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Individualization of the Immunosuppressive Therapy in Patients of Renal Transplantations through Pharmacodynamic and Pharmacogenetic Biomarkers

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This thesis is dedicated to my parents

For their endless love and support

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List of abbreviations

6-MP	6-mercaptopurine
ABCB1	ATP-binding cassette sub-family B member 1 (P-glycoprotein 1)
ACK buffer	Ammonium-Chloride-Potassium buffer
AMV	Avian myeloblastosis virus
AUC	Area under the curve
AZA	Azathioprine
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CNIs	Calcineurin inhibitors
CO2	Carbon dioxide
Cq	Quantification cycle
CsA	Cyclosporine A
CyP	Cyclophilin
CYP	Cytochrome P450
DGF	Delayed graft function
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
Evr	Everolimus
FDA	Food and Drug Administration
FKBP-12	FK binding protein-12
FOXP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN- γ	Interferon gamma
IL-2	Interleukin 2
LD	Linkage disequilibrium
LOD	Limit of Detection
LOQ	Limit of quantification
MDRD	Modification of Diet in Renal Disease
MPA	Mycophenolic acid
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORIs	Mammalian target of rapamycin inhibitors
NFAT	Nuclear factor of activated T-cells
NIH	National Institutes of Health
p70S6K1	70 kDa ribosomal protein S6 kinase1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PGx	pharmacogenetic
PI3K	Phosphoinositide 3-kinase
PK	Pharmacokinetic
PMA	Phorbol 12-Myristate 13-Acetate
p-p70S6K	phosphorylated p70 ribosomal protein S6 kinase1
pS6RP	Phosphorylated S6 ribosomal protein
qPCR	Quantitative real-time polymerase chain reaction
RBCs	Red blood cells

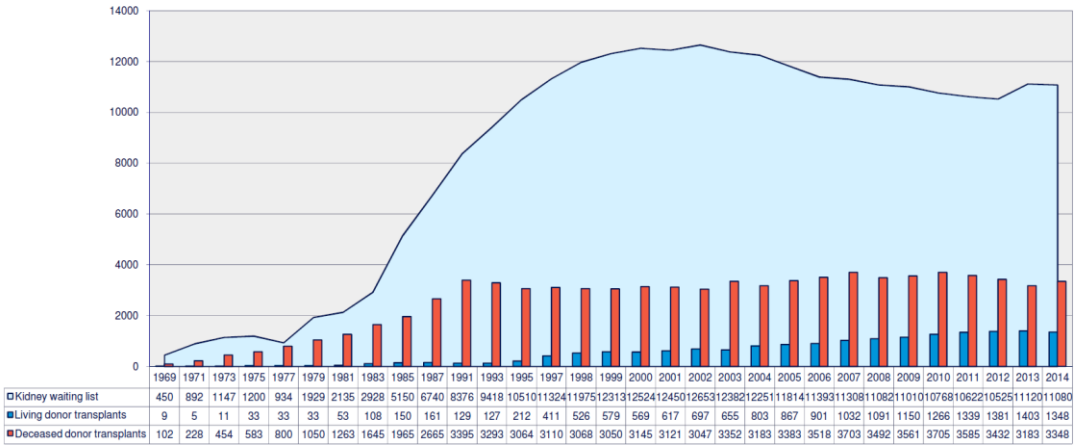
RGE	Residual gene expression
RNA	Ribonucleic acid
RPMI 1640 Medium	Roswell Park Memorial Institute 1640 Medium
rs Number	Reference SNP ID number
RT-qPCR	Reverse transcription–qPCR
RTx	Renal transplantation
S6RP	Ribosomal protein S6
Sir	Sirolimus
SNP	Single nucleotide polymorphisms
Tac	Tacrolimus
TDM	Therapeutic drug monitoring
TPMT	Thiopurine methyltransferase enzyme
VKORC1	Vitamin K epoxide reductase complex subunit 1
WBCs	White blood cells

1 Introduction

1.1 Renal transplantation (RTx)

Renal transplantation (RTx) is the treatment of choice for many patients with end-stage renal disease since it provides lower mortality rates and better quality of life compared to the conventional hemodialysis **(Tonelli et al., 2011)**. The first successful RTx was carried out in 1954 in Brigham hospital in Boston, USA by Murray and donor and recipient of the renal graft were identical twins (**Merrill et al., 1956)**.

Advanced surgical techniques, tissue typing and most importantly various immunosuppressive strategies over the past decades has led to increase in the number of transplants and improvement in transplantation outcomes. Nowadays, transplantation medicine is recognized to be part of the standard professional practice in developed countries **(Neipp et al., 2009)**. According to the German Foundation for Organ Transplantation (Deutsche Stiftung Organtransplantation – DSO), an average of ten organs are transplanted every day in Germany.



	1969	1971	1973	1975	1977	1979	1981	1983	1985	1987	1991	1993	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Kidney waiting list	450	892	1147	1200	934	1929	2135	2928	5150	6740	8376	9418	10510	11324	11975	12313	12524	12450	12653	12382	12251	11814	11393	11308	11082	11010	10768	10622	10525	11120	11080
Living donor transplants	9	5	11	33	33	33	53	108	150	161	129	127	212	411	526	579	569	617	697	655	803	867	901	1032	1091	1150	1266	1339	1381	1403	1348
Deceased donor transplants	102	228	454	583	800	1050	1263	1645	1965	2665	3395	3293	3064	3110	3068	3050	3145	3121	3047	3352	3183	3383	3518	3703	3492	3561	3705	3585	3432	3183	3348

Figure 1: Dynamics of the Eurotransplant kidney transplant waiting list and transplants between 1969 and 2014 (statistics of Eurotransplant Organization)

1.2 Immunosuppressive drugs

Transplantation of unrelated organs triggers immunological defense reaction, potentially resulting in rejection of the transplanted organ. Consequently, balanced suppression of the alloimmune response through the use of effective immunosuppressive protocols is an essential pre-requisite for successful organ transplantation.

Managing the host's immune response after organ transplantation has passed through many stages. Total body irradiation, corticosteroids and azathioprine were among the earliest immunosuppressive strategies used. However their use was complicated by considerable toxicity and high rejection rates. The first immunosuppressive protocol which showed reasonable results was a combination of azathioprine and steroids. This regimen resulted in a 1-year graft survival rate of around 50% and it was the dominant protocol for over 20 years. The introduction of cyclosporine A (CsA) in the early 1980s has opened a new avenue in the field of transplantation medicine with a 1-year graft survival of > 90% **(Taylor et al., 2005)**.

The discovery of CsA was followed by introduction of other efficacious agents and the number of immunosuppressive drugs increased rapidly over the last few decades. Currently, there are four main groups of drugs used as maintenance therapy after transplantation: calcineurin inhibitors (CNIs) (CsA and tacrolimus [Tac]), mammalian target of rapamycin inhibitors (sirolimus (Sir) and everolimus (Evr)), antiproliferative agents (azathioprine [AZA] and mycophenolic acid [MPA]) and corticosteroids. Furthermore, there are several biologic agents available for the initial induction of immunosuppression; two of them are used widely, anti-thymocyte globulin or interleukin-2 receptor antibody **(Kalluri and Hardinger, 2012)**.

Modern immunosuppressive protocols after organ transplantation are based on the combination of drugs which target the immune response at different levels. The aim of such combinations is to increase the overall efficacy and decrease the toxicity of the individual agents. Whereas different centers adopt different strategies, the most widely accepted regimen comprises a combination of a CNI (CsA or Tac), and anti-proliferative agent (MPA) or a mTOR inhibitor (Sir or Evr) with or without antibody induction and steroids **(Halloran, 2004)**.

1.2.1 Calcineurin inhibitors

CNIs include CsA and Tac. Both drugs are used extensively in transplantation medicine and most immunosuppressive protocols contain one of these CNI.

Despite the difference in chemical structure, both CsA and Tac target the same pathway (Figure 2) After entering the cell, they are complexed with an immunophilin. CsA binds

to cyclophilin while Tac forms a complex with the immunophilin known as FK binding protein-12 (FKBP-12). The drug-immunophilin complex binds to calcineurin inhibiting its phosphatase activity which in turn prevents the dephosphorylation of the transcription factor *nuclear factor of activated T-cells* (NFAT) impeding its translocation to the nucleus and subsequent down-regulation of critical cytokine genes **(Ho et al., 1996)**. Three genes were found to be mainly down-regulated, namely IL-2, IFN-γ and GM-CSF. The end-result is decreased activation of T-lymphocytes **(Giese et al., 2004)**.

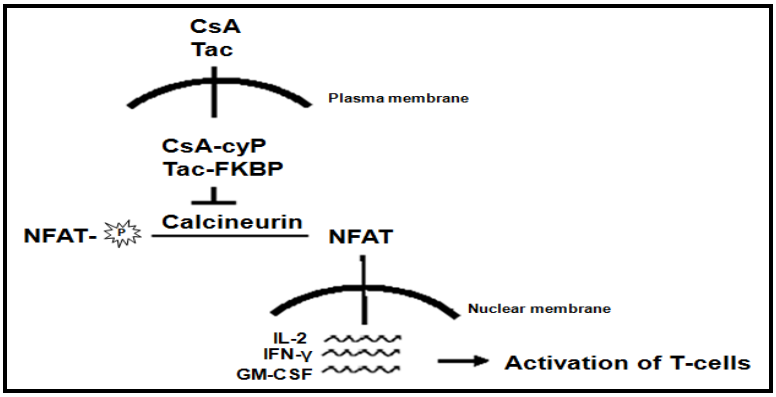


Figure 2: Mechanism of action of CNIs; CsA = cyclosporine A; CyP = cyclophilin; FKBP= FK506 binding protein; GM-CSF = granulocyte macrophage colony-stimulating factor; IFN- γ =interferon gamma; IL-2 = interleukin 2; NFAT = nuclear factor of activated T-cells; Tac = tacrolimus

The prolonged use of CNIs is associated with chronic suppression of the immune system with an increased risk for malignancy and infections. In addition, CNIs have a wide range of drug-specific adverse effects; many of them are dose-dependent. The most important adverse effect of both Tac and CsA is nephrotoxicity. Furthermore, CsA therapy is associated with increased incidence of hypertension, hyperlipidemia, hirsutism and gingival hyperplasia, while Tac therapy is associated more with the incidence of neurotoxicity, post-transplant diabetes mellitus and alopecia. However, the toxic profile of both drugs is overlapping **(Taylor et al., 2005)**.

