocytes have a higher cytoplasm-to-nucleus ratio than $CD3^+$ T-lymphocytes.[39] It is possible that the amount of FKBP-12 is higher in $CD14^+$ monocytes than in $CD3^+$ T-lymphocytes as a result of this difference in cell volume and cytoplasm-to-nucleus ratio. However, the Western blot results demonstrated that after normalization for the control protein (actin), $CD14^+$ monocytes still have a higher amount of FKBP-12 than $CD3^+$ T-lymphocytes indicating that this difference is not due to the difference in cell volume alone.

The result of the present study confirms that P-gp determines the intracellular tacrolimus concentration. By adding the P-gp inhibitor verapamil to the sample and every solution used during the cell isolation process, one can preserve the intracellular tacrolimus in $CD3^+$ T-lymphocytes. Since the cell isolation involves several washing steps and buffer incubations, the concentration measured in the presence of verapamil is likely to be the "snap-shot" concentration that is the closest to the *in vivo* concentration. The expert consensus from the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Expert Panel (IATDMCT) does not yet recommend the routine use of P-gp inhibitors for the measurement of the intracellular tacrolimus concentration but suggests lowering the temperature of the samples once PBMCs are separated to minimize the active transport.[11] Based on our findings, the magnitude of the difference in the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes ranges between 1.8- and 3.6-fold when compared to samples without added verapamil. Although the activity of P-gp can be inhibited at 4°C, the cell isolation procedure is much more difficult to process at this temperature. The addition of a P-gp inhibitor

should therefore be recommended to prevent the loss of tacrolimus by this efflux transporter during the pre-analytic work-up (Figure 6).

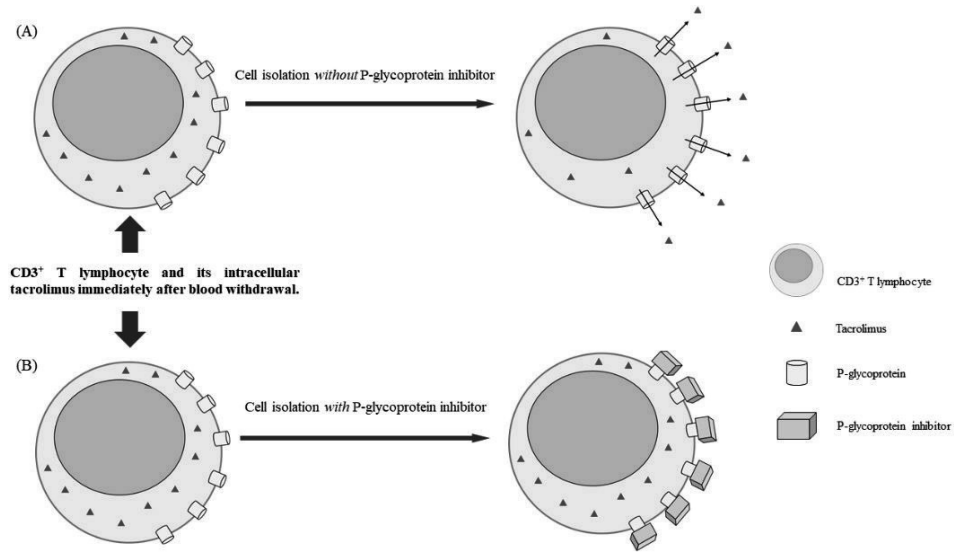

Figure 6: Summary of the effects of a P-gp inhibitor (in this case, verapamil) on intracellular tacrolimus concentrations in $CD3^+$ T-lymphocytes. (A) In the absence of the P-gp inhibitor (verapamil), a substantial amount of intracellular tacrolimus is lost during the cell isolation process. (B) The addition of a P-gp inhibitor allows the measurement of the intracellular tacrolimus concentration that reflects the *in vivo* concentration.

The present observations may also explain the lower intracellular tacrolimus concentration that was measured after cryopreservation of PBMCs compared with the freshly isolated PBMCs.[17] We believe the additional thawing and washing of cryopreserved samples resulted in a loss of a substantial amount of intracellular tacrolimus.

The addition of verapamil not only resulted in higher intracellular tacrolimus concentrations but also in a pharmacodynamic effect as demonstrated by the significantly lower proportion of cells producing intracellular IL-2 and IFN-γ. The percentage of cytokine-positive cells represent the results of *in vitro* stimulation of pure $CD3^+$ T-lymphocytes with CD3/CD28 magnetic beads. These percentages might be different from the *in vivo* stimulation by the alloantigen and in the presence of other stimulatory and regulatory cells.[40-43] The intracellular tacrolimus concentration (at the time of whole blood tacrolimus C_0 measurement) did not completely abrogate IL-2 and IFN-γ production, which is in line with previous studies.[44, 45] To assess the pharmacokinetic-pharmacodynamic relation, future studies should evaluate the intracellular tacrolimus concentration throughout a dosing interval and correlate this with

cytokine production.

This study has limitations. The effect of genetic polymorphisms in *ABCB1* was not investigated.[46] These polymorphisms may lead to differences in intracellular tacrolimus *in vivo* but we believe it is less likely that any differences resulting from *ABCB1* genotype cannot be overcome by verapamil in the concentration used in this study.[47-49] In addition, the main objective of this study was to demonstrate the mechanism behind the difference in the intracellular tacrolimus concentration between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes. Future studies should explore the population pharmacokinetics of intracellular tacrolimus with the use of verapamil to prevent the loss of intracellular tacrolimus, and include polymorphisms in the *ABCB1* gene as one of the potential factors responsible for the inter-individual variability. Moreover, the association between the intracellular tacrolimus concentration and acute rejection should be evaluated in future cohorts with the use of a P-gp inhibitor during the cell isolation process, to ensure the measurement of the true intracellular tacrolimus concentration. Currently, the role of intracellular tacrolimus concentration measurement is still in the area of research and development. Previous studies did not demonstrate an association between the intracellular tacrolimus concentration and clinical outcomes such as acute rejection in kidney transplantation.[17, 35] However, these studies suffered from methodological and analytical shortcomings. We believe the findings of the present study will increase the accuracy of the measurement of intracellular tacrolimus. Future studies should therefore use a P-gp inhibitor during the cell isolation process. Hopefully such a study may be able to answer the question if the intracellular tacrolimus concentration is a better TDM method than the monitoring of whole blood C_0.

In conclusion, the higher activity and expression of P-gp and the lower expression of FKBP12 in $CD3^+$ T-lymphocytes explain the lower intracellular tacrolimus concentration compared with $CD14^+$ monocytes. The addition of the P-gp inhibitor verapamil to the solutions and buffers used during the cell isolation process prevents the loss of intracellular tacrolimus from $CD3^+$ T-lymphocytes. This should therefore be recommended when measuring the intracellular tacrolimus concentration in leukocyte subsets.

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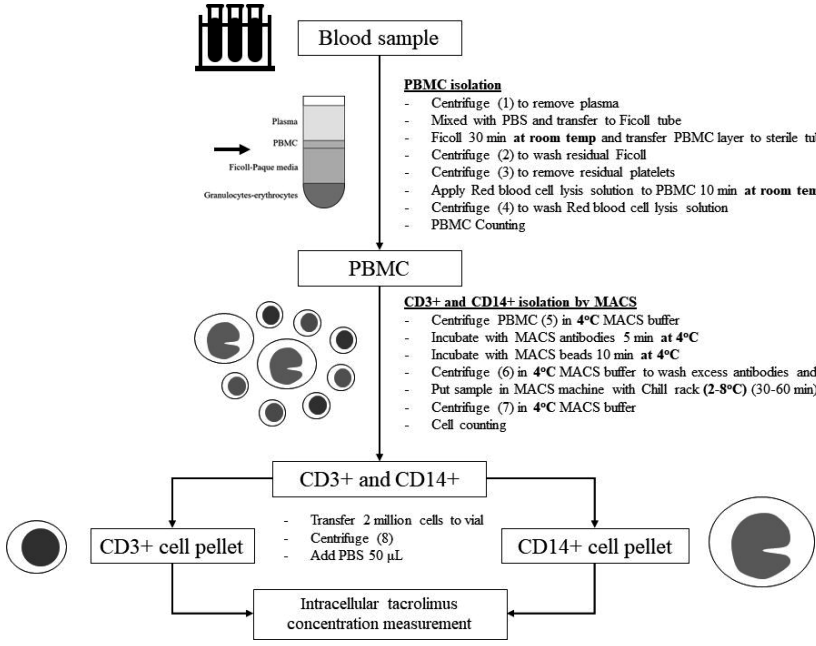
SUPPLEMENTARY DATA

Supplementary Table S1: Kidney transplant recipients included for the comparison of the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.

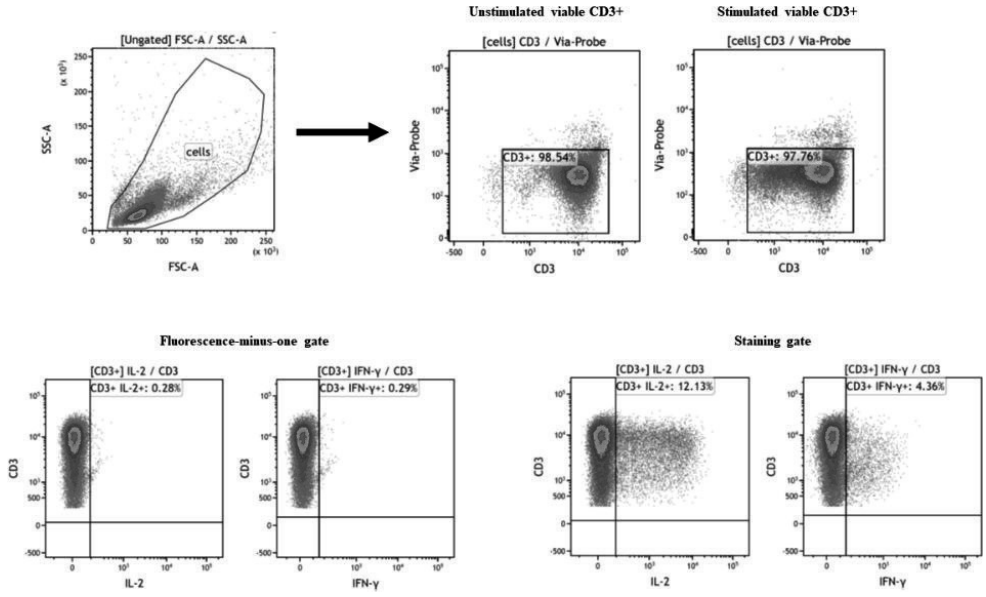
Patient characteristics	Value (n = 16)
Age, years (mean ± SD)	52.4 ± 15.4
Male, n (%)	13 (81%)
Time after transplantation, days (median with Q1 and Q3)	7 (6-10)
Living donor transplantation, n (%)	11 (69%)
Tacrolimus pre-dose whole blood concentration, ng/mL (median with Q1 and Q3)	9.5 (7.2-13.8)
Tacrolimus daily dose, mg/day (median with Q1 and Q3)	10 (8-19)

Supplementary Table S2: Kidney transplant recipients included to evaluate the effect of P-gp inhibitor on the intracellular tacrolimus concentration and cytokine production.

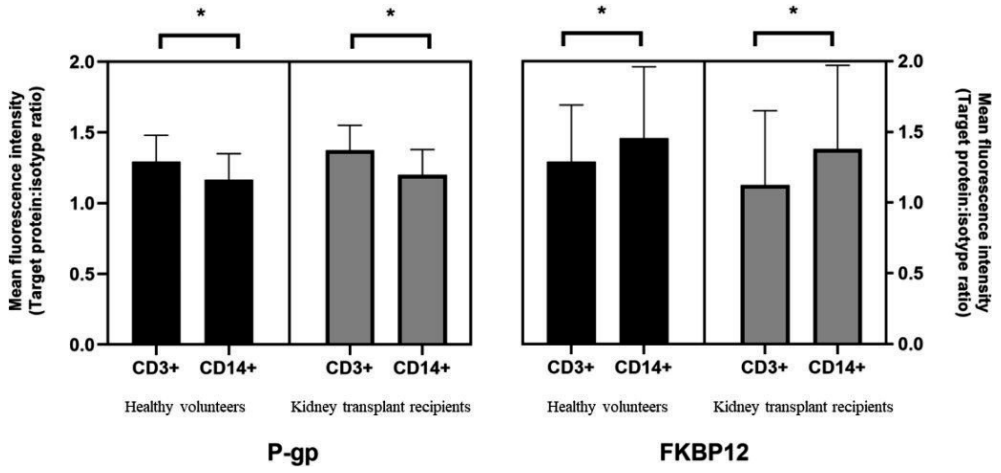
Patient characteristics	Value (n = 8)
Age, years (mean ± SD)	47.9 ± 16.2
Male, n (%)	5 (63%)
Time after transplantation, days (median with Q1 and Q3)	6.5 (4.3-15.8)
Living donor transplantation, n (%)	5 (63%)
Tacrolimus pre-dose whole blood concentration, ng/mL (median with Q1 and Q3)	7.2 (6.5-11.9)
Tacrolimus daily dose, mg/day (median with Q1 and Q3)	17 (7-20)



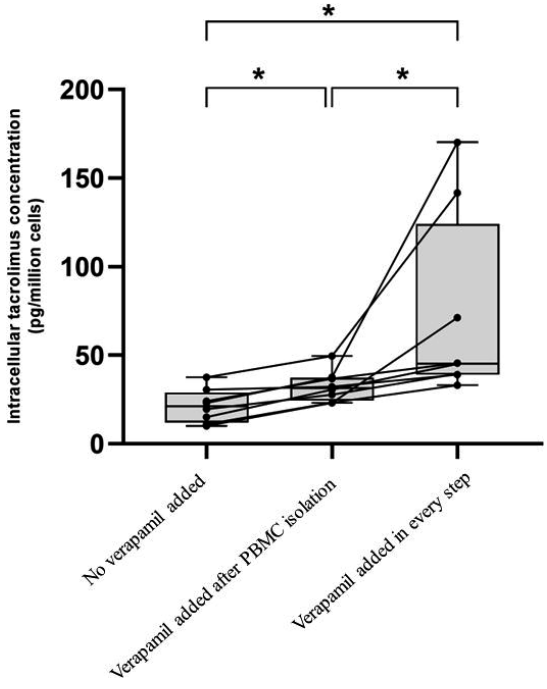
Supplementary Figure S1: Overview of the cell isolation process from whole blood to $CD3^+$ T-lymphocytes and $CD14^+$ monocytes.



Supplementary Figure S2: Gating strategy for cytokine-producing $CD3^+$ T-lymphocytes after MACS separation.



Supplementary Figure S3: Flow cytometric analysis of P-gp and FKBP-12 expression between $CD3^+$ T-lymphocytes and $CD14^+$ monocytes from 8 healthy volunteers and 8 kidney transplant recipients. * $p<0.05$



Supplementary Figure S4: The effect of adding verapamil at different cell isolation steps on the intracellular tacrolimus concentration in $CD3^+$ T-lymphocytes. * $p<0.05$

Chapter 10

Summary

SUMMARY

The overarching aim of the research described in this thesis was the development of various models and algorithms to predict post-transplantation complications and outcomes, as well as the response to immunosuppressive therapy, particularly tacrolimus. Although tacrolimus is the backbone of immunosuppression after kidney transplantation, treatment with this drug is complicated by its many side effects and its high intra- and inter-individual pharmacokinetic variability. Therapeutic drug monitoring (TDM) is routinely performed to optimize tacrolimus therapy, *i.e.* to increase its efficacy and to minimize tacrolimus' side effects. However, although the use of TDM enables a more personalized approach to pharmacologic immunosuppression, the current TDM strategy has important limitations.

In the introductory **Chapter 1** of this thesis, these limitations, as well as the unmet needs and challenges of current immunosuppressive therapy after kidney transplantation are described. This is followed by **Chapter 2** which describes the aims of the research in this thesis.

The outcomes of kidney transplantation have much improved because of progress in the field of transplant immunology, tissue typing and matching, organ allocation policy, and immunosuppressive drug therapy. However, post-transplant complications such as allograft rejection, infection, or toxicity of immunosuppressive drugs are obstacles for prolonged patient and allograft survival. The research described in **Part I** of this thesis focused on the prediction of these complications. One of the main limitations of tacrolimus is its nephrotoxicity which limits long-term kidney allograft survival and complicates the immediate post-transplant management. Tacrolimus is metabolized by cytochrome P450 3A5 (CYP3A5) which is expressed in the liver but also in the kidney. In **Chapter 3**, we investigated if local metabolism of tacrolimus in the kidney allograft is associated with the incidence of nephrotoxicity. To this end, 50 kidney transplant recipients and their respective donors, were genotyped for *CYP3A5* (the gene encoding CYP3A5 that is polymorphically expressed) and the association between genotype and the incidence of tacrolimus-nephrotoxicity was investigated. The main finding of this study was that kidney transplant recipients who expressed CYP3A5 (those with the *CYP3A5**1/*1 or *1/*3 genotype) were at higher risk for tacrolimus-associated nephrotoxicity when they received a kidney transplant from a non-CYP3A5 expressing donor (a donor with the *CYP3A5**3/*3 genotype) compared to other CYP3A5 donor-recipient combinations. This implies that recipients who need a high tacrolimus dose to achieve the target concentration (as found in expressor genotype recipients who are considered fast metabolizers) and were transplanted with a non-expressor kidney allograft (a slow metabolizer allograft) have an increased risk of tacrolimus-related nephrotoxicity.

We postulate that the relatively slower intra-allograft tacrolimus metabolism combined with the high tacrolimus dose requirement of recipients with the *CYP3A5* expressor genotype results in a high intra-allograft tacrolimus concentration-to-whole blood concentration ratio. A slower intra-renal tacrolimus metabolism combined with a high daily tacrolimus dose is responsible for the nephrotoxicity. This observation implies that the *CYP3A5* genotype of both donors and recipients should be determined to assess the risk of tacrolimus-associated nephrotoxicity. Recipients with a high risk of nephrotoxicity risk could benefit from targeting a lower tacrolimus exposure (*i.e*. a lower whole blood concentration) or a CNI-free immunosuppressive regimen such as a belatacept-based immunosuppressive regimen.

At present there are no clinical tools to assess the net state of immunosuppression of kidney transplant recipients. Such an "immunostat" would enable a personalized risk assessment for individual kidney transplant recipients and tailoring of their immunosuppressive therapy. The enzyme-linked immunosorbent spot (ELISPOT) assay is a laboratory test that quantifies memory cytokine-producing T lymphocytes reactive to a specific antigen. Several studies have assessed the possibility to predict acute rejection with ELISPOT. However, the value of this assay to predict rejection has not been completely clarified. Studies have mainly reported findings related to the interferon-γ (IFN-γ) ELISPOT assay in pre- and post-transplantation peripheral blood samples and using different cutoff levels of the cytokine-producing cells (*i.e.* different cut-offs for a positive and negative test result). Therefore, a meta-analysis of the performance of the donor-specific ELISPOT assay to predict acute rejection was conducted (**Chapter 4)**. This meta-analysis included a total of 32 studies that examined the performance of donor-specific ELISPOT in predicting both acute T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR). The main finding of this analysis is that the donor-specific IFN-γ ELISPOT can be used to reliably predict TCMR but not ABMR. The negative predictive value (NPV) of the IFN-γ ELISPOT assay is higher than the positive predictive value (PPV; 79-81% compared with 43-54%, respectively), when the prevalence of TCMR was 32%. The main advantage of using the IFN-γ ELISPOT assay therefore mainly lies in its possibility to exclude acute rejection.

In addition to predicting acute rejection, the IFN-γ ELISPOT assay can also be used to predict complications of immunosuppressive therapy. One such complication is the occurrence of BK virus (BKV) infection. In **Chapter 5**, the performance of the ELISPOT assay in predicting the clinical course of BKV infection was evaluated. Nine studies that utilized the BKV-specific IFN-γ ELISPOT assay were included in a meta-analysis. Our findings demonstrate that the BKV-specific IFN-γ ELISPOT assay can be used to discriminate kidney transplant recipients with an active (ongoing) BKV infection from recipients who will have a self-resolving BK viremia. BK viremic kidney transplant recipients with a negative BKV-specific ELISPOT were at 71.9-fold higher risk of having active, ongoing BKV repli-

cation, compared with viremic recipients who had a positive BKV-specific ELISPOT. The latter group cleared BK viremia. The findings of the meta-analysis described in **Chapter 5** suggest that the IFN-γ BKV-specific ELISPOT can be used to guide clinical management of patients suffering from BK viremia. Kidney transplant recipients with detectable BK viremia and a positive BKV-specific ELISPOT can be treated conservatively without any change in immunosuppression. On the contrary, BK viremic recipients with a negative BKV-specific ELISPOT are at risk for BKV-associated nephropathy (BKVAN) and their immunosuppressive therapy should be modified to prevent this complication.

Despite the growing knowledge of the effect of immunosuppressive drugs on kidney transplant outcomes, a long-term prediction model that includes the type of immunosuppressive medication is still lacking for the Asian population. Previous models were developed based on mainly Caucasian populations. In **Chapter 6**, the choice of immunosuppression (tacrolimus *versus* cyclosporine A) was included in a prediction model for long-term kidney transplant outcomes, along with pre-transplant and post-transplant information of both donor and recipient. Cox regression prediction models for 5- and 10-year patient and death-censored graft survival were built based on data of a total of 6,662 Thai kidney transplant recipients. The model demonstrated that tacrolimus was superior to cyclosporine A in terms of both patient and allograft survival. Other variables that were significantly associated with patient and allograft survival included age of both recipient and donor, primary kidney disease, the presence of cardiovascular co-morbidities in the recipients, the type of donor (living *vs*. traumatic head injury *vs*. cerebrovascular disease donor), peak panel reactive antibody titer, donor hepatitis C virus serology, serum creatinine before donor nephrectomy, cold ischemia time, and the presence of mycophenolic acid and prednisolone. With this model, kidney transplant recipients with high-risk of graft loss can be identified. The clinical use of this model may guide the monitoring of these patients and their immunosuppressive treatment in particular. In addition, patients and their families can be informed early of their general prognosis.

Regarding TDM of tacrolimus, the intracellular compartment of peripheral blood mononuclear cells (PBMCs) is an interesting matrix for the measurement of the tacrolimus concentration since it represents a more specific site of tacrolimus' action than whole blood, which is the matrix that is now most often used for the measurement of tacrolimus. In **Part II** of the thesis, several studies are described which investigated the intracellular tacrolimus concentration in-depth. First, the current peripheral blood mononuclear cell (PBMC) isolation process was evaluated. We investigated if the PBMC fraction is contaminated with granulocytes during acute rejection. The work described in **Chapter 7** demonstrates that, unlike other inflammatory conditions such as burn wounds or sepsis that cause a high rate of granulocyte contamination of the PBMC fraction, acute rejection does not result in granulo-

cyte contamination in PBMCs of kidney transplant recipients. This finding ensures that Ficoll density gradient separation can be used and interpreted confidently when isolating PBMCs from kidney transplant recipients.

The intracellular tacrolimus concentration is potentially a better target for tacrolimus TDM than the whole blood concentration. Theoretically, a lower intracellular tacrolimus concentration should associate with a higher acute rejection incidence. However, previous studies investigating the intra-PBMC tacrolimus concentration did not consistently demonstrate an association between the intracellular tacrolimus concentration and acute rejection. PBMCs consist of different immune cell subsets including T lymphocytes, monocytes, B lymphocytes, and NK cells. To examine a more specific matrix than the PBMC fraction, the measurement of the intracellular tacrolimus concentration in T lymphocytes and monocytes was performed for the first time and this work is described in **Chapter 8.** T lymphocytes and monocytes are key players in rejection and are involved in allorecognition and antigen presentation. A total of 61 samples from 53 kidney transplant recipients was included in our study. Of these, 28 samples were obtained during an episode of acute TCMR and 33 samples were obtained from non-rejecting patients. The intra-T lymphocyte and intra-monocyte tacrolimus concentrations were not different between rejecting and non-rejecting patients. However, two interesting observations were made. First, the intracellular tacrolimus concentration in T lymphocytes was significantly lower than in monocytes. Second, the intracellular tacrolimus concentration in freshly isolated samples was significantly higher than in cryopreserved samples. This observation suggests that PBMC should be freshly isolated and used for the measurement of the intracellular tacrolimus concentration.

In **Chapter 9** the mechanism behind the differences in the intracellular tacrolimus concentration between T lymphocytes and monocytes was explored. Our work focused on two important proteins involved in the pharmacokinetics of tacrolimus, namely P-glycoprotein (P-gp) and FK-binding protein-12 (FKBP-12). P-gp serves as an efflux pump that lowers the intracellular tacrolimus concentration, whereas FKBP-12 is the tacrolimus receptor which is located in the cytosol. The main finding of the research described in **Chapter 9** is that the expression of P-gp is higher and the expression of FKBP-12 is lower in T lymphocytes compared with monocytes. These differences explain the different intracellular tacrolimus concentration in these two cell types. In addition, it was demonstrated that the addition of the P-gp inhibitor verapamil to the blood sample immediately after venipuncture is necessary to maintain the intracellular tacrolimus concentration and prevent tacrolimus loss during the T lymphocyte isolation process. This procedure therefore leads to a more reliable measurement of the intracellular tacrolimus concentration which reflects the *in vivo* situation more closely

In the next part, the findings and the implications of the research described in Chap-

ters 3-9 are discussed, conclusions are drawn and recommendations for future research are made.

NEDERLANDSE SAMENVATTING

Het doel van het onderzoek zoals beschreven in dit proefschrift was het ontwikkelen van predictie modellen waarmee de uitkomsten na een niertransplantatie voorspeldt kunnen worden. Een tweede doel was het ontwikkelen van predictie modellen om de respons op de anti-afstotingsbehandeling (de zogenaamde *immunosuppressieve* therapie) en in het bijzonder die op het geneesmiddel tacrolimus, te kunnen inschatten. Het immunosuppressivum tacrolimus vormt de hoeksteen van de anti-afstotingsbehandeling na niertransplantatie. Het geneesmiddel is effectief maar heeft vele bijwerkingen. Het gebruik van tacrolimus in de klinische praktijk wordt daarbij bemoeilijkt door de sterk variabele farmacokinetiek van het geneesmiddel, zowel binnen een individuele patiënt (de zogenaamde *intra*-individuele variabiliteit) als tussen verschillende patiënten (de zogenaamde *inter*-individuele variabiliteit). Om de behandeling met tacrolimus zo goed mogelijk af te stemmen op de behoefte van een individuele patiënt, wordt dit geneesmiddel gedoseerd op geleide van de gemeten concentraties van tacrolimus in het bloed. Dit staat bekend als *Therapeutic Drug Monitoring* (TDM). Deze strategie heeft echter belangrijke beperkingen.