CNIs are characterized by a highly variable pharmacokinetic (PK) profile among individuals. The oral bioavailability of CsA and Tac is irregular and difficult to predict. The peak level in the blood is reached during the first 2 to 3 hours after the dose which is the time of maximal calcineurin inhibition. CNIs are metabolized by the cytochrome

P450 isoenzymes (mainly CYP3A4 and CYP3A5) in the gut and liver. They are also substrate for P-glycoprotein which acts as an efflux pump transporting the drug out of the intestinal or hepatic cells **(Kapturczak et al., 2004)**.

1.2.2 mTOR inhibitors

The mammalian target of rapamycin inhibitors (mTORIs) include Sir (known also as rapamycin) and Evr. These agents are used as alternative to CNIs in some patients particularly to decrease the risk of CNI-associated nephrotoxicity. They act by inhibiting the downstream signaling effects of the protein kinase mTOR which is a key regulator of cell growth and proliferation. After entering the cell, Sir and Evr bind to the FKBP-12 which acts as the drug receptor. The resulting complex binds to mTOR causing inhibition of its kinase activity with subsequent inhibition of cell proliferation. Their favorable effect as immunosuppressant comes from their ability to block cytokine-mediated T-cell proliferation **(Dowling et al. 2010)**.

The toxic profile of both Sir and Evr is similar and the adverse effects mostly comprise skin-, blood- and metabolism-related side effects. Anemia, leucopenia, thrombocytopenia, hyperlipidemia, skin rashes and mouth ulcers are among the most common adverse effects of both drugs **(Taylor et al., 2005)**.

Similar to the CNIs, the PK profile of mTORIs is also variable among individuals. While Sir is a naturally occurring compound, Evr was produced as a chemical modification of Sir by adding a hydoxyethyl group at position 40 of Sir. This modification resulted in differences in the PK properties. Evr is much more polar than Sir with a subsequent difference in tissue distribution. The elimination half life of Evr is also considerably lower than that of Sir (mean 28 h vs. 62 h in mean). Both drugs are metabolized by the CYP3A enzyme system **(Klawitter et al., 2015)**.

1.3 Monitoring of therapy with immunosuppressive drugs

1.3.1 The pharmacokinetic approach

The immunosuppressive drugs are characterized by having a narrow therapeutic window. Thus monitoring of the therapy is mandatory to keep the delicate balance between over-immunosuppression with increased risk of infection or malignancy and under-immunosuppression with increased the risk of rejection. The therapy with immunosuppressive drugs is further complicated by the great interindividual variability of pharmacokinetics which makes the "one dose fits all" strategy unsuitable for these drugs.

These facts constitute the rational of the traditional therapeutic drug monitoring (TDM) which is the currently used approach to guide dosing of the immunosuppressive drugs. TDM entails the use of the level of the drug in the blood or plasma, mostly the pre-dose level to adjust the dose of drug with the aim of keeping the drug level within a predefined therapeutic range **(Cattaneo et al., 2004)**.

The use of TDM as a tool to adjust therapy with immunosuppressive drugs allowed reducing the PK component of variability of drug response. This approach has, however, several shortcomings: a) TDM is applied only when therapy has been started so it cannot be used to predict the initial dose, b) It does not account for the inter-individual variability in the immune response i.e. the PD component, and c) It is of less value to predict the response in case of multiple drug therapy with possible interaction **(Budde and Glander, 2005)**.

All these limitations reveal the need to search for new approaches to help a better optimization of the therapy with the immunosuppressive drugs.

1.3.2 Pharmacodynamic Biomarkers

Owing to the great variability in response of patients to immunosuppressive drugs and the incompetence of the conventional approaches to account for such variability, the trend in the field of transplantation medicine is moving toward a more tailored approach or "individualized therapy". This approach is strongly linked to the development of

"biomarkers" which could reflect the individual response of the patients to the drug therapy **(Ashton-Chess et al., 2009)**.

According to the Biomarkers Definitions Working Group of the NIH, a biological biomarker can be defined as: *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* **(Strimbu and Tavel, 2010)**. There are different kinds of biomarkers which could be employed as predictors of the patient's response to treatment; pharmacodynamic (PD) and pharmacogenetic (PGx) biomarkers are among the most notable biomarker categories. While PD biomarkers are concerned with measurement of the physiologic effect of the immunosuppressive drugs on their targets, the PGx biomarkers investigate the effect of genetic variation on response to drugs including adverse drug reactions.

A wide range of PD biomarkers for immunosuppressive drugs have been proposed in the last decade indicating the general effect of the drug on the immune cells or more specific indicating the direct effect of the drug on specific drug targets **(Wieland et al., 2010).** However, only a very limited number of these biomarkers are currently accepted in clinical practice. This is because most of the biomarkers are neither analytically validated according to international guidelines nor suitable due to their complicated nature for routine use. From this perspective more efforts need to be spent to test the "fitness for purpose" of the candidate biomarker assays which has the potential to be implemented clinically.

Therefore, the analytical performance of two PD assays was tested in the current thesis: the first assay is related to the monitoring of CNI therapy and is based on the determination of variable expression levels of certain cytokine genes under the influence of CNIs (NFAT-regulated gene expression assay). The second assay monitors mTORI therapy and is based on measuring the various level of phosphorylation of a ribosomal protein that is decreased under the influence of mTORIs therapy (measurement of S6RP using phosphoflow).

A) NFAT-regulated gene expression assay

As stated before (section 1.2.1.), CNIs impede the action of the transcription factor NFAT and consequently down-regulate certain target genes (IL2-, IFN-γ and GM-CSF). Giese and colleagues **(Giese et al., 2004)** have established a method to estimate the residual transcriptional activity of NFAT through measuring the level of gene expression of IL-2, INF-γ and GM-CSF in mitogen stimulated whole blood at two different time points (before and after drug intake) using reverse transcription–qPCR (RT-qPCR). The extent to which the expression of these genes is inhibited after intake of CsA or Tac reflects the degree of immunosuppression exerted by these agents. This method could be used in addition to TDM to monitor CNI therapy particularly in long-term renal allograft recipients. The assay protocol has been established in the Institute of Immunology at the University of Heidelberg and a primer set for the amplification of the target genes is commercially available (Search-LC, Heidelberg).

The NFAT-regulated gene expression assay which utilizes RT-qPCR provides several advantages: a) The results are available within 24 hours; b) No sophisticated cell isolation and incubation steps are needed; c) A primer set for the amplification of the target genes is commercially available; d) The assay protocol is clearly described; and e) There are promising data from clinical trials including de novo and stable transplant patients **(Sommerer et al., 2012)**.

These data indicate that the NFAT-regulated gene expression assay is a promising PD biomarker assay that has the potential to be implemented clinically. However, data about the performance of this assay in an independent laboratory as well as data about inter-laboratory performance are, so far, lacking. In this work, the analytical performance of the NFAT-regulated gene expression assay was tested in the clinical laboratory of the Klinikum Stuttgart, Germany utilizing the commercially-available kit (Cyclosporine Immune Monitoring; Search-LC, Heidelberg). Furthermore an interlaboratory comparison with University Hospital Heidelberg was performed to ensure agreement of the results.

B) Measuring the level of S6RP through phosphoflow

mTOR is a serine/threonine kinase, which operates through phosphorylation of serine and threonine residues at downstream target molecules (Figure 3). One of its most notable targets is p70 ribosomal protein S6 kinase 1 (p70S6K), leading to its activation. The p70S6 kinase is in turn another protein kinase which, upon activation by mTOR, phosphorylates also downstream targets as the 40S ribosomal protein S6 (S6RP) that is involved in regulation of protein translation **(Asnaghi et al., 2004)**. Inhibition of mTOR through the action of mTORIs leads to decreased level of phosphorylation of its downstream targets whether direct or indirect **(Dowling et al., 2010)**. Therefore measurement of the level of phosphorylation of these molecules could be used as a biomarker of therapy with mTORIs.

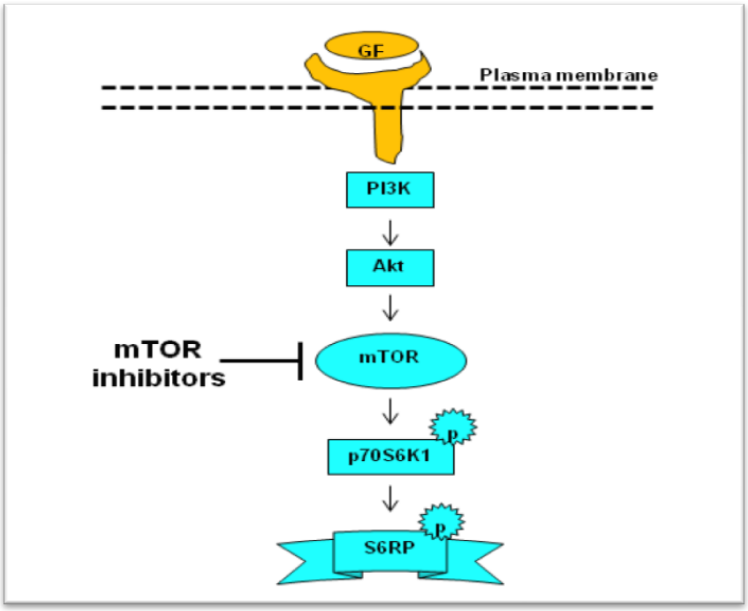


Figure 3: The mTOR pathway; the main upstream effector of mTOR is the PI3K-Akt pathway which responds to wide variety of signals including growth factors. mTOR is a protein kinase which phosphorylates p70S6K1which in turn phosphorylates S6RP.. mTOR inhibitors decrease the level of phosphorylation of p70S6K1 as well as S6RP through inhibiting mTOR. Akt = protein kinase B; GF = growth factor; mTOR = mammalian target of rapamycin p70S6K1 = 70kDa ribosomal protein S6 Kinase1; PI3K = phosphoinositide 3-kinase; S6RP = S6 ribosomal protein

Several research groups have addressed the use of the phosphorylation status of p70S6 kinase and its substrate S6RP as biomarkers of mTORI therapy. Different approaches have been attempted: western blot assay **(Hartmann et al., 2005)** and ELISA **(Dekter**

et al., 2010; Hartmann et al., 2013). However, these protocols are not suitable for routine clinical use since they entail the use of peripheral blood mononuclear cells (PBMCs) as a sample matrix which requires a long preparation time and the use of large sample volume in addition to the complicated nature of the assay protocol.