Hoofdstuk 1 van dit proefschrift vormt een inleiding. Hierin worden de beperkingen en behoeften van de huidige immunosuppressieve therapie na niertransplantatie beschreven. In **Hoofdstuk 2** worden de doelen gesteld van het in dit proefschrift beschreven onderzoek.

De uitkomsten na niertransplantatie zijn de afgelopen decennia sterk verbeterd. Dit is het gevolg van ontwikkelingen op het gebied van de transplantatie immunologie, verbeterde technieken voor het "*matchen*" van organen, nieuwe strategieën voor het alloceren van organen en verbeterde immunosuppressieve behandelingen. Complicaties treden echter nog steeds op. Transplantaat afstoting, infecties en toxiciteit van de verschillende immunosuppressieve geneesmiddelen zijn slechts enkele van de vele complicaties na transplantatie en deze beïnvloeden de lange termijn resultaten negatief. Het onderzoek zoals beschreven in **deel I** van dit proefschrift heeft zich gericht op het voorspellen van deze complicaties.

Een van de belangrijkste bijwerkingen van tacrolimus is haar nefrotoxiciteit. Dit schadelijke effect op de nier kan al in de vroege postoperatieve fase optreden maar laat zich met name gelden bij het chronisch gebruik van tacrolimus. Tacrolimus wordt gemetaboliseerd door het enzym cytochroom P450 3A5 (CYP3A5) dat tot expressie komt in de lever en darm maar ook in de nieren (en dus in het niertransplantaat). In het onderzoek beschreven in **Hoofdstuk 3** werd onderzocht of lokaal metabolisme van tacrolimus in het niertransplantaat is geassocieerd met het optreden van nefrotoxiciteit. Hiertoe werden 50 niertransplantatie pa-

tiënten en hun donoren ge-genotypeerd voor *CYP3A5* (het gen dat codeert voor het CYP3A5 eiwit). CYP3A5 komt polymorf tot expressie. Individuen met het *CYP3A5*1/*1* of **1/*3* genotype brengen CYP3A5 tot expressie en worden beschouwd als snelle metaboliseerders. Individuen met het *CYP3A5*3/*3* genotype brengen geen CYP3A5 tot expressie en zijn langzame metaboliseerders. De belangrijkste bevinding van het onderzoek beschreven in **Hoofdstuk 3** is dat niertransplantatie patiënten die CYP3A5 tot expressie brengen een hoger risico hebben op het ontwikkelen van tacrolimus-gerelateerde nefrotoxiciteit wanneer zijn een nier krijgen van een donor die CYP3A5 niet tot expressie brengt. De hypothese is dat deze patiënten een hoge dosis tacrolimus nodig hebben om hun streef-expositie te bereiken (vanwege hun eigen snelle metabolisme) en dat deze hoge tacrolimus dosis vertraagd wordt gemetaboliseerd in het niertransplantaat. Hierdoor kunnen lokaal hoge tacrolimus concentraties ontstaan die nefrotoxisch zijn. Deze observatie suggereert dat het *CYP3A5* genotype van zowel de ontvanger als de donor voorafgaand aan de niertransplantatie dient te worden bepaald om een inschatting te kunnen maken van het risico op nefrotoxiciteit. Patiënten met een verhoogd risico op deze bijwerking zouden voordeel kunnen hebben van een lagere blootstelling aan tacrolimus of van een immunosuppressieve behandeling zonder tacrolimus. Dit laatste zou kunnen worden gerealiseerd door hen belatacept in plaats van tacrolimus voor te schrijven.

Er bestaan momenteel geen goede methodes om vast te stellen in hoeverre het immuunsysteem van een individuele patiënt is onderdrukt. Met een dergelijke "immunometer" zou het mogelijk zijn om het risico op afstoting of infectie voor een patiënt in te schatten en de immunosuppressieve therapie hierop aan te passen. De ELISPOT test, wat staat voor *E*nzyme-*L*inked *I*mmunosorbent *SPOT*, is een laboratorium test waarmee de hoeveelheid antigeen-specifieke, cytokine (ontstekingseiwitten)-producerende geheugen T cellen kan worden gemeten. In diverse eerder uitgevoerde studies is onderzocht of met behulp van deze ELISPOT test het optreden van acute afstoting kan worden voorspeld. Desondanks is de waarde van deze test nog steeds onvoldoende duidelijk. De meeste onderzoeken beperkten zich namelijk tot het meten van de γ-interferon (INF-γ) ELISPOT en er werden verschillende afkapwaarden gebruikt om een positief van een negatief testresultaat te onderscheiden. Daarom werd een meta-analyse uitgevoerd naar de waarde van de donor-specifieke ELISPOT test bij het voorspellen van acute afstoting (**Hoofdstuk 4**). In deze meta-analyse werden 32 studies opgenomen die elk de waarde onderzochten van de donor-specifieke ELISPOT test voor het voorspellen van acute T cel-gemedieerde afstoting (TCMR) of antistof-gemedieerde afstoting (ABMR). De belangrijkste bevinding van de meta-analyse is dat het mogelijk is om met de donor-specifieke INF-γ ELISPOT betrouwbaar TCMR te voorspellen. Met de donor-specifieke INF-γ ELISPOT kan ABMR echter niet goed worden voorspeld. De negatief voorspellende waarde (NPV) van de donor-specifieke INF-γ ELISPOT is hoger dan haar

positief voorspellende waarde (PPV): 79-81% *versus* 43-54% bij een prevalentie van TCMR van 32%. De toegevoegde waarde van de donor-specifieke INF-γ ELISPOT ligt daarom met name in het kunnen uitsluiten van een acute afstoting.

De INF-γ ELISPOT kan ook worden gebruikt om complicaties van de immunosuppressieve therapie te voorspellen. Eén van deze complicaties is een infectie met het BK virus (BKV). In **Hoofdstuk 5** is onderzocht in hoeverre de ELISPOT test het klinisch beloop van een infectie met BKV kan voorspellen. Er werd een tweede meta-analyse uitgevoerd waarin negen onderzoeken werden opgenomen die elk de voorspellende waarde van de BKV-specifieke INF-γ ELISPOT onderzochten. De belangrijkste bevinding van deze meta-analyse is dat met behulp van de BKV-specifieke INF-γ ELISPOT een onderscheid kan worden gemaakt tussen patiënten die een doorgaande BKV infectie zullen hebben en patiënten bij wie de BKV infectie vanzelf opklaart. Patiënten met een BK viremie en een negatief BKV-specifiek INF-γ ELISPOT test resultaat hadden een 71.9-maal hoger risico op doorgaande BKV replicatie in vergelijking met patiënten met een BK viremie en een positief test resultaat in de BKV-specifieke INF-γ ELISPOT. De laatstgenoemde patiënten klaarden het BK virus. De bevindingen van het onderzoek beschreven in **Hoofdstuk 5** laten zien dat de BKV-specifieke INF-γ ELISPOT kan worden gebruikt om het klinisch beleid te bepalen. Niertransplantatie patiënten met een BK viremie en een positieve BKV-specifieke INF-γ ELISPOT kunnen conservatief worden behandeld zonder een verlaging van hun immunosuppressieve therapie. Niertransplantatie patiënten met een BK viremie en een negatieve BKV-specifieke INF-γ ELISPOT hebben daarentegen een hoger risico op het ontwikkelen van een BKV-geassocieerde nefropathie (BKVAN). Hun immunosuppressieve behandeling moet worden aangepast om deze complicatie te voorkomen.

Momenteel bestaat er geen model dat de lange termijn uitkomsten van Aziatische niertransplantatie patiënten voorspelt en daarbij ook de immunosuppressieve therapie in ogenschouw neemt. Dergelijke modellen werden eerder wel ontwikkeld voor de Kaukasische bevolking. In **Hoofdstuk 6** wordt de ontwikkeling van een predictie model voor de lange termijn uitkomsten van Aziatische patiënten beschreven. In dit model werden factoren zoals het type immunosuppressie (tacrolimus of cyclosporine A) en andere klinische data van zowel de donor als de ontvanger meegenomen. Met behulp van Cox regressie analyse werd de 5 -en 10-jaars patiënt -en transplantaat overleving (gecensureerd voor overlijden) voorspeld. Hierbij baseerden wij ons op data van 6.662 Thaise niertransplantatie patiënten. Het model liet zien dat de beste patiënt -en transplantaat overleving werd bereikt wanneer patiënten werden behandeld met tacrolimus in plaats van met cyclosporine A. Andere variabelen die significant waren geassocieerd met patiënt -en transplantaat overleving waren leeftijd van de donor en ontvanger, de primaire nierziekte, de aanwezigheid van cardiovasculaire co-morbiditeit bij de ontvanger, het type donor (levend *versus* een donor met traumatisch schedel-hersenletsel

versus een donor met een cerebrovasculair accident), de hoogst gemeten titer *panel reactive antibodies*, de hepatitis C serostatus van de donor, de laatst bekende serum kreatinine concentratie voor donor nefrectomie, de koude ischemietijd en het gebruik van mycofenolzuur en prednisolon.

Met behulp van dit model kunnen niertransplantatie patiënten met een hoog risico op het verlies van hun transplantaat worden geïdentificeerd. Het model kan derhalve richting geven aan de opvolging van deze patiënten en hun immunosuppressieve behandeling. Daarnaast kunnen patiënten en hun familie beter worden geïnformeerd over hun algehele prognose.

Het bepalen van de intracellulaire tacrolimus concentratie in mononucleaire cellen in het perifere bloed (PBMCs) is mogelijk een goede manier om de behandeling met dit geneesmiddel te vervolgen. De huidige standaard voor TDM van tacrolimus is het meten van de concentratie in volbloed. Het merendeel van de tacrolimus aanwezig in volbloed is echter in erythrocyten gelokaliseerd en deze cellen spelen geen rol van betekenis in het afstotingsproces. De concentratie tacrolimus aanwezig in PBMCs is daarom mogelijk relevanter. In **Deel II** van dit proefschrift worden diverse studies beschreven die tot doel hadden de methode voor het meten van tacrolimus in PBMCs en de klinische relevantie daarvan te bestuderen.

Als eerste werd de huidige methode voor het isoleren van PBMCs geëvalueerd. Wij onderzochten in hoeverre de PBMC fractie na Ficoll-Paque isolatie gecontamineerd is met granulocyten ten tijde van een acute afstoting. In **Hoofdstuk 7** tonen wij aan dat een acute afstoting niet leidt tot een relevante contaminatie van de PBMC fractie met granulocyten. Ten tijde van andere toestanden die gepaard gaan met inflammatie, zoals na een verbranding of een sepsis, is dit wel het geval. De huidige Ficoll-Paque PBMC isolatie methode kan dus gebruikt worden om PBMCs van niertransplantatie patiënten te isoleren en bestuderen, ook ten tijde van een afstoting.

Het meten van de intracellulaire tacrolimus concentratie is mogelijk een betere methode voor TDM van tacrolimus dan het meten van de volbloed concentratie (de huidige standaard methode). In theorie zou een lagere intracellulaire tacrolimus concentratie geassocieerd moeten zijn met een verhoogd risico op transplantaatafstoting. In eerdere onderzoeken werd echter geen consistente relatie tussen de intracellulaire tacrolimus concentratie in PBMCs en acute afstoting gezien. De PBMC fractie bestaat uit verschillende soorten immuun cellen en bevat onder andere T lymfocyten, B lymfocyten, monocyten en NK cellen. Het onderzoek beschreven in **Hoofdstuk 8** beschrijft de eerste methode voor het meten van tacrolimus in T lymfocyten en monocyten. Mogelijk correleert de intracellulaire tacrolimus concentratie in deze twee celtypen beter met klinische uitkomsten dan de intra-PBMC concentratie. T lymfocyten en monocyten spelen een centrale rol in allo-recognitie, antigeen

presentatie en afstoting. In het onderzoek werden 61 bloedmonsters van 53 niertransplantatie patiënten geïncludeerd. Van deze 61 monsters werden er 28 afgenomen ten tijde van een acute TCMR en 33 monsters werden afgenomen bij patiënten zonder een (histologisch-bewezen) afstoting. De intracellulaire tacrolimus concentratie in T lymfocyten en monocyten was echter niet verschillend tussen patiënten met en patiënten zonder een acute afstoting. Twee interessante waarnemingen werden gedaan. Als eerste bleek dat de intracellulaire tacrolimus concentratie veel lager was in T lymfocyten dan in monocyten. Ten tweede bleek dat de intracellulaire tacrolimus concentratie veel hoger was in vers geïsoleerde bloedmonsters in vergelijking met monsters die diep gevroren waren. Dit laatste suggereert dat alleen vers geïsoleerde bloedmonsters gebruikt moeten worden voor de isolatie van cellen en de bepaling van intracellulaire tacrolimus concentraties.