The use of flow cytometry provides many advantages over the other traditional analytical techniques since it is rapid, sensitive and high throughput. Therefore, the use of flow cytometry in the clinical laboratory showed remarkable increase in the last decade. The advent of antibodies against phosphoproteins turned the phosphoflow technique into a powerful tool for analysis of cell signaling events (phosphoflow technique).

Previous work showed the possibility to measure the level of p70S6K in the context of PBMCs with satisfactory analytical performance using phosphoflow technique **(Hoerning et al., 2015; Wang and Fan, 2015)**. Despite the promising results of the use of phosphoflow as a measure of phosphorylation status of mTOR targets, these protocols employ the use of PBMCs as well, which render them not appropriate for the clinical setting

The use of whole blood samples would, therefore, represents a better alternative when it comes to the routine clinical practice. Previously, this would be confronted with the artifactual changes that occur during sample processing since protein phosphorylation is highly dynamic. This hurdle has been overcome by the introduction of a protocol that entails fixation of the leucocytes in whole blood samples before lysis of red blood cells (RBCs) **(Chow et al., 2005)**. This concept was first addressed by Dieterlen et al. who established a phosphoflow protocol to measure the level of phosphorylation of S6RP using whole blood as a sample matrix. The assay showed satisfactory analytical performance *in vitro* **(Dieterlen et al., 2012)**. However, there are so far no published data regarding the performance of the assay using clinical patient samples. Furthermore, the fixation-permeabilization steps were conducted employing an in-house established protocol. Replication of the results in absence of an optimized commercial kit is difficult.

In this work, the technique of phosphoflow was used to measure the level of phosphorylation of S6RP using whole blood samples as a biomarker for mTOR inhibitors' activity. The assay protocol was set up in the Central Institute for Clinical Chemistry and Laboratory Medicine, Klinikum Stuttgart and entails the use of a commercial kit (PerFix-p Kit, Beckman Coulter) to fix and permeabilize white blood cells (WBCs) in whole blood samples before lysis of RBCs and anti-human p-S6RP (S235/S236) as a phosphoantibody. The analytical performance of the assay protocol was then tested both *in vitro* using blood samples spiked with different immunosuppressive drugs and *ex vivo* with residual clinical samples of different patient groups and three volunteers who took a single dose of Evr. In a separate set of experiments and to verify the measured values with a conventional method, the results of phosphoflow cytometry were compared with western blot technique.

1.3.3 Pharmacogenetic biomarkers

The variability of response to drugs among individuals is multifactorial. One important factor is the difference in the genetic make-up of the patients. It has been shown that genetic variation can be responsible for up to 60% of interindividual variability of drug response including genes coding for drug targets, drug metabolizing enzymes and drug transporters **(Godman et al., 2013)**.

Pharmacogenetics and pharmacogenomics aim to elucidate underlying mechanisms for inter-individual variability in drug response with specific focus on genetic variation. While pharmacogenetics refers to the effect of a single gene on the response to a drug, pharmacogenomics is a broader term which refers to the impact of the genome as a whole on the response of a drug. Nevertheless, the two terms are used interchangeably **(Yagil and Yagil, 2002)**.

Genetic variants which are found in > 1% of a population are termed genetic polymorphisms. The most common type of polymorphisms is the single nucleotide polymorphism (SNP) which refers to a single nucleotide alteration in a certain DNA sequence. The impact of genetic polymorphisms on immunosuppressive drug therapy can be well illustrated by azathioprine which is among the earliest immunosuppressive drugs used after organ transplantation. Azathioprine is a purine analogue which is

inactivated by methylation through the action of the enzyme thiopurine methyltransferase (TPMT). Patients who carry a variant allele of TPMT (with consequent impaired ability to deactivate the drug) may be at increased risk of severe drug toxicity if a prior dose adjustment was not performed **(de Jonge and Kuypers, 2008)**.

Genetic variation can also contribute significantly to the inter-individual variability in disposition and response to other immunosuppressive drugs like the widely used CNIs. Several SNPs in drug metabolizing enzymes and transporting proteins of CNIs have been described to date and which could impact CNI pharmacokinetics **(Hasselink et al., 2014)**. In this work, the pharmacogenetic part focused on Tac since it is the standard CNI used after renal transplantation in the Klinikum Stuttgart.

As Tac is metabolized by CYP3A4 and CYP3A5 isoenzymes and is substrate for P-glycoprotein, genetic variations in these genes could be a significant determinant of inter-individual variability in PK of Tac. Except for the *CYP3A5*3* polymorphism, the effects of other SNPs were inconsistent (Table 1 and 2). A special concern is the newly discovered *CYP3A4*22* polymorphism which was found to have a minor allele frequency of 5-7% in Caucasians and to impact the PK of CNIs **(Elens et al., 2013b)**. However, more evidence needs to be accumulated to validate the role of this SNP related to Tac PK . Similarly, numerous SNPs have been described for the *ABCB1* gene encoding for P-glycoprotein, however only three of them were particularly of interest regarding Tac PK namely the variants 3435T>C, 2677T>G/A and 1236T>C SNPs. Many studies have addressed the influence of these individual SNPs or the specific haplotype T-T-T on the PK of CNIs (Table 3)

In the PGx part of this thesis, the influence of seven SNPs in *CYP3A4*/5 and *ABCB1* on Tac pharmacokinetics were investigated in a cohort of 121 renal transplant patients in the early period after transplantation. The investigated alleles are *CYP3A5*3*, *CYP3A4*22*, *CYP3A4*1B*, and four SNPs in the *ABCB1* gene (3435T>C, 2677T>G/A and 1236T>C).

Table 1: Effect of *CYP3A4*22* single nucleotide polymorphism on Tac pharmacokinetics after organ transplantation

SNP	Cohort			Pharmacokinetic Parameter(s)	Time point	Outcome	Ref.
	Patients	Place of study	n				
*CYP3A4*22*	RTx	France	186	Tac trough level (C_0) Dose-adjusted C_0 Tac dose-requirement	Days 10, 14, 30, 60 and 90 post-transplantation. *N.B. Day 10 postoperative is equivalent to day 3 after initial dose of Tac which is started at day 7 postoperative*	Tac C_0 was higher in carriers of *CYP3A4*22* compared to homozygous wild type at day 10 postoperative ($P< 0.001$). Compared to the homozygous wild type, carriers of *CYP3A4*22* showed higher dose-adjusted C_0 of Tac as well as lower Tac dose requirement (by about 30%) over time in the first 3 months after surgery	Pallet et al., 2015
*CYP3A4*22*	RTx	Belgium	96	Tac C_0 Dose-adjusted C_0 Tac dose-requirement	First 2 weeks post-operative	During the first 2 weeks after transplantation, carriers of *CYP3A4*22* showed higher Tac C_0 (significant at days 2 and 10), higher dose-adjusted C_0 (significant at days 2,3 and 10) and lower Tac doses (significant at days 3,4 and 14)	Elens et al., 2013a
*CYP3A4*22*	RTx	Netherlands Belgium	185	Tac C_0 Tac dose-requirement	Day 3, day 10 and month1, 3, 6 and 12 after transplantation *N.B. Tac was administered 2 days before surgery*	At day 3, Tac C_0 was significantly higher in carriers of *CYP3A4*22*. This difference was not translated to a clinical relevance regarding the incidence of DGF From day 10 to month 6, the Tac dose requirement was found to be 33% lower in carriers of *CYP3A4*22*	Elens et al., 2011a
*CYP3A4*22*	RTx	Netherlands	49	Tac C_0 Dose-adjusted C_0 Tac dose-requirement	Patients in the stable phase (> 1 year after transplantation)	*CYP3A4*22* was associated with a significant increase in dose-adjusted C_0 as well as a significant decrease in dose requirements	Elens et al., 2011b

*CYP3A4*22*	RTx	Poland	241	Tac C_0 Dose-adjusted C_0 Tac dose-requirement	Day 7 and at month 1, 3, 6 and 12 after transplantation	Carriers of *CYP3A4*22* showed a trend to exhibit higher Tac C_0 and dose-adjusted C_0, however the difference was significant **only** at month 3 (dose-adjusted C_0) and month 6 (C_0) after transplantation	Kurzawski et al., 2014
*CYP3A4*22*	RTx	Norway	123	Dose-adjusted C_0	2 to 7 weeks after transplantation	**No** difference was noticed regarding the dose-adjusted C_0 between carriers *CYP3A4*22* and the wild type homozygote	Lunde et al., 2014
*CYP3A4*22*	RTx	Netherlands	101	Population PK analysis		*CYP3A4*22* was associated with 16% lower clearance of Tac , however this effect was regarded to be clinically **insignificant**	Moes et al., 2014
*CYP3A4*22*	RTx	Spain	206	Tac C_0 Dose-adjusted C_0 Tac dose-requirement	1 Week 6 Months	Carriers of *CYP3A4*22* **did NOT** show significance difference compared to the wild type homozygote regarding the Tac C_0, dose-adjusted C_0 and dose requirements at the selected time points	Tavira et al., 2013
*CYP3A4*22*	RTx	Brazil	140	Dose-adjusted C_0	3 months after transplantation	*CYP3A4*22* was found to have **No** association with the dose-adjusted Tac blood concentration at the examined time point	Santoro et al., 2013
*CYP3A4*22*	Pediatric heart Tx	Netherlands (cohort form Canada)	60	Tac C_0 Dose-adjusted C_0 Tac dose-requirement	First 2 weeks after transplantation	Tac dose requirements were 30% lower in carriers of *CYP3A4*22* during the first 2 weeks after surgery, however No significant difference was seen regarding Tac trough level or dose-adjusted concentration in the same period.	Gijsen et al., 2013

Table 2: Effect of *CYP3A5 single nucleotide polymorphism on Tac pharmacokinetics after renal transplantation**

Cohort				Time point after transplantation	Findings Described as the change in pharmacokinetics parameter between the expressers (who express at least allele; *1) and non-expressers (CYP3A5*3/*3)	Ref.
Type of Tx	Place of study	Number of study population	Percentage of non-expressers (CYP3A5*3/*3) within the study population			
RTx	Netherlands	64	70%	Months 3, 12	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Hesselink et al., 2003
RTx	France	80	84%	Month 1	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Thervet et al., 2003
RTx	Belgium	50	78%	Stable phase	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Haufroid et al., 2004
RTx	Japan	30	57%	Day 28	↓ dose-adjusted C0 of Tac ↓ dose-adjusted AUC0-12 of Tac ↑ Tac dose requirements to reach target concentration	Tsuchiya et al., 2004
RTx	UK	180	69%	Month 3	↓ dose-adjusted C0 of Tac Longer time to reach target concentration	Macphee et al., 2005
RTx	China	118	59%	Week 1 Month 1, 3	↓ dose-adjusted C0 of Tac	Zhang et al., 2005
RTx	China	30	63%	Month 3,6, 12	↓ dose-adjusted C0 of Tac	Zhao et al., 2005
RTx	Canada	44	84%	Week 1 Month 3	↓ dose-adjusted C0 of Tac Longer time to reach target concentration	Roy et al., 2006
RTx	Germany	134	87%	Stable phase (> one year post-transplantation)	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Renders et al., 2007
RTx	China	63	57%	Day 28	↑ Tac clearance ↓ dose-adjusted AUC0-12 of Tac	Rong et al., 2010
RTx	Brazil	151	61%	Week 1 Month 1, 2 and 3	↓ dose-adjusted C0 of Tac	Santoro et al., 2011
RTx	France	209	82%	Days 15, 30, 90 Years 1, 2	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Glowacki et al., 2011
RTx	Spain	103	90%	Week 1	↓ dose-adjusted C0 of Tac	Gervasini et al.,

				Months 1, 5 Year 1	↑ Tac dose requirements to reach target concentration	2012
RTx	Morocco	10	60%	First 3 month after transplantation	↑ Tac dose requirements to reach target concentration	Elmachad et al., 2012
RTx	Mexico	291 124 adults 167 pediatric	52%	Month 6	↑ Tac dose requirements to reach target concentration	García-Roca et al., 2012
RTx	Japan	39	56%	Day 28	↑ Tac clearance ↓ dose-adjusted AUC0-12 of Tac ↑ Tac dose requirements to reach target concentration	Tada et al., 2005
RTx	Belgium	59	83%	After first Tac dose	↓ dose-adjusted C0 of Tac ↓ weight-adjusted C0 of Tac	Mourad et al., 2006
RTx	Korea	70	63%	Months 1, 3, 6, 12	↓ dose-adjusted C0 of Tac ↑ Tac dose requirements to reach target concentration	Cho et al., 2012

Table 3: Effect of *ABCB1* SNPS and haplotypes on Tac pharmacokinetics after organ transplantation

Cohort			SNPs investigated	Time point	Findings	Ref.
Transplant	**Place**	**n**				
RTx	France	81	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Month 1	2677GG: ↓ C0/D C-G-C haplotype: ↓ C0/D	Anglicheau et al., 2003
Liver Tx	France	42	3435C>T	1-3 days post-transplantation	3435TT: ↑ C0/D	Bonhomme-Faivre et al., 2009
Liver Tx	Belgium	150	1236C>T, 2677G>T/A, 3435C>T	Day 7	1236CC, 2677GG: ↓ Tac hepatic conc.	Elens et al., 2007
RTx	UK	206	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Month 3	2677GG, 3435CC: ↓ C0/D	Fredericks et al., 2006
Liver Tx Paediatric	UK	51	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Month 6 Year 1,2,3,4,5	Carriers of 2677G>T or 3435C>T: ↑ C0/D only 3 years post-transplantation T-T-T haplotype: ↑ C0/D	Hawwa et al., 2009
RTx	Spain	35	2677G>T/A, 3435C>T	First 6 weeks after transplantation	3435CC: ↓ C0/D	López-Montenegro et al., 2010
RTx	UK	180	3435C>T	Month 3	3435CC: ↓ C0/D	MacPhee et al., 2002
Liver/Renal Tx	Italy	101 Liver 50 Renal 51	2677G>T/A, 3435C>T	Month 1,3,6	Carriers of 2677T/A: ↑ dose requirements only in RTx recipients	Provenzani et al., 2010
RTx	Canada	44	2677G>T/A, 3435C>T, T-129C	Week 1 Month 3	Less than three copies of T-129C, 2677T, 3435T: ↓ C0/D	Roy et al., 2006
Lung Tx	USA	91	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Month 1,3,6,9,12	1236TT/2677TT/3435TT: ↑ C0/D	Wang et al., 2006
Liver Tx	China	50 Tx 50 donors	3435C>T	First month after transplantation	3435CC: ↑ Tac dose requirements	Wei-lin et al., 2006
RTx	Korea	70	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Month 1,3,6,12	**No association** between *ABCB1* polymorphisms and Tac concentrations	Cho et al., 2012
RTx	Spain	103	1236C>T, 2677G>T/A, 3435C>T and haplotypes	Week 1 Month 1,5,12	**No association** between *ABCB1* polymorphisms and Tac concentrations or dose requirements.	Gervasini et al., 2012
Liver/Renal Tx and liver donors	Korea	506 Tx 62 donors	2677G>T/A, 3435C>T	when conc. of Tac in blood level reached steady state	**No association** between *ABCB1* polymorphisms and Tac concentrations	Jun et al., 2009

RTx	Belgium	304	2677G>T/A, 3435C>T	Month 3,12	**No association** between *ABCB1* polymorphisms and Tac concentrations or dose requirements.	Kuypers et al., 2010b
RTx candidates	Belgium	19	1236C>T, 2677G>T/A, 3435C>T	After the first dose of Tac	**No association** between *ABCB1* polymorphisms and Tac PK parameters	Haufroid et al., 2006
RTx	Belgium	59	1236C>T, 2677G>T/A, 3435C>T	After the first dose of Tac ?	**No association** between *ABCB1* polymorphisms and Tac concentrations	Mourad et al., 2006
RTx	Japan	39	3435C>T	Day 28	**No association** between *ABCB1* polymorphisms and Tac PK parameters	Tada et al., 2005

2 Aims of the thesis:

The aim of this thesis was to investigate PD and PGx biomarkers which may have the potential to be implemented in the clinical practice for better individualization of immunosuppressive therapy after renal transplantation. The following work is to be performed:

1. Validation of the "NFTA-regulated gene expression assay" as a pharmacodynamic biomarker to monitor therapy with CNIs in an independent laboratory as well as testing its inter-laboratory performance

2. Testing the analytical suitability of a phosphoflow assay protocol based on a commercial kit to measure the level of p-S6RP as a biomarker of therapy with mTORIs

3. Investigating the frequency and influence of seven relevant SNPs on Tac pharmacokinetics early after transplantation in a cohort of 121 renal transplant recipients.

3 Materials, patients and methods

3.1 Materials

3.1.1 Reagents

Reagent	**Supplier**
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich, Munich, Germany
Ionomycin	AppliChem GmbH, Darmstadt, Germany
Cyclosporine A	Sigma-Aldrich, Munich, Germany
Tacrolimus	Sigma-Aldrich, Munich, Germany
Everolimus	Sigma-Aldrich, Munich, Germany
Mycophenolic acid	Sigma-Aldrich, Munich, Germany
2 Mercapto-ethanol	Merck, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Munich, Germany
Fetal Calf Serum (FCS)	PAN Biotech GmbH, Aidenbach, Germany
Penicillin/Streptomycin(10,000 U/mL)	Life Technologies, Paisley, UK
Hepes buffer	Life Technologies, Paisley, UK
L- glutamine	Life Technologies, Paisley, UK
Bovine serum albumin (BSA)	VWR International GmbH, Darmstadt, Germany
Phosphate-Buffered Saline (PBS)	Life Technologies, Paisley, UK
RPMI 1640 medium	Life Technologies, Paisley, UK
ACK (Ammonium-Chloride-Potassium) lysing buffer	Life Technologies, Paisley, UK
RNaseOUT	Invitrogen, Darmstadt, Germany
High Pure RNA Isolation Kit	Roche Diagnostics GmbH, Mannheim-Germany
1st Strand cDNA Kit for RT-PCR (AMV)	Roche Diagnostics GmbH, Mannheim-Germany
LightCycler FastStart DNA Master SGI	Roche Diagnostics GmbH, Mannheim-Germany
Primer set (Cyclosporine Immunemonitoring)	Search-LC, Heidelberg, Germany
PerFix-p Kit	Beckman Coulter, Marseille, France
CD3-PC7	Beckman Coulter, Brea, CA, USA
CD4-ECD	Beckman Coulter, Brea, CA, USA
CD8-PC5	Beckman Coulter, Brea, CA, USA
Rabbit IgG Isotype Control Alexa Fluor® 488 Conjugate	Beckman Coulter, Brea, CA, USA
Phospho-S6 Ribosomal Protein (Ser235/236) Alexa Fluor® 488	Beckman Coulter, Brea, CA, USA
QIAamp DNA Blood Mini Kit	Qiagen, Hilden, Germany
TaqMan Genotyping Assay Mix	Thermo Fischer Scientific, Darmstadt, Germany
TaqMan Universal PCR Master Mix	Thermo Fischer Scientific, Darmstadt, Germany