In **Hoofstuk 9** werd onderzocht waarom T lymfocyten een lagere intracellulaire tacrolimus concentratie hebben dan monocyten. Dit werk richtte zich op twee eiwitten die een belangrijke rol spelen in de farmacokinetiek van tacrolimus, namelijk P-glycoproteïne (P-gp) en het zogenaamde FK-binding protein-12 (FKBP-12). P-gp is een efflux pomp die de intracellulaire tacrolimus concentratie verlaagd door het geneesmiddel uit het cytosol te transporteren. FKBP-12 is de receptor van tacrolimus en is in het cytosol gelokaliseerd. De belangrijkste bevinding van het onderzoek beschreven in **Hoofdstuk 9** is dat de expressie van P-gp hoger en die van FKBP-12 lager is in T lymfocyten dan in monocyten. Deze verschillen in eiwit expressie verklaren de eerder gemeten verschillen tussen deze celtypen in de intracellulaire tacrolimus concentratie. Een tweede belangrijke bevinding is dat de toevoeging aan de bloedmonsters van het geneesmiddel verapamil, dat P-gp remt, noodzakelijk is om verlies van tacrolimus uit het intracellulaire compartiment tegen te gaan tijdens de isolatie van T lymfocyten. De toevoeging van verapamil dient onmiddellijk na het afnemen van het bloed bij een patiënt te gebeuren. Wij denken dat deze verbeterde methode leidt tot een betrouwbaarder meetresultaat dat de *in vivo* situatie beter benadert.

In de nu volgende Algemene Discussie worden de bevindingen en implicaties van het onderzoek beschreven in hoofdstukken 3-9 besproken. Wij eindigen deze discussie met een aantal conclusies en aanbevelingen voor vervolgonderzoek.

THAI SUMMARY

วิทยานิพนธ์นี้มีวัตถุประสงค์คือการศึกษาและพัฒนาองค์ความรู้เพื่อการทำนายผลของการปลูกถ่ายไต ซึ่งหมายรวมถึงผลที่เกี่ยวข้องกับการอยู่รอดของผู้ป่วยและไตปลูกถ่าย รวมไปจนถึงภาวะแทรกซ้อนต่าง ๆ ที่พบได้ เช่น การเกิดการปฏิเสธหรือการสลัดไต (rejection) ภาวะติดเชื้อ และการเกิดความเป็นพิษจากการใช้ยากดภูมิคุ้มกัน ปัจจุบัน tacrolimus เป็นยากดภูมิคุ้มกันหลักที่ใช้ในผู้ป่วยปลูกถ่ายไต โดยใช้ร่วมกับ mycophenolic acid (MPA) และ corticosteroid เนื่องจาก tacrolimus นั้นมี intra- และ inter-individual variability สูง จึงต้องมีการตรวจติดตามยาเพื่อปรับขนาดยาที่ใช้ให้เหมาะสมสำหรับผู้ป่วยแต่ละราย (therapeutic drug monitoring; TDM) เพื่อให้ได้ประสิทธิภาพในการรักษาด้วยยา tacrolimus อย่างเต็มที่ และลดอัตราการเกิดผลข้างเคียงให้น้อยที่สุด อย่างไรก็ตามการตรวจระดับ pre-dose concentration (C_0) ของ tacrolimus ในเลือดซึ่งเป็นวิธีการตรวจติดตามยา tacrolimus ที่ใช้กันทั่วไปในเวชปฏิบัตินั้นมีข้อจำกัดหลายประการ รวมไปถึงไม่สามารถทำนายการเกิด rejection และการเกิดพิษของยาได้อย่างแม่นยำในผู้ป่วยทุกราย

ใน**บทที่ 1** ของวิทยานิพนธ์นี้ได้กล่าวถึงหลักการทำ TDM สำหรับยากดภูมิคุ้มกัน โดยเน้นประเด็นที่ยา tacrolimus เป็นหลัก รวมไปถึงการกล่าวถึงข้อจำกัดของวิธีการทำ TDM ในปัจจุบัน และความเป็นไปได้ในการปรับปรุงการทำ TDM ในอนาคต สำหรับใน**บทที่ 2** นั้นเกี่ยวข้องกับวัตถุประสงค์ของวิทยานิพนธ์ในภาพรวมและในรายละเอียดของแต่ละบทย่อย

ผลลัพธ์ของการการปลูกถ่ายไตในปัจจุบันนั้นดีขึ้นเป็นอย่างมากเมื่อเปรียบเทียบกับการปลูกถ่ายไตในยุคสมัยแรก อันเนื่องมาจากความก้าวหน้าของวงการ transplant immunology, การตรวจเนื้อเยื่อและความเข้ากันได้ระหว่างผู้บริจาคและผู้รับบริจาค, การจัดการและการบริหารแจกจ่ายอวัยวะจากผู้ที่เสียชีวิต, และการพัฒนายากดภูมิคุ้มกัน อย่างไรก็ตามภาวะแทรกซ้อนหลังการปลูกถ่ายไตนั้นยังคงพบได้บ่อย เช่น การปฏิเสธหรือการสลัดอวัยวะปลูกถ่าย, การติดเชื้อ, และความเป็นพิษจากยากดภูมิคุ้มกันที่ใช้หลังการปลูกถ่ายอวัยวะ ภาวะแทรกซ้อนเหล่านี้ส่งผลให้อัตราการรอดชีวิตของผู้ป่วยและอวัยวะปลูกถ่ายสั้นลงเมื่อเปรียบเทียบกับผู้ที่ไม่มีภาวะแทรกซ้อน ในส่วนแรกของวิทยานิพนธ์นี้มีวัตถุประสงค์เพื่อค้นหาวิธีการในการทำนายภาวะแทรกซ้อนเหล่านี้ และคัดกรองผู้ป่วยที่มีความเสี่ยงสูงเพื่อให้การตรวจติดตามที่เหมาะสม หนึ่งในข้อจำกัดของการใช้ tacrolimus คือภาวะความเป็นพิษต่อไตปลูกถ่าย (nephrotoxicity) ซึ่งมีผลลดอายุขัยของไตปลูกถ่าย โดย tacrolimus นั้นถูก metabolized โดย cytochrome P450 3A5 (CYP3A5) ซึ่งเป็นเอนไซม์ที่ทำงานหลักที่ตับ อย่างไรก็ตามมีรายงานว่าสามารถตรวจพบที่เนื้อเยื่อของไตได้ ใน**บทที่ 3** นั้นศึกษา local metabolism ของยา tacrolimus ในไตปลูกถ่ายว่ามีความสัมพันธ์กับการเกิดความเป็นพิษของยาต่อเนื้อไตหรือไม่ ผู้ป่วยที่ได้รับการปลูกถ่ายไตทั้งหมด 50 รายได้รับการตรวจ genotype ของ *CYP3A5* ทั้งของผู้ป่วย (ซึ่งเป็น genotype ที่ใช้ในการ metabolism ของยา tacrolimus ในระดับ systemic) และ *CYP3A5* ของผู้บริจาคไต (ซึ่งเป็น genotype ของเนื้อไตปลูกถ่ายและสัมพันธ์กับ local metabolism ของ tacrolimus ในเนื้อไตปลูกถ่าย) ผลการศึกษาหลักพบว่าความเสี่ยงในการเกิด tacrolimus-associated nephrotoxicity สูงที่สุดในผู้รับบริจาคที่มี *CYP3A5* expressor (*CYP3A5**1/*1 หรือ *CYP3A5**1/*3) ที่ได้รับไตบริจาคจาก *CYP3A5* non-expressor donor (*CYP3A5**3/*3) เมื่อเปรียบเทียบกับ recipient-donor *CYP3A5* combination รูปแบบอื่น ๆ ผลการศึกษานี้แสดงให้เห็นว่าในผู้รับบริจาคที่ต้องการขนาดยา tacrolimus ในขนาดสูงเพื่อให้ได้ระดับยา tacrolimus ในเลือดอยู่ใน therapeutic level (ดังที่พบใน recipient *CYP3A5* expressor) เมื่อได้รับไตปลูกถ่ายที่เป็น non-expressor *CYP3A5* ซึ่งมี local metabolism ที่น้อยนั้น ไตปลูกถ่ายที่มี local metabolism น้อยอาจไม่สามารถกำจัด

tacrolimus ในเนื้อไตได้ทันกับขนาดยา tacrolimus ขนาดสูงที่ผู้ป่วยได้รับ (เพื่อให้ระดับ tacrolimus ในเลือดได้ตามมาตรฐาน) หรืออาจเรียกได้ว่ามี intra-allograft tacrolimus concentration-to-whole blood ratio สูง และนำไปสู่การเกิดความเป็นพิษที่ไตตามมา ผลการศึกษาจากงานวิจัยนี้สามารถนำไปใช้ในการประเมินความเสี่ยงของการเกิดความเป็นพิษต่อไตจากยา tacrolimus ในผู้ป่วยแต่ละราย ผู้รับบริจาคไตที่มีความเสี่ยงสูงในการเกิดความเป็นพิษที่ไตอาจได้ประโยชน์จากการใช้ยา tacrolimus โดยตั้งเป้าหมายให้ระดับยาในเลือดต่ำกว่าผู้รับบริจาคไตที่มีความเสี่ยงน้อยหรือปานกลาง รวมไปถึงอาจพิจารณาการใช้ calcineurin inhibitor (CNI)-free regimen เช่น belatacept-based immunosuppression

ในปัจจุบันยังไม่มีเครื่องมือทางคลินิกหรือวิธีการตรวจทางห้องปฏิบัติการที่สามารถใช้ตรวจติดตาม net state ของ immunosuppression ในผู้ป่วยได้ หากสามารถคิดค้นเครื่องมือดังกล่าวซึ่งทำหน้าที่เป็น immunostat ได้นั้นจะสามารถปรับขนาดของยากดภูมิคุ้มกันให้เหมาะสมเฉพาะกับผู้ป่วยแต่ละรายได้ ซึ่งเป็นเป้าหมายของการรักษาในอนาคต (precision medicine) ในการปลูกถ่ายไตนั้นการตรวจ enzyme-linked Immunosorbent spot (ELISPOT) ซึ่งเป็นการตรวจทางห้องปฏิบัติการที่วัดปริมาณของ cytokine-producing memory T lymphocytes ในการตอบสนองต่อ antigen ที่สนใจ มีการศึกษาหลายการศึกษาที่พบว่าการใช้ ELISPOT นั้นสามารถทำนายการเกิด acute rejection หลังการปลูกถ่ายไตได้ อย่างไรก็ตามการศึกษาเหล่านี้มีความหลากหลายทั้งการใช้ cutoff ที่แตกต่างกัน รวมไปถึงเวลาที่ทำการตรวจ ELISPOT ก็แตกต่างกันในแต่ละการศึกษา ดังนั้นใน**บทที่ 4** ของวิทยานิพนธ์นี้จึงได้ทำการศึกษา meta-analysis เพื่อหาความสามารถในการทำนายการเกิด kidney allograft rejection โดยการใช้ donor-specific interferon-γ (IFN-γ) ELISPOT การศึกษา meta-analysis นี้รวบรวมการศึกษาทั้งหมด 32 การศึกษาของ donor-specific IFN-γ ELISPOT ในการทำนายภาวะ acute T cell-mediated rejection (TCMR) และ antibody-mediated rejection (ABMR) ผลการศึกษาที่สำคัญคือ donor-specific IFN-γ ELISPOT สามารถใช้ทำนายการเกิด TCMR ได้อย่างแม่นยำ แต่ไม่สามารถใช้สำหรับการทำนาย ABMR ได้ โดยมีค่า negative predictive value (NPV) อยู่ที่ร้อยละ 79-81 เมื่อเปรียบเทียบกับค่า positive predictive value (PPV) ที่ร้อยละ 43-54 เมื่อความชุกของการเกิด TCMR อยู่ที่ร้อยละ 32 ดังนั้นประโยชน์หลักของการใช้ donor-specific IFN-γ ELISPOT คือการใช้เพื่อการคัดแยกภาวะ TCMR ออก มากกว่าการนำไปใช้เพื่อยืนยันการวินิจฉัย TCMR

นอกจากภาวะ acute rejection แล้ว IFN-γ ELISPOT ยังสามารถนำไปใช้เพื่อการทำนายภาวะแทรกซ้อนอื่นของการใช้ยากดภูมิคุ้มกันได้ หนึ่งในภาวะแทรกซ้อนที่สำคัญคือการติดเชื้อ BK virus (BKV) ใน**บทที่ 5** ของวิทยานิพนธ์นี้ ได้รวบรวมการศึกษาที่ใช้ IFN-γ ELISPOT สำหรับการทำนาย clinical course ของการติดเชื้อ BKV หลังการปลูกถ่ายไต และวิเคราะห์โดยการใช้ meta-analysis ผลการศึกษาที่สำคัญพบว่า BKV-specific IFN-γ ELISPOT สามารถใช้แยกการดำเนินโรคของผู้ป่วยที่มี BK viremia ว่าจะมีการดำเนินโรคเป็น active ongoing BKV infection หรือเป็น self-limiting BKV infection ผู้ป่วยที่มี BK viremia และมีผลการตรวจ BKV-specific IFN-γ ELISPOT เป็นลบนั้นมีโอกาสดำเนินโรคเป็นในลักษณะ active ongoing BKV infection มากกว่าผู้ป่วยที่มี BK viremia และมีผลการตรวจ BKV-specific IFN-γ ELISPOT เป็นบวกถึง 71.9 เท่า ในกลุ่มผู้ป่วยที่ตรวจพบ BK viremia และมี BKV-specific IFN-γ ELISPOT เป็นบวกนั้นเป็นผู้ที่สามารถสร้างภูมิคุ้มกันต่อต้าน BKV ได้และสามารถกำจัด BKV replication ในที่สุด การดูแลรักษาผู้ป่วยกลุ่มนี้คือการตรวจติดตามระดับ BK viremia ในเลือด โดยอาจไม่จำเป็นต้องลดยากดภูมิคุ้มกันเพิ่มเติม ในทางตรงกันข้าม ผู้ป่วย BK viremia ที่มีผลของ BKV-specific IFN-γ ELISPOT เป็นลบนั้นเป็นกลุ่มที่ยังไม่สามารถสร้างภูมิคุ้มกันต่อ BKV ได้จึงอาจจำเป็นต้องได้รับการลดยากดภูมิคุ้มกันหรือให้การรักษาอื่น ๆ เพื่อป้องกันการดำเนินของโรคที่รุนแรงมากขึ้นอันนำไปสู่การเกิด BKV-associated nephropathy (BKVAN) ต่อไป