3.1.2 Buffers and solutions

Name	Constituents	Concentration
Culture medium	RPMI 1640 Medium Inactivated FCS Penicillin / Streptomycin Hepes buffer 1 M L Glutamine 200 mM β-mercaptoethanol (BME) in medium: : 50 ml of RPMI Medium + 35 µl BME	500 ml 50 ml 5 ml 5 ml 5 ml 2.5 ml
Wash buffer 2%	Phosphate-buffered saline Bovine serum albumin	500 ml 10 g

3.1.3 Equipment

Instrument	Supplier
PP tubes natur 12 ml 16/100 mm	Hain Lifescience GmbH, Nehren, Germany
PS Tubes 5 ml	Greiner Bio-One GmbH, Kremsmünster, Austria
Tube 50ml, 115x28mm, PP	Sarstedt AG & Co, Nümbrecht, Germany
Safe-lock tubes 1.5ml	Eppendorf AG, Hamburg, Germany
Multiply-Pro cup 0.2ml, PP	Sarstedt AG & Co, Nümbrecht, Germany
Pipettes (2µl, 10 µl, 100µl, 200µl, 1000µl)	Mettler-Toledo GmbH, Gießen, Germany
Multichannel Pipette	Mettler-Toledo GmbH, Gießen, Germany
Pipettes (10 µl, 100 µl, 1000 µl)	Eppendorf AG, Hamburg, Germany
Biosphere Filter Tips 10 µl	Sarstedt AG & Co, Nümbrecht, Germany
SafeSeal-Tips (100 µl, 1 ml)	Biozym Scientific GmbH, Oldendorf, Germany
Serological pipettes 5ml, 10ml	Greiner Bio-One GmbH, Kremsmünster, Austria
Rubber pipette filler	Sigma-Aldrich, Munich, Germany
Eppendorf 5417C Centrifuge	Eppendorf, Hamburg, Germany
Universal 320 R benchtop centrifuge	Hettich GmbH & Co. KG, Tuttlingen, Germany
Hettich MIKRO 20 centrifuge	Hettich GmbH & Co. KG, Tuttlingen, Germany
Universal 16R centrifuge	Hettich GmbH & Co. KG, Tuttlingen, Germany
REAX 2000 vortex	Heidolph GmbH & Co. KG, Schwabach, Germany
Julabo R5 water bath	Julabo GmbH, Seelbach, Germany
Mini galaxy A CO2 incubator	RS Biotech Inc., Irvine, UK
96-well Plate, Thermo-Fast®	Abgene, Hamburg, Germany
384-well plate, Thermo-Fast®	Abgene, Hamburg, Germany
Veriti 384-well Thermal Cycler	Thermo Fischer Scientific, Darmstadt, Germany
ABI PRISM 7900HT Real-Time PCR System (TaqMan)	Thermo Fischer Scientific, Darmstadt, Germany
Nanodrop 2000c	Peqlab Biotechnology GmbH, Erlangen, Germany
LightCycler Capillaries (20 µl)	Roche Diagnostics GmbH, Mannheim-Germany
Primus 25 advanced thermal cycler	peQlab, Erlangen, Germany
LightCycler 2.0 instrument	Roche Diagnostics GmbH, Mannheim-Germany

Cytomics FC 500 Flow cytometer	Beckman Coulter GmbH, Krefeld, Germany

3.1.4 Software and web servers

Name	**Supplier**
ABI 7900HT v 2.4	Applied Biosystems (California, USA)
R-3.2.3	www.r-project.org
GraphPad Prism version 5.00	GraphPad Software, San Diego California, USA
MedCalc Version 14.12.0.	MedCalc Software, Ostend, Belgium
Microsoft Excel Worksheet (2007)	Microsoft Corp., Redmond, USA
PubMed	http://www.ncbi.nlm.nih.gov/pubmed

3.2 Patients and methods

3.2.1 NFAT-regulated gene expression assay

A protocol for the analysis of the expression of the NFAT-regulated genes has been established in Heidelberg by Giese et al. **(Giese et al., 2004)** and a primer set for amplification of the target genes is commercially available (Search-LC, Heidelberg). The analytical performance of the assay was tested at the laboratory of Klinikum Stuttgart and an interlaboratory comparison with the renal transplant center in Heidelberg was conducted. The readout of the assay is the residual gene expression (RGE) which is the ratio of the expression level before and after drug intake.

3.2.1.1 Patients

Experiments were approved by the ethics committee of the Eberhard-Karls-University Tübingen. The analytical verification of the assay at the laboratory of Klinikum Stuttgart was done *in vitro* with anonymized drug-free left over whole blood samples which were spiked with Tac. For the inter-laboratory comparison of the assay, heparinized blood samples from 10 patients under CsA therapy were compared. The samples were collected and analyzed in Heidelberg and then sent to the laboratory of Klinikum Stuttgart to be re-analyzed within 24 hours.

3.2.1.2 Methods

3.2.1.2.1 Sample processing

Sample processing was performed essentially according to the previously published protocol. A stimulation solution was prepared consisting of PMA (100 ng/ml) and ionomycin (5 µg/ml) in complete culture medium (RPMI-1640). To activate lymphocytes, 1 ml heparinized whole blood was pipetted into polypropylene plastic tubes and was incubated with 1ml stimulation solution at 37°C in a CO2 incubator (with 7% CO2) for 3 hours. RBCs were then lysed using Ammonium-Chloride-Potassium (ACK) lysing buffer. Total RNA was extracted with High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim-Germany) according to the manufacturer's instructions. The elution volume was 50 µl, to which 1µl RNaseOUT (InvitrogenTM) was added. RNA samples were frozen at -80° C until further processing. At the time of reverse transcription, RNA samples were allowed to thaw and from each sample a volume of 8.2 µl was used to synthesize cDNA using First Strand cDNA Synthesis Kit for

RT-PCR (Roche Diagnostics GmbH, Mannheim-Germany) utilizing avian myeloblastosis virus (AMV) reverse transcriptase and oligo-(dT) primer according to the protocol provided. Reverse transcription was done on Primus 25 advanced thermal cycler (peQlab, Erlangen, Germany). The generated cDNA was diluted with ultra-pure H2O to a final volume of 500ml and stored at -20° C until PCR analysis. Real-time PCR was carried out on the LightCycler 2.0 instrument (Roche Diagnostics GmbH, Mannheim-Germany) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim-Germany) and the LightCycler - Primer Set; Cyclosporine Immune Monitoring (Search-LC, Heidelberg). Quantification cycle (Cq) values were determined by the Fit-Points Method and a baseline at -0.6.

In the original protocol, Giese et al. used MagNA Pure lysis buffer to lyse leucocytes and a MagNA Pure-LC device to isolate mRNA, and they did not add RNase out to the eluted RNA.

For *in vitro* experiments, blood was spiked with the desired concentration of Tac prior to the stimulation step. Spiking of samples with the drug was done at 37° C and 7% CO2 for 30 min

3.2.1.2.2 Testing the analytical performance

The analytical performance of the NFAT-regulated gene expression assay has been tested in terms of concentration-effect relationship, precision (within- and between-run), limit of quantification and stability. An inter-laboratory comparison with the University Hospital Heidelberg has been also performed.

i. ***Concentration-effect relationship:*** The effect of different concentrations of Tac on the expression of NFAT-regulated genes was determined. Heparin anti-coagulated drug free whole blood (5 ml) was used. The blood was divided into 5 plastic tubes (1 ml each) and each tube was spiked with a specific concentration of Tac while one tube was left untreated. The concentrations tested were 50µg/L, 25µg/L, 12.5µg/L and 6.25µg/L of Tac.

ii. ***Within-run precision:*** The within-run precision of the NFAT-regulated gene expression assay was tested at three different concentrations of Tac (50 µg/L, 25 µg/L, and 12.5 µg/L) using 3 blood samples from 3 different individuals. The blood was processed in a way that allowed 6 independent measurements of RGE of each blood

sample. Whole blood was divided into 12 plastic tubes. Six of the samples were spiked with Tac while the other 6 were left untreated. At the end of the stimulation step RNA was isolated from all samples in parallel and stored at -80°C. The next day, cDNA was synthesized and stored at -20° C. Finally, 6 consecutive runs of PCR (treated versus non-treated) were performed and RGE was determined. Mean, standard deviation and coefficient of variation were calculated (n=6).

iii. ***Between-run precision:*** Evaluation of the between-run precision by investigating the same blood sample on six different days is inapplicable due to sample instability over this period of time. Thus the between-run precision was tested starting from the step of the isolated RNA. The between-run precision was also tested at 3 different Tac concentrations (50 µg/L, 25 µg/L, and 12.5 µg/L) using remaining RNA samples of previously processed blood stored at -80°C. RNA samples were allowed to thaw and were pooled. One pool formed from RNA of Tac treated blood (50 µg/L, 25 µg/L, or 12.5 µg/L) and another pool from RNA of non-treated blood. These 2 pools of RNA were reverse transcribed 6 times on 6 different days and 6 PCR runs were done on 6 different days in parallel to calculate RGE. The mean, standard deviation and coefficient of variation were calculated (n=6).

iv. ***Limit of quantification (LOQ):*** To determine LOQ of the NFAT-regulated gene expression assay, blank cDNA samples were spiked with known concentrations of IL-2, INF-γ and GM-CSF cDNA standards (LightCycler Primer Set kit, Search-LC). The number of cDNA copies in each sample was determined using qPCR. Serial dilutions of the standards were applied until reaching a number of copies that could not be detected anymore. The lowest number of copies that showed a CV ≤ 20% (n=6) was set to be the LOQ **(Armbruster and Pry, 2008)**.

v. ***Stability:*** To assess the stability of the samples, 3 anonymized left over blood samples from routine diagnostics free of Tac or CsA were used. Each sample was divided into 2 aliquots. One aliquot was analyzed at the day of blood collection, where it was incubated with Tac in a concentration of 25 µg/L and further processed as previously described. The other portion was stored at room temperature and processed after 24 hours like the first day and by applying the same concentration of Tac. The results

were then compared and the % differences between the measurements in both days were calculated.

vi. ***Inter-laboratory comparison:*** For the inter-laboratory comparison of the NFAT-regulated gene expression assay, heparinized blood samples from 10 patients under CsA therapy were compared. For each patient 2 whole blood samples were collected at the University Hospital Heidelberg, one sample before and another sample 2 hours after CsA intake. Patient samples were analyzed at the University Hospital Heidelberg for NFAT-regulated gene expression using the validated protocol of Giese et al. **(Giese et al., 2004)** and then the whole blood samples were sent to the laboratory of the Klinikum Stuttgart to be re-analyzed within 24 hours. Patients gave their informed consent to this biomarker study in Heidelberg.

3.2.1.2.3 Calculation of mean residual gene expression (RGE)

A calculation sheet provided by Search-LC GmbH was used to calculate the residual expression of the NFAT-regulated target genes after drug intake. This calculation included a beta actin standard and normalization to 2 reference genes (β-actin and cyclophilin B). Mean RGE of IL-2, INF-γ and GM-CSF represents the ratio between the expression level before and 2 hours after CsA intake (in case of patient samples) or the ratio between the expression levels in treated versus non-treated blood samples (in case of *in vitro* experiments).

3.2.1.2.4 Statistical analysis

Statistical analyses were done using Microsoft Excel Worksheet 2007 and MedCalc Version 14.12.0. (MedCalc, Oostende, Belgium) The Spearman rank correlation coefficient test was utilized to determine the correlation between the measurements in the two laboratories. Passing-Bablok regression analysis was applied to calculate the agreement between the measurements in both laboratories. Method bias was also investigated using a Bland-Altman difference plot based on expression of differences as percentages.

3.2.2 Phosphoflow assay of S6RP

To measure the level of phosphorylation S6RP by flow cytometry, a commercial kit for fixation and permeabilization of the cells in the context of whole blood samples and anti-

phospho Ser 235/236 antibody were employed. The following protocol is part of published work by our group (**Abdel-Kahaar et al., 2016)**.

3.2.2.1 Patients

Anonymized left over immunosuppressant drug-free EDTA whole blood samples and residual EDTA whole blood samples containing immunosuppressive drugs were from the routine laboratory at the Klinikum Stuttgart. In addition, three volunteers took a single dose of everolimus (0.5 mg tablet) and blood was collected 1 and 3 hours afterwards. Approval from the local ethics committee of the Ärztekammer Stuttgart was obtained. Complete blood count and measurement of blood concentrations of immunosuppressive drugs were done as a part of the routine tests in our department. Immunosuppressants were determined by a validated LC-MS/MS procedure **(Valbuena et al, 2015)**.

3.2.2.2 Methods

3.2.2.2.1 Sample processing

Aliquots of 100 µl whole blood were pipetted into round-bottomed test tubes (4 tubes for each patient) and placed in a 37ºC water bath for 10 min. The PMA and the blank solution, which served as negative control, were freshly prepared. To decrease assay imprecision, aliquots that received PMA were performed as a duplicate where the mean of p-S6RP from both measurement is to be finally used. Stimulation was performed for exactly 6 min with 150 µg/L PMA before samples were fixed with 65 µl of the fixation buffer. After adding the fixation buffer, the tubes were vortex mixed and further incubated for 10 min. Then 1 ml lysis buffer was added, the tubes were vortexed again and placed again in the water bath at 37ºC for 15 min. After complete lysis of RBCs, the samples were washed twice through adding cold (4°C) wash buffer (2% BSA in PBS), centrifugation at 500 g for 4 min and discarding the supernatant. Then antibodies were added as an antibody mix containing PC7-conjugated anti CD3, ECD-conjugated anti CD4, PC5-conjugated anti CD8 and Alexa Fluor® 488 conjugated anti p-S6RP (S235/S236). To one set of tubes anti p-S6RP was replaced with Rabbit IgG Isotype Control Alexa Fluor® 488. The tubes were incubated in the dark for 30 min at room temperature then washed with 2 ml cold wash buffer and then resuspended in 350 µl resuspension buffer. Fixation and permeabilization of the cells were done using a PerFix-p Kit according to the manufacturer's protocol.

3.2.2.2.2 Testing the analytical performance

The following experiments have been performed:

i. ***Concentration-effect relationship:*** anonymized left-over drug-free whole blood samples (n=2) were supplemented with varying concentrations of Evr and incubated for 30 min in a CO2 incubator with 7% CO2 at 37°C. The clinically relevant concentrations of 2.7 µg/L, 9.1 µg/L and 27.4 µg/L were tested.

ii. ***Verification of the effect of Evr on p-S6RP in vivo:*** three healthy volunteers took a single dose of Evr (0.5 mg tablet) and blood was collected 1 h and 3 h afterwards for assessment of the p-S6RP.

iii. ***Specificity:*** specificity of the assay was investigated *in vitro* and *ex vivo*
In vitro**:** anonymized left-over drug-free blood samples (n=4) were supplemented with different immunosuppressive agents (other than mTORIs) at high concentrations. Therefore, blood was spiked with Tac (25 µg/L), CsA (500 µg/L) or MPA (25 mg/L) in separate aliquots, while one aliquot was left untreated to serve as a control.
Ex vivo**:** To determine the specificity of S6RP phosphorylation as a biomarker of mTORIs PD effects *in vivo*, we examined phosphorylation of S6RP in left-over samples from kidney transplant recipients receiving Evr (n=20), Sir (n=12), Tac (n=12) or CsA (n=12); dialysis patients but not receiving immunosuppressants (n=5); and patients with inflammatory conditions indicated by an elevated C-reactive protein (CRP) concentration of >10 mg/L (n=5). The levels of p-S6RP in these groups were compared to the levels in a control group (n=10) that did not belong to one of the patient groups mentioned above or show any abnormalities in their laboratory results.

iv. ***Within-run precision:*** To test within-run precision, 4 blood samples (2 drug free and 2 containing Evr) were processed 5-6 times in parallel.

v. ***Stability of the analyte p-S6RP:*** stability was tested using blood samples without immunosuppressive drugs (n=3), blood samples with Sir (n=2) and one blood sample with CsA (n=1). The samples were processed on the day of blood collection, and

aliquots from these samples were stored at room temperature for repeated analysis after 24 h

vi. ***Method comparison:*** in a separate set of experiments, a method comparison between phosphoflow assay for p-S6RP and an established state-of-the-art western blot protocol for PD monitoring of mTORIs based on the determination of p-p70S6K was performed **(Hartmann et al., 2005)**. For this comparison, anonymized left-over blood samples from RTx patients receiving Evr (n=7), Sir (n=2) or CsA (n=1) and healthy volunteers (n=5) were used. Blood samples were divided into two aliquots; one aliquot was used to measure the level of p-S6RP by phosphoflow cytometry, while PBMCs isolated from the second aliquot were stimulated with PMA for 30 min and stored at -20°C before sending to a reference laboratory for western blot analysis.

3.2.2.2.3 Flow cytometric analysis

Samples were analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter). Phosphorylation of S6RP was examined separately in CD3+CD4+ and CD3+CD8+ cells. We used the median S6RP phosphorylation (from the histogram of the flow cytometer) as a measure of fluorescence intensity and an index of median S6RP phosphorylation in stimulated cells versus median S6RP phosphorylation in unstimulated cells as a read out of the net effect of the drug on the phosphorylation status of S6RP in the cells of interest.

3.2.2.2.4 Western blot analysis

In a separate set of experiments and to verify the measured values with a conventional method, we compared the results of phosphoflow cytometry with western blot technique. Blood samples of subjects receiving mTORIs (n=15) were divided into 2 aliquots; one aliquot was used to measure the level of p-S6RP by phosphoflow cytometry in our laboratory while PBMC were isolated from the second aliquot and stimulated with PMA for 30 minutes and stored at - 20° C before they were sent to the University Hospital Ulm to be analyzed by Western blot. While we used anti p-S6RP (S235/S236) in the phosphoflow protocol, an anti-phospho-p70S6 kinase (Thr389) antibody was employed in the western blot protocol. Western blot analysis was performed as previously described **(Hartmann et al., 2005)**.

3.2.2.2.5 Statistical analysis

Statistical analyses were done using Microsoft Excel Worksheet 2007 and MedCalc statistical software (Ostende, Belgium). Comparisons of blood samples from different patient groups were made using the Mann-Whitney U test. For method comparison Passing & Bablok regression was employed. A p value <0.05 was considered significant.

3.2.3 Genotyping methods for pharmacogenetics

Single nucleotide polymorphisms (SNPs) in the genes of *CYP3A4, CYP3A5* and *ABCB1* were investigated to be correlated with the Tac pharmacokinetics.