ถึงแม้ว่าความรู้เกี่ยวกับยากดภูมิคุ้มกันจะพัฒนาขึ้นเป็นอย่างมากในปัจจุบัน แต่ยังไม่มีสมการการทำนายผลของการปลูกถ่ายไตในระยะยาวในกลุ่มประชากรชาวเอเชีย สมการการทำนายผลของการปลูกถ่ายไตในการศึกษาที่ผ่านมาล้วนเป็นการสร้างขึ้นจากฐานข้อมูลของผู้ป่วย Caucasian เป็นหลัก ใน**บทที่ 6** ของวิทยานิพนธ์นี้ ได้นำเสนอสมการเพื่อการทำนายผลของการปลูกถ่ายไตในระยะยาว คือ อัตราการรอดชีวิตของผู้ป่วยและไตปลูกถ่าย โดยคำนึงถึงตัวเลือกของการใช้ยากดภูมิคุ้มกันเข้ามาในสมการร่วมด้วย (tacrolimus หรือ cyclosporine A) ข้อมูลจากผู้ป่วยชาวไทยที่ได้รับการปลูกถ่ายไตจำนวน 6,662 คนนั้นได้ถูกนำมาทำการวิเคราะห์โดยการใช้ Cox regression prediction model เพื่อหาผลลัพธ์คืออัตราการรอดชีวิตของผู้ป่วยและไตปลูกถ่าย ณ เวลา 5 และ 10 ปีหลังการปลูกถ่ายไต สมการการทำนายนี้แสดงให้เห็นว่าการใช้ยา tacrolimus นั้นส่งผลในระยะยาวมากกว่าการใช้ cyclosporine A ทั้งในด้านอัตราการรอดชีวิตของผู้ป่วยและในอัตราการรอดของไตปลูกถ่าย สำหรับตัวแปรอื่นที่มีผลต่อผลลัพธ์ดังกล่าว ได้แก่ อายุของผู้บริจาคไตและผู้รับบริจาคไต, โรคไตเดิมของผู้ป่วย, การมีโรคร่วมทางระบบหลอดเลือดและหัวใจของผู้ป่วย, ชนิดของการปลูกถ่ายไต (การปลูกถ่ายจากผู้บริจาคที่มีชีวิตหรือผู้เสียชีวิตจากอุบัติเหตุหรือโรคทางหลอดเลือดสมอง), ค่า panel reactive antibody ที่สูงที่สุดก่อนการปลูกถ่ายไต, ผลการตรวจ hepatitis C virus ของผู้บริจาค, ค่า serum creatinine ของผู้บริจาคไตก่อนการตัดไตบริจาค, cold ischemia time, และการใช้ MPA หรือ prednisolone หลังการปลูกถ่ายไต ด้วยสมการการทำนายนี้ทำให้สามารถคัดกรองผู้ป่วยที่มีความเสี่ยงสูงต่อการเสียชีวิตหรือการสูญเสียไตปลูกถ่ายได้ตั้งแต่ช่วงสัปดาห์แรกหลังการผ่าตัดปลูกถ่ายไต ผู้ป่วยที่มีความเสี่ยงสูงนี้ควรได้รับการตรวจติดตามการทำงานของไตปลูกถ่ายรวมไปถึงการติดตามระดับของยากดภูมิคุ้มกันอย่างใกล้ชิดมากกว่าผู้ป่วยที่มีความเสี่ยงต่ำ นอกจากนั้นประโยชน์ของสมการเพื่อการทำนายผลของการปลูกถ่ายไตในระยะยาวนี้ยังสามารถใช้แจ้งข้อมูลเบื้องต้นให้แก่ผู้ป่วยและญาติได้ตั้งแต่ในระยะแรกหลังการผ่าตัด เพื่อให้มีความรู้ความเข้าใจเกี่ยวกับแนวทางการดำเนินโรคและการป้องกันภาวะแทรกซ้อนต่าง ๆ ที่อาจเกิดขึ้นหลังการผ่าตัด

สำหรับการตรวจติดตามระดับยา tacrolimus ในเวชปฏิบัตินั้น การตรวจระดับของ tacrolimus ภายใน peripheral blood mononuclear cells (PBMCs) เป็นทางเลือกที่น่าสนใจในการทำ TDM เนื่องจากเป็นบริเวณที่ tacrolimus มีการออกฤทธิ์ในการกดภูมิคุ้มกันโดยตรง และอาจจะแสดงส่วน active ของ tacrolimus ได้ดีกว่าการตรวจระดับของ tacrolimus ใน whole blood เหมือนดังที่ทำกันเป็นมาตรฐานในปัจจุบัน ในส่วนที่ 2 ของวิทยานิพนธ์นี้ประกอบไปด้วยการศึกษาหลายการศึกษาเพื่อวิเคราะห์การตรวจระดับของ tacrolimus ภายในเซลล์ต่าง ๆ ของ PBMCs โดยในขั้นตอนแรกนั้นกระบวนการแยก PBMCs จากเลือดของผู้ป่วยที่ได้รับการปลูกถ่ายไตได้ถูกทำการวิจัยว่ามีการปนเปื้อนของ granulocytes ระหว่างการเกิด acute rejection หรือไม่ เนื่องจากมีรายงานการศึกษาว่าการมีภาวะการอักเสบในร่างกาย เช่น การติดเชื้อในกระแสเลือดหรือการมีแผลไฟไหม้ขนาดรุนแรงนั้นเพิ่มอัตราการปนเปื้อนของ granulocytes ใน PBMCs ได้ ใน**บทที่ 7** ของวิทยานิพนธ์นี้แสดงให้เห็นว่าการใช้ Ficoll density gradient separation เพื่อการแยก PBMCs ออกจาก whole blood ของผู้ป่วยที่ได้รับการปลูกถ่ายไตไม่ได้ทำให้เกิดการปนเปื้อนของ granulocytes มากไปกว่าที่พบในการแยก PBMCs ในประชากรทั่วไป การใช้เทคนิค Ficoll density gradient separation นี้จึงเป็นวิธีที่สามารถนำมาใช้แยก PBMCs ออกจาก whole blood ของผู้ที่ได้รับการปลูกถ่ายไตได้

ในทางทฤษฎีแล้วระดับของยา tacrolimus ใน PBMCs ควรมีความสัมพันธ์ที่ผกผันกับการเกิด acute rejection กล่าวคือหากระดับยา tacrolimus ใน PBMCs มีขนาดสูง อัตราการเกิด acute rejection ควรจะลดลง อย่างไรก็ตามหลายการศึกษาในอดีตนั้นพยายามทดสอบสมมติฐานนี้แต่ก็ไม่สามารถแสดงให้เห็นถึงความสัมพันธ์นี้ได้ เนื่องจาก PBMCs ประกอบไปด้วยเซลล์ในระบบภูมิคุ้มกันที่หลากหลาย ได้แก่ T lymphocytes, monocytes, B lymphocytes, และ natural killer (NK) cells การศึกษาใน**บทที่ 8** ของวิทยานิพนธ์นี้จึงมุ่งเป้าไปที่การตรวจวัดระดับของ tacrolimus ภายใน T lymphocytes และ monocytes ซึ่งเป็น

เซลล์ในระบบภูมิคุ้มกันที่มีจำนวนมากเป็นลำดับแรกและลำดับสองของ PBMCs และยังเป็นเซลล์ที่มีส่วนสำคัญในการเกิด alloimmune recognition และ acute rejection process ตามมา โดยข้อมูลจากตัวอย่าง 61 ตัวอย่างจากผู้ป่วยที่ได้รับการปลูกถ่ายไต 53 รายได้ถูกนำมาวิเคราะห์เพื่อหาความสัมพันธ์ระหว่างระดับ tacrolimus ภายใน T lymphocytes และ monocytes และการเกิด acute rejection โดยถึงผลการศึกษาหลักนั้นจะไม่สามารถแสดงให้เห็นความสัมพันธ์นี้ได้ แต่ก็พบผลการศึกษาสำคัญ 2 ประการได้แก่ การที่ระดับของยา tacrolimus ใน T lymphocytes นั้นมีค่าต่ำกว่า monocytes อย่างมีนัยสำคัญ และการที่ระดับยา tacrolimus ในตัวอย่างที่เป็น cryopreserved samples น้อยกว่า freshly isolated samples ข้อสรุปของการศึกษานี้ที่สำคัญคือความจำเป็นที่ต้องใช้ freshly isolated cells ในการตรวจระดับของ tacrolimus ภายในเซลล์

บทที่ 9 ของวิทยานิพนธ์นั้นได้อธิบายกลไกของการที่ระดับยา tacrolimus ภายใน T lymphocytes นั้นต่ำกว่าระดับยา tacrolimus ภายใน monocytes โดยใช้การศึกษาที่วัดปริมาณของโปรตีนที่สำคัญต่อ intracellular pharmacokinetics ของยา tacrolimus คือ FK-binding protein-12 (FKBP-12) และ P-glycoprotein (P-gp) โดย FKBP-12 นั้นเป็น binding protein ของ tacrolimus ที่อยู่ภายใน cytosol ของเซลล์ และ P-gp นั้นเป็น efflux transporter ของยา tacrolimus ผลการศึกษาหลักพบว่า T lymphocytes นั้นมี P-gp ที่มากกว่า และ FKBP-12 ที่น้อยกว่า monocytes ซึ่งความแตกต่างนี้สามารถอธิบายระดับของยา tacrolimus ที่แตกต่างกันในเซลล์ทั้ง 2 ชนิดนี้ได้ นอกจากนั้นการใช้ verapamil ซึ่งเป็น P-gp inhibitor ใส่ในน้ำยาที่ใช้ระหว่างกระบวนการ cell isolation ยังส่งผลให้ระดับของยา tacrolimus ใน T lymphocytes ของผู้ป่วยที่ได้รับการปลูกถ่ายไตนั้นสูงกว่าระดับยา tacrolimus ใน T lymphocytes จากผู้ป่วยรายเดียวกันแต่ไม่ได้ใช้ verapamil ระหว่างกระบวนการ cell isolation อีกด้วย ซึ่งเป็นสิ่งที่ยืนยันว่ามีการสูญเสีย intracellular tacrolimus ใน T lymphocytes ไปในระหว่างกระบวนการ cell isolation ผ่าน P-gp efflux transporter ดังนั้นในการศึกษาที่เกี่ยวข้องกับการวัดระดับของยา tacrolimus ภายใน T lymphocytes หรือใน PBMCs (ซึ่งมี T lymphocytes เป็นกลุ่มหลัก) ในอนาคตนั้นจำเป็นต้องใช้ P-gp inhibitor ระหว่างกระบวนการ cell isolation เพื่อให้สามารถวัดระดับของ tacrolimus ในเซลล์ได้อย่างแม่นยำ

ในลำดับถัดไปจะกล่าวถึงการวิเคราะห์ผลการศึกษาทั้งหมดตั้งแต่บทที่ 3 ถึงบทที่ 9 ของวิทยานิพนธ์นี้ และแนวทางการประยุกต์เพื่อนำไปใช้ในเวชปฏิบัติ ร่วมกับแนวทางในดำเนินการศึกษาเพื่อต่อยอดในอนาคต

Chapter 11

General Discussion and Future Perspectives

GENERAL DISCUSSION

The outcome of patients who receive a kidney transplant is superior to those of patients who are treated with other forms of renal replacement therapy, especially in terms of quality of life and survival.[1] However, although acute rejection rates and 1-year allograft survival have much improved, the long-term outcomes of transplantation, including allograft and patient survival, have not improved to a comparable degree.[2-5] Multiple complications after transplantation negatively affect the survival and function of the kidney allograft. Clinicians have to deal with the consequences of rejection (both acute and chronic), cardiovascular comorbidities, infections, and the adverse effects (toxicity) of immunosuppressive drugs. It is important to identify and stratify patients at risk for these complications. This can be done by using various clinical tools and with the help of laboratory investigations. The research described in this thesis aimed to develop prediction models and to optimize monitoring of the immunosuppressant tacrolimus, which remains the cornerstone of immunosuppressive therapy after transplantation. Here the findings of the work described in this thesis are discussed.