3.2.3.1 Patients

A cohort of 121 renal transplant patients who underwent transplantation between 2009 and 2015 at Klinikum Stuttgart were invited to participate in this study. The study was approved by ethics committee of the Eberhard-Karls-University Tübingen and all participants gave written informed consent. The standard maintenance therapy after renal transplantation at Klinikum Stuttgart is a triple therapy of Tac, MPA and a corticosteroid. Tac therapy is started at the day of renal transplantation using a dosage of 0.1mg/kg/day and subsequently adjusted to achieve a pre-defined target trough blood Tac concentration of 6-8 µg/L in the first three months after transplantation and 4-6 µg/L thereafter. Drug concentrations and administered doses were retrospectively collected from patients' records at Klinikum Stuttgart at the first 2 weeks after transplantation. Dose-adjusted trough concentrations per body weight were calculated for the C0 (µg/L) level. Tac blood concentrations were routinely measured by a validated LC-MS/MS procedure. Tac measurements were not always available for all patients at all time points. Delayed graft function was defined as the need for dialysis therapy within the first postoperative week.

3.2.3.2 Methods

3.2.3.2.1 Sample processing

For preparation of the samples for genotyping, DNA was isolated from the whole blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In summary, 200µl whole blood is mixed with 20µl QIAGEN protease and 200µl buffer AL in 1.5 ml microcentrifuge tube and incubated at 56°C for 10

min to allow for lysis of the cells. Then 200µl ethanol is added to the previous mixture and the whole volume is transferred to the QIAamp Mini spin column supported by 2ml collection tubes. Ethanol is added to ensure optimal binding of DNA to the QIAamp membrane. The spin columns with the supporting tubes are centrifuged at 8000rpm for 1min. At this step the DNA will be adsorbed onto the QIAamp silica membrane while the rest will pass through. The collection tube containing the filtrate is discarded and the spin column is placed in a clean 2ml collection tube. This is followed by 2 wash steps; the first with adding 500µl buffer AW1 followed by centrifugation at 8000rpm for 1 min and the second wash step with adding 500µl buffer AW2 and centrifugation at 14.000 rpm for 3 min. DNA is then eluted in a clean 1.5ml microcentrifuge tube using 200 µl buffer AE. The spin column loaded with buffer AE is incubated for 5 min at room temperature to increase the DNA yield. The last step is to centrifuge the spin columns with the collecting tubes at 8000rpm for 1 min. to receive the eluted DNA in the clean collection tubes.

Concentrations of nucleic acid samples were assessed using Nanodrop 2000c (Peqlab Biotechnology GmbH, Erlangen, Germany)

3.2.3.2.2 Selection of polymorphisms for genotyping

A literature search regarding relevant candidate genes which may explain interindividual variability of calcineurin pharmacokinetics resulted in three genes which are *CYP3A4*, *CYP3A5* and *ABCB1*. The samples were genotyped for seven selected known SNPs in these genes for which previously functional consequences have been reported. Predesigned TaqMan allelic discrimination assays (ThermoFischer Scientific, Darmstadt, Germany) were used for genotyping of these variants

Gene	**SNP**	**rs Nr.**	**Assay ID**
CYP3A4	*CYP3A4*22*	rs35599367	C__59013445_10
CYP3A4	*CYP3A4*1B*	rs2740574	C___1837671_50
CYP3A5	*CYP3A5*3*	rs776746	C__26201809_30
ABCB1	*3435T>C*	rs1045642	C___7586657_20
ABCB1	*2677T>G*	rs2032582	C_11711720D_40
ABCB1	*2677T>A*	rs2032582	C_11711720C_30
ABCB1	*1236T>C*	rs1128503	C___7586662_10

3.2.3.2.3 Taqman genotyping

The seven selected SNPs were genotyped using TaqMan allelic discrimination assays (Applied Biosystems, Darmstadt, Germany) as follows: Genomic DNA was placed in a 384-well plate (Thermo-Fast® Abgene, Hamburg, Germany) so that the final amount of genomic DNA in each well was 10ng. The samples were allowed to completely dry down by evaporation at room temperature in a dark, amplicon-free location. Amplification was performed on Veriti®384-well Thermal Cycler (Applied Biosystems) in a final volume of 5µl containing 10ng dried genomic DNA, 2.5µl of 2X TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.25µl of 20X TaqMan® Genotyping Assay Mix (Applied Biosystems) and 2.5µl of DNase-free water. After PCR amplification, an endpoint plate read was performed on ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems).

3.2.3.2.4 Statistical analysis

All statistical analyses were performed with R-3.2.3 software (www.r-project.org). The differences in quantitative variables (Area under the curve; AUC, of dose-adjusted Tac levels and Tac dose) among individuals with different genotypes were investigated using Kruskal-Wallis tests or Wilcoxon-Mann-Whitney tests as appropriate. Associations between each of the seven SNPs and delayed graft function or acute rejection were investigated by Fisher's exact test. Linear modeling was used to investigate the association between *ABCB1* haplotypes (based on rs1128503, rs2032582, rs1045642) and log-transformed AUC of dose adjusted as well as dose and weight adjusted Tac levels. A P-value of less than 0.05 was considered statistically significant.

4 Results

4.1 NFAT-regulated gene expression assay

4.1.1 Concentration-effect relationship

Tac decreased the expression of NFAT-regulated genes in a concentration-dependent manner. The measured RGE values were of 15%, 47%, 71%, and 89% when the spiked Tac concentrations were 50 µg/L, 25 µg/L, 12.5 µg/L, and 6.25 µg/L, respectively (Figure 4).

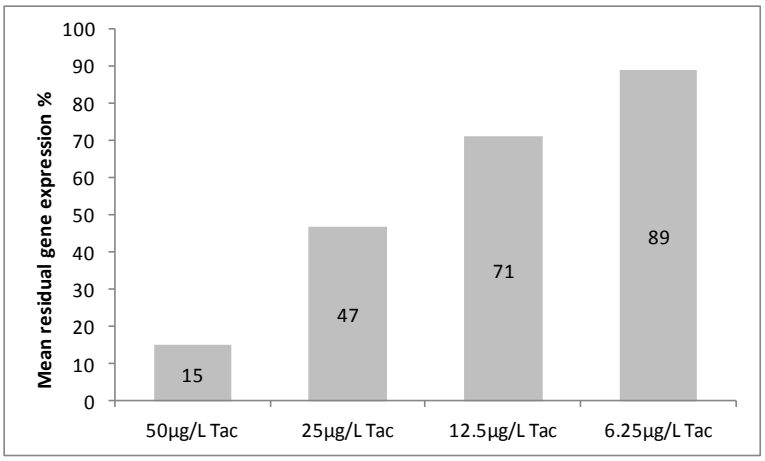


Figure 4: Effect of different concentrations of Tac on the expression of NFAT-regulated genes, *in vitro* (Single determinations). Tac= tarolimus

4.1.2 Precision

The results are illustrated in Table 4.

Table 4: The within- and between-run imprecision expressed as coefficient of variation of the NFAT-regulated gene expression assay at 3 different concentrations of Tac.

	Within-run precision			**Between-run precision**		
	Tac concentration			**Tac concentration**		
	50 µg/L	**25 µg/L**	**12.5 µg/L**	**50 µg/L**	**25 µg/L**	**12.5 µg/L**
Mean RGE (n=6)	15.9	36.1	82.6	16.5	34.0	87.5
SD	2.6	5.0	2.3	2.3	1.7	6.0
CV (%)	16.3%	13.9%	2.8%	13.7%	4.9%	6.8%

CV = coefficient of variation; RGE= residual gene expression; SD= standard deviation; Tac = tacrolimus

The within-run coefficient of variation tested at 3 different concentrations was < 17%. The within-run CVs represent the imprecision of the whole assay and not only the PCR step. The between-run coefficient of variation at 3 different concentrations was < 14%. The between-run CVs reflect the imprecision of the reverse transcription and PCR steps.

4.1.3 Limit of quantification (LOQ)

The LOQ of the NFAT-regulated genes was below 200 copies per reaction (Table 5).

Table 5: The Limit of quantification (LOQ) of NFAT-regulated genes

cDNA	Mean (n=6)	SD	CV (%)
IL-2	194	22.1	11.4
IFN-γ	154	23.5	15.2
GM-CSF	131	17.5	13.4

CV = coefficient of variation; GM-CSF = granulocyte macrophage colony stimulating factor; IFN-γ = interferon gamma; IL-2 = interleukin 2; SD= standard deviation

4.1.4 Stability

Regarding stability of the samples, the difference between RGE of the same samples (n=3) measured twice within 24 hours was in mean 6% (range -15% - +19%). Figure 5 shows the RGE after 24 hours as a % of the measurement at the first day.

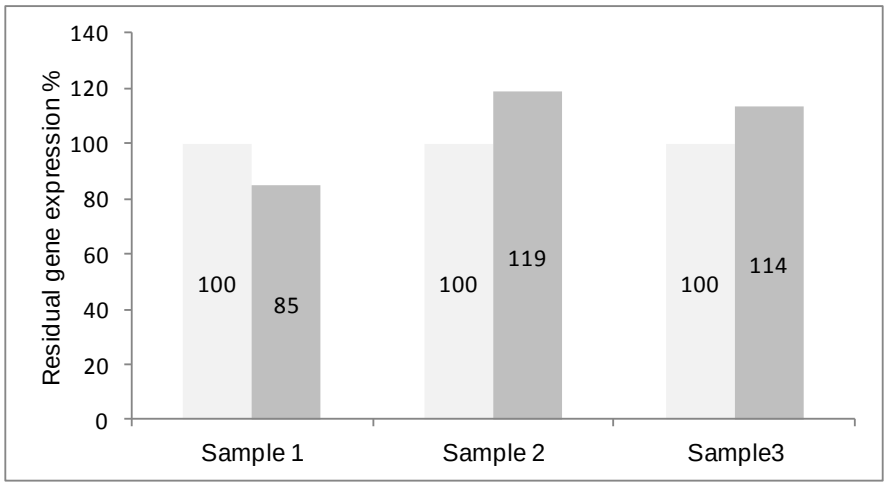


Figure 5: Stability of the NFAT-regulated gene expression assay: the residual gene expression measured after 24 h was expressed as % in relation to the RGE measured at the day of blood collection which was set to be 100% (n=3).