The prediction of kidney transplantation outcomes

Predicting of acute rejection

The incidence of acute rejection after kidney transplantation has decreased since triple maintenance immunosuppression consisting of tacrolimus, mycophenolic acid (MPA), and corticosteroids has become standard of care.[6-8] However, acute rejection occurs in as much as 10-20% of kidney transplant recipients and acute rejection therefore still is an important complication. The importance of acute rejection lies in the fact that it negatively affects long-term transplantation outcome.[2] The analysis of a large kidney transplant registry showed that acute rejection was associated with allograft failure, death from cardiovascular disease and cancer.[3] The mechanism behind this association is that poor kidney allograft function and proteinuria after an episode of acute rejection increase the risk of cardiovascular disease and death.[9] The impairment of anti-tumor surveillance and immunity to oncogenic viruses after high-intensity immunosuppressive treatment for acute rejection is responsible for cancer development.[10]

Several risk factors for acute rejection have been identified and these include deceased donor kidney transplantation (as opposed to live donor transplantation), a higher number of human leukocyte antigen (HLA) mismatches, repeat transplantation, age of the recipient (higher risk in younger patients), black ethnicity, the use of cyclosporine A (compared with tacrolimus), and poor compliance.[11-14] However, these epidemiologic data and clinical information are not accurate enough to predict acute rejection and to differentiate recipients with a high rejection risk from those with a low rejection risk.[15]

The adaptive immune response plays a central role in T cell-mediated rejection (TCMR).[12, 16] Laboratory investigations that explore biomarkers involved in allo-recognition and rejection initiation have been evaluated in cohorts of kidney transplant recipients to determine their performance to diagnose acute rejection.[12] These biomarkers include urinary molecules such as granzyme B or perforin, urinary or blood messenger RNA (mRNA) of gene-encoding chemokines such as CXCL9 or CXCL10, donor-derived cell-free DNA (dd-cfDNA), urinary extracellular vesicles (uEV), or gene expression panels such as the Kidney Solid Organ Response Test (kSORT).[17-19] Many of these biomarkers are still in the developmental phase and have not been validated in follow-up studies, while their efficacy to diagnose acute rejection in the real-world remains to be demonstrated.[18, 20] Importantly, most of these biomarkers do not predict (future) acute rejection.

The enzyme-linked immunosorbent spot (ELISPOT) assay has been proposed as a biomarker to predict acute rejection in kidney transplantation.[21-23]. ELISPOT is an assay that quantifies the amount of cytokine-producing T lymphocytes in response to a specific antigen which can then be used to evaluate the immune status in transplant recipients.[22, 24] Previous studies reported a good performance of the donor-specific ELISPOT assay in predicting acute rejection.[22-25] However, these studies differed in terms of the type of acute rejection that was studied, the cutoff values that were used to define positive and negative results, and the timing of ELISPOT assay testing (pre-transplantation *vs*. early post-transplantation to predict later allograft rejection).[21-26] Therefore, a meta-analysis of the donor-specific ELSIPOT assay to predict acute rejection was conducted (Chapter 4). Interestingly, the odds ratios (OR) of the recipients with a positive donor-specific interferon-γ (IFN-γ) ELISPOT to develop TCMR were 2.81 when the assay was performed before transplantation and 12.65 when ELISPOT testing was performed after transplantation. This demonstrates the good predictive performance of IFN-γ ELISPOT for TCMR. However, because of its high negative predictive value (NPV; 81% for the pre-transplant ELISPOT and 79% for the post-transplant ELISPOT) and poor positive predictive value (PPV) (43% for the pre-transplant ELISPOT and 54% for the post-transplant ELISPOT), the ELISPOT assays are best used for the exclusion of acute rejection rather than its confirmation. An example of how the IFN-γ ELISPOT assay may be used in clinical practice is the situation when a patient has allograft dysfunction. Patients who then test positive in the donor-specific IFN-γ ELISPOT will need a second and confirmatory test (such as an allograft biopsy) to demonstrate acute rejection. In contrast, whenever such a patient tests negative in the donor-specific IFN-γ ELISPOT, other causes than rejection likely explain graft deterioration. Unnecessary increases of the immunosuppressive drugs can thus be avoided. This proposed management strategy should be tested in a future prospective cohort or randomized, controlled trial.[27] The results of the ongoing clinical trials are expected to elucidate more information on the use of donor-specific IFN-γ ELISPOT assay (Clinical-

Trials.gov identifier NCT03465397 and NCT03652402).

Furthermore, the results from this donor-specific IFN-γ ELISPOT assay can be applied to adjust the immunosuppression regimen. A patient with a positive donor-specific IFN-γ ELISPOT might require more intensive immunosuppression and minimization of immunosuppressants should be avoided. On the contrary, a patient with a negative donor-specific IFN-γ ELISPOT result may benefit from a lower dose of immunosuppressants. However, since the ELISPOT results can change dynamically after transplantation,[25] the assay should be periodically assessed (for example at 3 months and 6 months after transplantation) and the optimal cutoff according to the local laboratory must be identified. With such scheduled measurements, the ELISPOT assay could serve as an "immunostat" for kidney transplant recipients, which can continuously monitor a patient's immune status. The immunosuppressive therapy can then be adjusted accordingly.

Predicting the clinical course of BK virus infection

BK virus (BKV) is one of the most common opportunistic viral infections after kidney transplantation.[28] It is also a common cause of infection-related kidney allograft failure.[29-31] BKV infection is found in more than 90% of healthy individuals and has an extremely low morbidity.[32] However, in immunocompromised patients, such as kidney transplant recipients, BKV can replicate (either as a result of a re-activation of a latent infection of the recipient or as a donor-derived *de novo* infection) resulting in BK viremia. BK viremic recipients can progress to BKV-associated nephropathy (BKVAN) which is an important cause of allograft loss.[33] Multiple risk factors for BKV reactivation or viremia have been identified and include donor and recipient serostatus, dosage and exposure to immunosuppressants, the use of T cell-depleting agents, a previous rejection episode, the number of HLA mismatches, and a ureteral stent.[31] The best practice is to detect BKV reactivation early to prevent disease progression and the development of BKVAN. There is no specific anti-BKV drug available and post-transplant screening for BK viremia is recommended in all kidney transplant recipients. Once BK viremia is detected, reduction of immunosuppression is recommended. This can be done by reducing the dose of MPA or the calcineurin inhibitor (CNI) by 50% or withdraw MPA altogether. This immunosuppression reduction strategy result in approximately 80% of cases in clearance of the BK viremia.[34] Unfortunately, 10-20% of patients will have persistent BK viremia and these patients need further immunosuppressive drugs reduction or a second line treatment.[34] However, none of these second line treatments (which include leflunomide, cidofovir, fluoroquinolones, or intravenous immunoglobulin) has proven to be effective.[31, 32] Importantly, BK viremic recipients can develop acute rejection after immunosuppression reduction which also significantly increases the risk of allograft loss.[35] Taken together, BKV infection is a problem and may result in allograft loss. A clinical tool to guide

the management of immunosuppressive therapy in case of BK viremia is needed.

To defend the host against BKV, BKV-specific $CD4^+$ T lymphocytes produce and secrete of pro-inflammatory cytokines including IFN-γ. With this knowledge, a BKV-specific IFN-γ ELISPOT assay was developed and investigated.[32, 36, 37] By using the BKV-specific IFN-γ ELISPOT assay in patients with BK viremia, clinicians may be able to predict the clinical course and adjust the immunosuppressants accordingly. However, these studies were limited by the low number of included patients. Furthermore, the cutoff value to define negative and positive ELISPOT results in each study was different. For these reasons, a meta-analysis of the BKV-specific IFN-γ ELISPOT was performed (described in Chapter 5). This meta-analysis demonstrated that BK viremic patients with a negative BKV-specific IFN-γ ELISPOT were at 71.9-fold higher risk for an ongoing, active BKV replication, compared with a BK viremic recipient with a positive ELISPOT result. Patients with a positive BKV-specific IFN-γ ELISPOT are the patients who have immune reactivity against the virus. These patients are more likely to clear the virus without the need for additional immunosuppressant reduction or any second line treatment. In contrast, BK viremic patients with a negative BKV-specific IFN-γ ELISPOT are at risk for an ongoing BKV infection and the development BKVAN. In this group, immunosuppressants can be reduced more aggressively or second line therapy can be initiated earlier.

According to the guideline from the American Society of Transplantation Infectious Diseases Community and Practice, BK viral load screening is recommended monthly during the first 6-9 months post-transplantation and every three months thereafter until 2 years.[34] BKV-specific IFN-γ ELISPOT could be implemented and assessed every 3-6 months, at the same time of BK viral load screening. When interpreted together, these two tests might benefit the patient and guide the immunosuppressive management. Patients without BK viremia but who have a recent change in their BKV-specific IFN-γ ELISPOT result, such as a drop in the number of IFN-γ producing cells or a change from a previously positive to a negative BKV-specific ELISPOT, may need more frequent BK viral load testing or empirical reduction of immunosuppression. Once patients have developed BK viremia, the BKV-specific IFN-γ ELISPOT can inform on the current immune status of the patient and if further immunosuppression reduction is needed. With this strategy, the optimal immunosuppression could be achieved in order to clear the virus, while avoiding unnecessary immunosuppression reduction and the risk of acute rejection.

In addition to BKV, a meta-analysis of the cytomegalovirus (CMV)-specific IFN-γ ELISPOT assay has shown an acceptable sensitivity (73-84%) but a relatively lower specificity (46-61%) in predicting active CMV viremia after kidney transplantation. This indicates that the CMV-specific IFN-γ ELISPOT assay is good at identifying high-risk patients

although the number of false positive patients is high.[38] ELISPOT assays to predict other common virus infections such as those with Epstein-Barr virus, herpes simplex virus, or varicella-zoster virus are still in development.[39-41]

Prediction of tacrolimus-nephrotoxicity

Since tacrolimus was first used in liver transplant recipients in 1989,[6] it has become the cornerstone immunosuppressant after all types of solid organ transplantation (kidney, liver, heart, lung and small intestine) and is likely to remain so for the next decade.[7, 8] Due to its narrow therapeutic window and high inter-patient variability, therapeutic drug monitoring (TDM) of tacrolimus is required to ensure therapeutic efficacy and limit toxicity.[42] Whole blood pre-dose concentration (C_0) monitoring is the routine TDM strategy for tacrolimus in clinical practice.[43, 44] However, the association between tacrolimus C_0 and clinical outcomes after kidney transplantation is far from perfect. Many patients develop acute rejection despite having an adequate C_0 and other develop tacrolimus-related toxicity despite their C_0 being on target.[45]

One of the strategies to adjust the dosage of tacrolimus is the use of clinical pharmacogenetics. Cytochrome P450 3A5 (CYP3A5) is the main tacrolimus metabolizing enzyme and is expressed in the liver and small intestine. Kidney transplant recipients with the *CYP3A5*1/*1* or *CYP3A5*1/*3* genotype (the expressor genotype and fast metabolizer phenotype) require a significantly higher dosage of tacrolimus to achieve the same target (concentration) compared with the recipients with *CYP3A5*3/*3* (the non-expressor genotype and the slow metabolizer phenotype).[46] A population pharmacokinetic analysis demonstrated that recipients with the *CYP3A5**1/*1 or *CYP3A5**1/*3 genotype require a 1.62-times higher dose of tacrolimus compared with recipients who have the *CYP3A5**3/*3 genotype to achieve the same tacrolimus exposure.[47] These observations have been reported by many other researchers, both in adults and children and in different types of solid organ transplant recipients.[46, 48-52] When the tacrolimus starting dose was guided by this population pharmacokinetic model in *de novo* kidney transplant recipients, 58% of the recipients successfully achieved the target tacrolimus C_0 on day 3, compared with only 39% "on target" when the tacrolimus starting dose was based on body weight alone.[53] Because of the difference in the rate of tacrolimus metabolism between patients with different *CYP3A5* genotypes, recipients with the *CYP3A5* expressor genotype might be at higher risk for acute rejection (resulting from sub-therapeutic tacrolimus concentrations) although this observation requires confimation.[54-56] In addition, *CYP3A5* genotype may also relate to the risk of developing tacrolimus-associated nephrotoxicity.

The pathophysiology of tacrolimus-associated nephrotoxicity relates to the drug's effects on the renal vasculature and tubulo-epithelium. The vascular effects of tacrolimus

are the vasoconstriction of the afferent renal arterioles that result from increased endothelin and thromboxane production, the activation of the renin-angiotensin-aldosterone system (RAAS), and a reduction of vasodilatory factors such as prostacyclin, prostaglandin, and nitric oxide.[56] This in turn causes endothelial dysfunction, platelet aggregation, and thrombotic microangiopathy. If this process continues for a prolonged period of time, glomerular ischemia, glomerulosclerosis, arteriolar hyaline thickening, tubulo-interstitial ischemia, interstitial fibrosis, and tubular atrophy will develop and decreased kidney function ensues.[57] Tacrolimus also causes so-called isometric tubular vacuolization.[58, 59] The actual pathogenesis of isometric vacuolization is still unknown but it is thought to result from the disruption of the endoplasmic reticulum and/or mitochondria.[58]

Previous studies showed that kidney transplant recipients with a fast metabolizer phenotype were at risk for tacrolimus-associated nephrotoxicity although this association is inconsistent.[59-63] Since kidney tissue also expresses CYP3A5,[64] the allograft (donor) *CYP3A5* genotype might also have an effect on tacrolimus-associated nephrotoxicity. The role of different combinations of donor-recipient *CYP3A5* genotype in terms of the risk of tacrolimus-associated nephrotoxicity has not been established previously. This was investigated in Chapter 3. We observed that kidney transplant recipients with a *CYP3A5* expressor genotype who received an allograft from a non-expressing donor (and who therefore donated a non-CYP3A5 expressing kidney allograft) were at the highest risk to develop tacrolimus-nephrotoxicity compared with other *CYP3A5* donor-recipient combinations. This finding suggests that the rate of intra-allograft tacrolimus metabolism in the non-expressor kidney was not rapid enough to clear the high tacrolimus dose that is prescribed to recipients with the *CYP3A5* expressor genotype (who need more tacrolimus to get to target). This may result in the accumulation of tacrolimus in the allograft and cause nephrotoxicity. The monitoring of C_0 only will not prevent tacrolimus-associated nephrotoxicity in such cases. The measurement of tacrolimus concentration and possibly its metabolites, in the kidney allograft itself would give more information and further elucidate the role of renal CYP3A5 on the intra-allograft tacrolimus metabolism and nephrotoxicity.

So how should this finding be integrated into clinical decision making? We believe that recipients who are intrinsic *CYP3A5* expressors should have the option to not receive a kidney allograft from a *CYP3A5* non-expressor living donor if multiple potential donors are available. If genotyping is not done during the pre-transplantation evaluation, *CYP3A5* genotyping after transplantation might still be beneficial. Kidney transplant recipients with a high nephrotoxicity risk (from donor-recipient *CYP3A5* mismatching) might need a lower target tacrolimus C_0 compared with other *CYP3A5* combinations. The use of CNI-free or CNI minimization regimens should also be considered in this group. However, this has to be balanced with the risk of acute rejection. We believe that the possibility to reduce the nephrotox-

ic effects of tacrolimus based on donor-recipient *CYP3A5* matching should be investigated.

Predicting long-term outcome of kidney transplantation

Most but not all, kidney transplant recipients have adequate allograft survival at 5-10 years after transplantation despite the fact that the post-transplant clinical course is often complicated by rejection, infection, recurrent kidney disease, cardiovascular disease, or malignancy. Each of these complications may directly or indirectly affect the survival of the patient or kidney allograft and its function. These complications are related to multiple factors including the immunosuppressive regimen, the recipient's comorbidities and underlying kidney disease, any donor's disease or pre-transplant donor kidney function, and the immunologic interactions between donor tissue and the recipient's immune system. To date, many kidney transplantation models have been developed to predict allograft and/or patient survival. These models were often developed by analyzing both pre-transplant and post-transplant information to make them as accurate as possible.[65-69] The development and validation cohorts in these studies yielded a good or satisfying predictive performance for kidney transplant outcomes, including allograft and patient survival. Sadly, none of these models were developed based on populations outside North America or Europe. A prediction model for the Asian kidney transplant population, which represents approximately 60% of the global population,[70] is lacking. Differences in socio-economic factors, culture, healthcare systems, practices in transplant centers, organ allocation systems in place, and genetic variability between the Caucasian and Asian population suggest that these models cannot be used to accurately predict outcomes in the Asian population.

In Chapter 6, we describe the development of the first such model for an Asian (Thai) population. Pre-transplant information, perioperative factors, and immunosuppressive therapy were included in the model. The endpoints were 5- and 10-year allograft and patient survival.[71] The significant variables that were included in the final models were the age of the recipient and donor, the primary kidney disease, recipient cardiovascular comorbidities, the donor type, donor hepatitis C virus serology, peak panel reactive antibody, serum creatinine before donor nephrectomy, cold ischemia time, the use of tacrolimus *vs*. cyclosporine A, and the presence of mycophenolic acid and prednisolone. The model can be accessed free of charge at https://www.nephrochula.com/ktmodels/.

The 5-year patient survival in Thailand was 93-96% and the 5-year allograft survival was 88-93%. These numbers are comparable with the Japanese Transplant Registry and the European Renal Association-European Dialysis and Transplant Association Registry,[72, 73] and slightly better than the Scientific Registry of Transplant Recipients in United States.[74] We demonstrated (Chapter 6) that the use of a model developed for a Caucasian population[67] does not have an adequate performance when applied to an Asian population. This emphasiz-

es the importance to develop kidney transplant prediction models for one's own population and ethnicity. With such a model, clinicians will be able to consult patients and their families regarding transplantation outcomes. Moreover, the developed model can serve as a risk-stratifying tool for kidney transplant recipients. Recipients with an expected short allograft survival should receive more close monitoring of their immunosuppression and kidney allograft function, adherence should be ensured, and frequent surveillance for potential complications is required. This model might also be useful for the TELE-clinic for kidney transplant recipient who have a low risk of allograft failure.

Since the clinical course after transplantation can be influenced and altered by several complications that result in a dynamic change of allograft function, the model called DynPG integrated serum creatinine at multiple post-transplant time points to predict patient-graft survival probability.[68] iBox is another model that includes post-transplant information such as proteinuria, allograft function, biopsy results, and the presence of donor-specific anti-HLA antibodies. With iBox allograft survival can be accurately predicted at 3-, 5-, and 7-years in the future.[69] However, as mentioned earlier, these models and tools (DynPG, iBox) were developed based on Caucasian populations which might not directly apply to the Asian population. Besides differences in donor allocation systems and the frequency of HLA types, the clinical pharmacokinetics of immunosuppressive medications are also different between Asian and Caucasian patients. For example, the optimal dose of MPA in Asian transplant recipients is 20-40% lower than in Caucasian recipients.[75] The frequency of various *CYP3A5*1* allele is different across ethnicities; 85% in African-American, 56% in Asian, and 16% in Caucasian.[76, 77] Next-generation prediction models should therefore include patient data at a global level and include sufficient patients from different ethnicities. With the use of machine learning a model may be developed that includes variables that are dynamically changed during post-transplant follow-up.

Advances in intracellular tacrolimus concentration measurement

Tacrolimus binds with the cytosol protein, FK-binding protein-12 (FKBP12) and inhibits the enzyme calcineurin, thereby preventing de-phosphorylation of the nuclear factor of activated T cells (NFAT) and cytokine production (including interleukin (IL)-2).[78] Because of its high inter- and intra-individual pharmacokinetic variability, TDM is needed to optimize tacrolimus treatment. The parameter most widely used for tacrolimus TDM is the whole blood C_0. However, despite TDM, over and under-exposure to tacrolimus occur frequently and rejection still occurs. In fact, many patients develop acute rejection even when the whole blood tacrolimus C_0 falls within the therapeutic range.[45] Tacrolimus is 83% bound to erythrocytes, 16% to plasma proteins, and only 1% is unbound (the free fraction) which is considered the pharmacologically active tacrolimus.[79]

Since the main site of action of tacrolimus lies within the immune cells (where the tacrolimus receptor FKBP-12 is located), monitoring the intracellular tacrolimus concentration in peripheral blood mononuclear cells (PBMCs) has been proposed as a potentially better strategy for TDM than whole blood concentrations.[80-82] However, only a single study in liver transplant recipients could demonstrate an association between the intracellular tacrolimus concentration in PBMCs and acute rejection.[83] Other studies in heart, liver, or kidney transplantation could not replicate this finding.[80, 84-88] These conflicting findings may be explained by several factors. First, most of the studies did not include a red blood cell lysis step into the cell isolation protocol. As the majority of tacrolimus is located within erythrocytes, contamination of PBMCs with erythrocytes may lead to an overestimation of the intra-PBMC tacrolimus concentration. Second, the timing of PBMC sampling was different from the timing of acute rejection in many studies. Third, PBMCs may not be the best matrix for the intracellular tacrolimus concentration measurement as PBMCs consist of many immune cell subsets including T lymphocytes, monocytes, B lymphocytes, and NK cells, which all have different functions in alloimmunity.[89]

$CD3^+$ T lymphocytes play a major role in allorecognition, the initiation of acute rejection, and providing the help to B lymphocytes for the humoral response against the kidney allograft.[90-93] In Part II of this thesis, a more specific matrix for tacrolimus concentration measurement than PBMCs was explored, namely the T lymphocyte and monocyte compartments. The current method for isolating PBMC uses Ficoll gradient density separation. Before the measurement of the intracellular tacrolimus concentration in the various immune cell subsets can be conducted, the purity of PBMCs after cell isolation has to be checked. The proportion of immune cells subsets in PBMCs from kidney transplant recipients with and without acute rejection was investigated in Chapter 7. This was the first experiment of our efforts to develop and optimize an assay for the measurement of the intracellular tacrolimus concentration in T lymphocytes and monocytes. We observed that the percentage of T lymphocytes, monocytes, B lymphocytes, and NK cells was not different between kidney transplant recipients (with or without acute rejection) and healthy volunteers. In addition, acute rejection did not result in increased granulocyte contamination after Ficoll density gradient separation which was previously reported for other inflammatory conditions such as burn wounds or sepsis.[94, 95] This finding supports the use of Ficoll gradient density separation as the method to isolate PBMC and its subsets in kidney transplant recipients.

For the first time, the intracellular tacrolimus concentration in $CD3^+$ T lymphocytes and CD14+ monocytes was measured and the association with acute rejection after kidney transplantation was investigated. We hypothesized that patients with rejection would have lower intracellular tacrolimus concentrations compared with non-rejectors. Although this association could not be established (contrary to our expectations), other important findings

were revealed. First, $CD3^+$ T lymphocytes were found to have a significantly lower intracellular tacrolimus concentration compared with $CD14^+$ monocytes. Second, the intracellular tacrolimus concentration in cryopreserved samples was lower than in freshly isolated samples. These observations raised further questions regarding the optimization of the cell isolation and other pre-analytical processes for the measurement of the intracellular tacrolimus concentration.

Based on the above-mentioned findings, another study was conducted to explain the mechanism behinds the different intracellular tacrolimus concentration in $CD3^+$ T lymphocytes and $CD14^+$ monocytes. Various experiments, including flow cytometric analysis, Western blot, and the Rhodamine-123 assay were performed. The results of the experiments all pointed in the same direction and showed a higher expression of P-glycoprotein (P-gp) and a lower expression of FKBP-12 in $CD3^+$ T lymphocytes than $CD14^+$ monocytes. P-gp is the efflux transporter of tacrolimus and this transporter appears to be much more abundant on $CD3^+$ T lymphocytes than in $CD14^+$ monocytes. This likely results in a lower intracellular tacrolimus concentration in $CD3^+$ T lymphocytes via enhanced tacrolimus efflux form the T lymphocyte cytosol to the cell exterior. By adding the P-gp inhibitor verapamil to the washing solutions and buffers used during the cell isolation, the intracellular tacrolimus concentration in $CD3^+$ T lymphocytes was increased by approximately 2-fold. It can be concluded that a substantial amount of the intracellular tacrolimus is being pumped out during the cell isolation process and before the measurement of the intracellular tacrolimus concentration, and the addition of the P-gp inhibitor verapamil helps to preserve this intracellular tacrolimus. This concentration (*i.e.* measured in the presence of verapamil) is likely to represent the "true" *in vivo* intracellular tacrolimus concentration and we believe the concentration thus measured should be used to evaluate the association with acute rejection in a future study. In addition, P-gp activity can explain the difference in the intracellular tacrolimus concentration between cryopreserved and fresh samples. Cells in the former group probably lose their intracellular tacrolimus during the additional thawing and washing steps (in the absence of a P-gp inhibitor). Taken together, we have improved our technique for the measurement of tacrolimus in immune cell subsets and have increased the knowledge of the intracellular pharmacokinetics of tacrolimus.

CONCLUSIONS

- Kidney transplant recipients with a positive pre-transplant or post-transplant donor-specific IFN-γ ELISPOT assay are at higher risk for acute rejection and worse allograft function than recipients with a negative test result. The donor-specific IFN-γ ELISPOT assay is best used for the exclusion of a high acute rejection risk due to its high negative predictive value.
- Kidney transplant recipients with the *CYP3A5* *1/*1 or *1/*3 genotype are at the highest risk for tacrolimus-associated nephrotoxicity when they receive allograft with the *CYP3A5**3/*3 genotype as compared with other donor-recipient *CYP3A5* combinations.
- BK viremic kidney transplant recipients with a negative BKV-specific IFN-γ ELISPOT test result are at higher risk for ongoing BKV replication. Recipients with a positive BKV-specific IFN-γ ELISPOT test result are more likely to have a self-limiting BKV infection.
- The developed Asian kidney transplantation prediction model for allograft and patient survival has a better predictive performance in this population, compared with models developed with Caucasian patients. This emphasizes the necessity to use a model that is developed based on one's own population.
- The higher P-gp and lower FKBP-12 expression in T lymphocytes contributes to the lower intracellular tacrolimus concentration in these cells compared with monocytes.
- The addition of the P-gp inhibitor verapamil prevents the loss of intracellular tacrolimus from T lymphocytes. Verapamil should be used in future experiments measuring the intracellular tacrolimus concentration in T lymphocytes.

FUTURE PERSPECTIVES

The donor-specific IFN-γ ELISPOT assay should be tested in a randomized, controlled trial or a prospective cohort study to demonstrate whether immunosuppressive drugs can be safely adjusted based on the result of this assay in comparison to standard, physician-based adjustment. For example, the result of ELISPOT testing may help choosing the type of induction therapy (based on pre-transplant ELISPOT) and the maintenance immunosuppressive regimen (based on post-transplant ELISPOT). Recipients with a negative donor-specific IFN-γ ELISPOT might be good candidates for CNI minimization. In con-

trast, recipients with a positive pre-transplant IFN-γ ELISPOT assay might need anti-thymocyte globulin induction (as opposed to no induction therapy or IL-2 receptor blockers) and should be targeted to a relatively higher tacrolimus concentration. Recently, a randomized controlled trial of tacrolimus monotherapy *versus* standard immunosuppression in kidney transplant recipients with a negative pre-transplant donor-specific IFN-γ ELISPOT and a negative pre-transplant donor-specific anti-HLA antibody (DSA) was conducted.[27] The trial was terminated due to the low recruitment rate (only 167 patients were recruited where 673 patients were targeted). The authors found that the incidence of biopsy-proven acute rejection (BPAR) was significantly higher in the tacrolimus monotherapy group than in standard triple immunosuppression group. In addition, the tacrolimus monotherapy group had a lower kidney allograft function at 12-months after transplantation. The authors concluded that the higher BPAR rate outweighs any potential benefit of tacrolimus monotherapy based on the negative ELISPOT and DSA only. However, the ELISPOT assay used in this study was performed only in pre-transplantation. It would be interesting if the post-transplant donor-specific ELISPOT assay could be done at 3- or 6-month interval, to continually monitor patients' immune status. The immunosuppressive regimen could then be more tailored in each individual patient regarding the post-transplant ELISPOT result. In addition, maintenance with only tacrolimus monotherapy might be too aggressive immunosuppression reduction. Other CNI-minimization strategies, such as using CNI with mammalian target of rapamycin inhibitor (mTORi), might be used.

The BKV-specific ELISPOT assay has the capability to predict the clinical course of a BKV infection in kidney transplant recipients. Future studies should be conducted with a randomized, controlled design and compare the use of ELISPOT to the standard of care. ELISPOT assays should be assessed at multiple time points, for example every 6-8 weeks during the episode of BK viremia. These serial measurements will provide a more dynamic insight of the recipient's immune status than a single time point measurement. It should be elucidated if the adjustment of immunosuppressive medication based on BKV-specific IFN-γ ELISPOT assay will increase the rate of BKV clearance and decrease superimposed acute rejection and BKVAN.

To further evaluate the role of *CYP3A5* genotype in tacrolimus-nephrotoxicity, the effect of *CYP3A5* matching between donor and recipient should be investigated in a prospective study or a randomized, controlled trial. These studies should target the tacrolimus concentration differently based on the *CYP3A5* combinations of donor and recipient. The *CYP3A5* expressing recipients who receive a non-expressor allograft should be targeted to a lower tacrolimus C_0 and this may prevent nephrotoxicity. Other immunosuppressive regimens such as CNI-free or CNI-minimization might be assigned to this group, and compared with the standard tacrolimus, MPA, and prednisolone regimen. The intra-allograft tacrolimus

concentration (and its metabolites) should also be explored and correlated with the *CYP3A5* matching between donor and recipient.[96, 97] An extra allograft tissue is required from the biopsy to analyze and measure the intra-allograft tacrolimus concentration and its association with nephrotoxicity or rejection features in the biopsy tissue.

The Asian kidney transplant prediction models presented in this thesis utilize the pre-transplant information, perioperative information, and choice of immunosuppressive medication to predict long-term outcomes. Since the long-term outcomes are significantly affected by the post-transplant clinical course and events, the addition of post-transplant information such as *de novo* donor-specific anti-HLA antibody levels, acute rejection episodes, kidney allograft biopsy results, allograft function, changes in immunosuppression, infectious complications, and cardiovascular-metabolic comorbidities after transplantation will probably improve the performance of the model. However, this requires extensive data collection of thousands of recipients and this can only be done by using multicenter or multinational transplant registries that include sufficient numbers of recipients of all major ethnicities. In addition, the diversity of socio-economic status of the patients across different countries, including the reimbursement and costs of transplantation, might also affect the outcomes of kidney transplantation and should be taken into consideration. The use of machine learning algorithms should be the primary focus of the next generation prediction model since it has shown the potential to improve the performance of traditional model.[98, 99]

The measurement of the intracellular tacrolimus concentration is a promising strategy for TDM of tacrolimus. In future cohort studies the association between the intracellular tacrolimus concentration in $CD3^+$ T lymphocytes should be explored with the addition of a P-gp inhibitor during the cell isolation process. Then the association between intracellular tacrolimus concentrations and clinical events such as acute rejection and allograft survival should be explored. If an association between the intracellular tacrolimus concentration and acute rejection is established, then the appropriate therapeutic range of the intracellular tacrolimus concentration should be identified. In addition, since the intracellular tacrolimus concentration measurement is still a labor-intensive and time-consuming procedure, the development of a population pharmacokinetic model to predict the intracellular tacrolimus concentration from whole blood concentration or several intracellular measurements might be necessary to increase the generalizability of the test. The intracellular tacrolimus concentration should also be evaluated in other immune cell subsets, including B lymphocytes and NK cells. The function of these cells could then be assessed and its can be explored whether they correlate with the intracellular concentrations. Moreover, it is possible that not only the intracellular tacrolimus concentration is important but the intracellular concentration of MPA or mammalian target of rapamycin inhibitor (mTORi) might also have a significant role in preventing acute rejection. Otherwise, combining the intracellular tacrolimus concentration

with pharmacodynamic monitoring of MPA might improve the performance of the prediction of acute rejection.[100, 101] Analytical methods for the measurement of the intracellular concentration of other immunosuppressants than tacrolimus are needed.

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Appendices

PHD PORTFOLIO

Name PhD student:	Suwasin Udomkarnjananun
Erasmus MC department:	Internal Medicine, Section Nephrology and Transplantation
Research school:	Postgraduate School Molecular Medicine
PhD period:	November 2020 – October 2022
Supervisors:	Prof. dr. Carla C. Baan (promotor) Dr. D.A. Hesselink (co-promotor)
Total workload:	39.7 ECTS

Courses and workshops	Year	ECTS
Erasmus MC - Survival Analysis	2020	0.6
Erasmus MC - Microsoft Excel 2010: Basic	2020	0.3
Erasmus MC - Microsoft Excel 2010: Advanced	2020	0.4
Erasmus MC - Molecular Medicine	2021	0.7
Erasmus MC - PowerPoint Tricks you didn't know	2021	0.3
Erasmus MC - OneNote Organize your digital information	2021	0.3
2021 Virtual Advanced Course in Basic and Clinical Immunology	2021	1.5
Erasmus MC - Biomedical English Writing	2021	2.0
Erasmus MC - Introduction in GraphPad Prism Version 7	2021	0.3
Erasmus MC - Scientific Integrity	2021	0.3
Conferences		
Asian Pacific Congress of Nephrology - Speaker "Prediction tool in kidney transplantation"	2020	5.0
Asian Pacific Congress of Nephrology - Attendee	2020	2.0
Thai Transplantation Society Congress - speaker "Difficult case in kidney transplantation"	2020	5.0
Thai Transplantation Society Congress - speaker "HIV infection and transplantation"	2020	5.0
ESOT oral presentation "Donor ELISPOT and acute kidney transplant rejection: A systematic review and meta-analysis"	2021	3.0
ESOT oral presentation "BK virus-specific ELISPOT assay in kidney transplantation: A systematic review and meta-analysis"	2021	3.0
IATDMCT oral presentation "The use of BK virus-specific ELISPOT as an immune monitoring tool in kidney transplantation: A systematic review and meta-analysis"	2021	3.0

Others	Year	ECTS
Transplantation lab meeting presentation	2021	2.5
Transplantation lab journal club	2021	2.5
Transplantation lab meeting presentation	2022	1.0
Teaching a master student	2021-2022	1.0

Awards	Year	ECTS
IATDMCT travel grant award	2022	-

ABOUT THE AUTHOR

Suwasin Udomkarnjananun was born on the 27th of February 1986 in Bangkok, Thailand. He finished his primary and secondary school education at Saint Gabriel's College, Bangkok, in 2003. He began studying medicine at The Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, in Bangkok, graduating with first class honors in 2009. He initially worked as a physician at Queen Savang Vadhana Memorial Hospital under the Thai Red Cross Society, Chonburi, Thailand from 2009 to 2012. Thereafter, he began his residency training in internal medicine at King Chulalongkorn Memorial Hospital, completing his training in 2015 before entering the fellowship training program in nephrology at the same hospital. In 2017, he became a nephrologist and started his fellowship training in kidney transplantation which he finished in 2018.

From 2018, Suwasin has been an attending nephrologist at the Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital under the Thai Red Cross Society, Bangkok, Thailand. In November 2020, he received a grant from the Thai Red Cross to conduct research at Erasmus MC Transplant Institute, Rotterdam, the Netherlands. He started his PhD study in the field of transplant immunology under the supervision of Professor Dr. Carla C. Baan and Dr. Dennis A. Hesselink. His research focuses on the prediction of immunosuppressant responses in kidney transplantation, and measurement of intracellular tacrolimus concentrations.

LIST OF PUBLICATIONS

Related to this thesis

Udomkarnjananun S, Francke MI, Dieterich M, van De Velde D, Litjens NHR, Boer K, De Winter BCM, Baan CC, Hesselink DA. P-Glycoprotein, FK-Binding Protein-12, and the Intracellular Tacrolimus Concentration in T-Lymphocytes and Monocytes of Kidney Transplant Recipients. Transplantation. 2022

Udomkarnjananun S, Dieterich M, Boer K, Hesselink DA, Baan CC. Immune Subsets from Ficoll Density Gradient Separation in Kidney Transplant Recipients. Transplant Direct. 2022 Apr 15;8(5):e1319.

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Udomkarnjananun S, Kerr SJ, Francke MI, Avihingsanon Y, van Besouw NM, Baan CC, Hesselink DA. A systematic review and meta-analysis of enzyme-linked immunosorbent spot (ELISPOT) assay for BK polyomavirus immune response monitoring after kidney transplantation. J Clin Virol. 2021 Jul;140:104848.

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Other publications

Takkavatakarn K, Phannajit J, **Udomkarnjananun S**, Tangchitthavorngul S, Chariyavilaskul P, Sitticharoenchai P, Praditpornsilpa K, Eiam-Ong S, Susantitaphong P. Association Between Indoxyl Sulfate and Dialysis Initiation and Cardiac Outcomes in Chronic Kidney Disease Patients. Int J Nephrol Renovasc Dis. 2022 Mar 26;15:115-126.

Udomkarnjananun S, Phannajit J, Takkavatakarn K, Tumkosit M, Kingpetch K, Avihingsanon Y, Praditpornsilpa K, Eiam-Ong S, Susantitaphong P. Effects of phosphate binders on bone biomarkers and bone density in haemodialysis patients. Nephrology (Carlton). 2022 May;27(5):441-449.

Francke MI, Andrews LM, Lan Le H, van de Velde D, Dieterich M, **Udomkarnjananun S**, Clahsen-van Groningen MC, Baan CC, van Gelder T, de Winter BCM, Hesselink DA. Monitoring intracellular tacrolimus concentrations and its relationship with rejection in the early phase after renal transplantation. Clin Biochem. 2022 Mar;101:9-15.

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Kengsakul M, Nieuwenhuyzen-de Boer GM, Bijleveld AHJ, **Udomkarnjananun S**, Kerr SJ, Niehot CD, van Beekhuizen HJ. Survival in Advanced-Stage Epithelial Ovarian Cancer Patients with Cardiophrenic Lymphadenopathy Who Underwent Cytoreductive Surgery: A Systematic Review and Meta-Analysis. Cancers (Basel). 2021 Oct 7;13(19):5017.

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Laowalert S, Khotavivattana T, Wattanachanya L, Luangjarmekorn P, **Udomkarnjananun S**, Katavetin P, Eiam-Ong S, Praditpornsilpa K, Susantitaphong P. Bone turnover markers predict type of bone histomorphometry and bone mineral density in Asian chronic haemodialysis patients. Nephrology (Carlton). 2020 Feb;25(2):163-171.

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Udomkarnjananun S, Townamchai N, Iampenkhae K, Petchlorlian A, Srisawat N, Katavetin P, Sutherasan M, Santingamkun A, Praditpornsilpa K, Eiam-Ong S, Avihingsanon Y. Furosemide Stress Test as a Predicting Biomarker for Delayed Graft Function in Kidney Transplantation. Nephron. 2019;141(4):236-248.

Vanichanan J, **Udomkarnjananun S**, Avihingsanon Y, Jutivorakool K. Common viral infections in kidney transplant recipients. Kidney Res Clin Pract. 2018 Dec;37(4):323-337.

Udomkarnjananun S, Townamchai N, Virojanawat M, Avihingsanon Y, Praditpornsilpa K. An Unusual Manifestation of Calcineurin Inhibitor-Induced Pain Syndrome in Kidney Transplantation: A Case Report and Literature Review. Am J Case Rep. 2018 Apr 14;19:442-446.

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"Names don't matter. What's important is how you live your life."

Ramza Beoulve, 1997