4.1.5 Inter-laboratory comparison

The agreement between the RGE for the 10 patients measured in the two laboratories is shown in Figure 6. The Passing-Bablok regression analysis showed no significant deviation

from linearity. However, the regression line was tilted and no constant bias was observed over the measuring range. Low RGE values were higher in the Stuttgart lab and higher RGE values were lower in the Stuttgart lab compared to Heidelberg lab. However, this lead to an acceptable mean difference of 4.5 % when data from both laboratories were analyzed using a Bland-Altman method comparison plot. Spearman's rank correlation analysis revealed an excellent and highly significant overall correlation ($r=0.951$; $p<0.0001$, $n=10$).

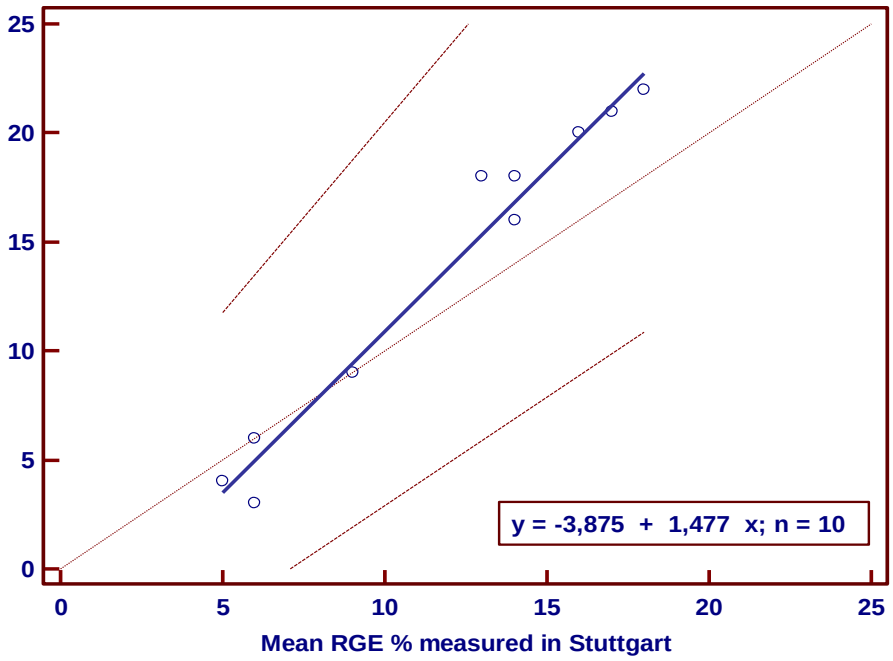


Figure 6: Passing-Bablok regression analysis of residual gene expression (RGE) measured in Heidelberg and RGE measured in Stuttgart. Regression line (solid line), the confidence interval for the regression line (dashed lines) and identity line (x=y, dotted line) are displayed

4.2 Phosphoflow assay of S6RP

4.2.1 Concentration effect relationship

Evr decreased the level of phosphorylation of S6RP in both CD3+CD4+ and CD3+CD8+ cells *in vitro* in a concentration-dependent manner as indicated in Figure 7.

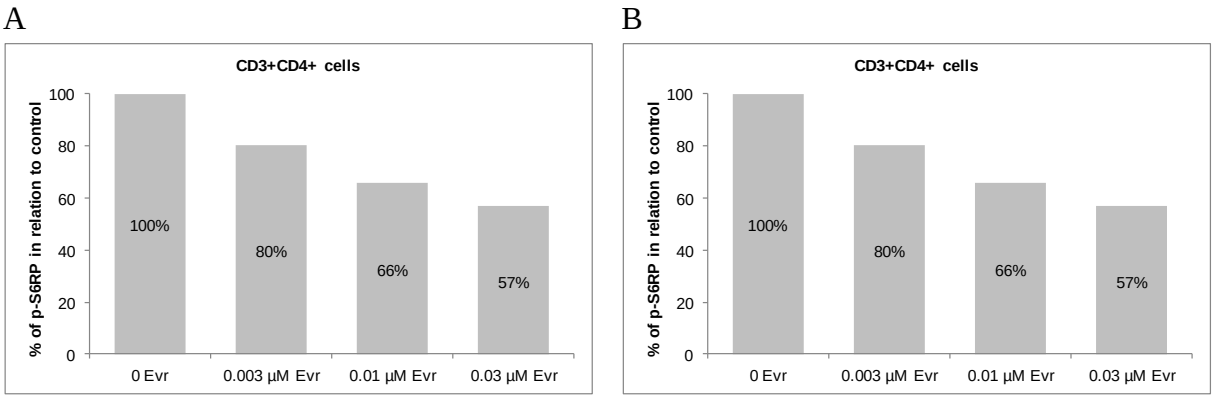


Figure 7: Effect of different everolimus (Evr) concentrations on p-S6RP in CD3+CD4+ cells (A) and CD3+CD8+ cells (B). The level of p-S6RP at each concentration of Evr was expressed as % of the p-S6RP at control sample (drug-free) which was set to be 100%. This Figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

4.2.2 Verification of the *in vivo* effect of a single dose of Evr on p-S6RP

Figure 8 indicates the changes in the level of S6RP phosphorylation in CD3+CD4+ and CD3+CD8+ cells at 1 and 3 h after a single dose of Evr (0.5 mg) in 3 healthy individuals. It can be seen that S6RP showed the greatest suppression at a time point that corresponds to the highest Evr concentration.

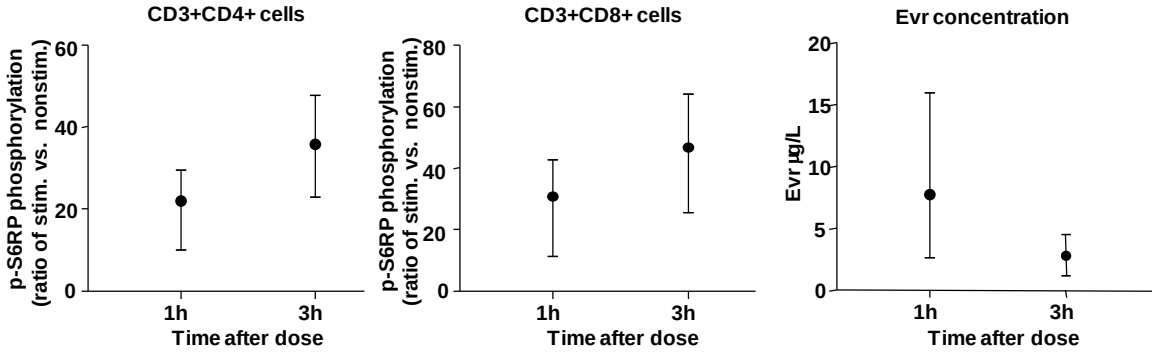


Figure 8: Time course of the effect of a single dose of everolimus (Evr) on the level of phosphorylation of S6RP in CD4+ and CD8+ cells and the concentration of Evr in blood. This Figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

4.2.3 Specificity

Specificity of the assay was investigated using samples spiked with different immunosuppressants and using samples from various patient groups, including RTx patients treated with Evr and Sir.

- ***In vitro***

High concentrations of non-mTORI immunosuppressants fail to elicit a significant effect on the level of p-S6RP *in vitro* when compared to the controls, except for CsA in CD3+CD8+ cells (Figure 9)

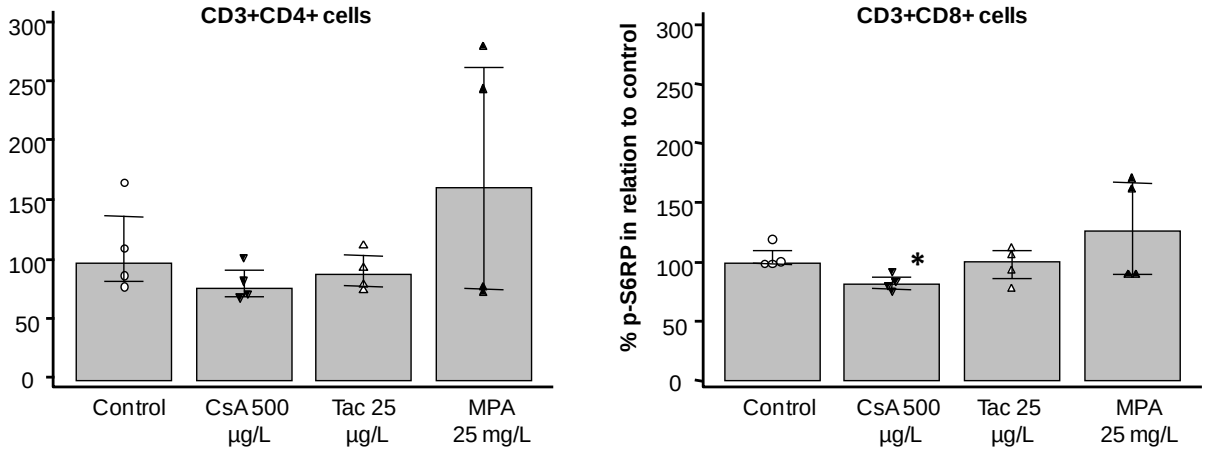


Figure 9: *In vitro* effect of tacrolimus (Tac), cyclosporine A (CsA) and mycophenolic acid (MPA) on inhibition of S6RP phosphorylation in CD3+CD4+ cells and CD3+CD8+ cells. Controls did not contain immunosuppressants. Columns show median, and bars show 25th and 75th percentiles (n=4). Median of control = 100%, *=p<0.05. This Figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

- **Ex vivo**

The results are illustrated in Figure 10.

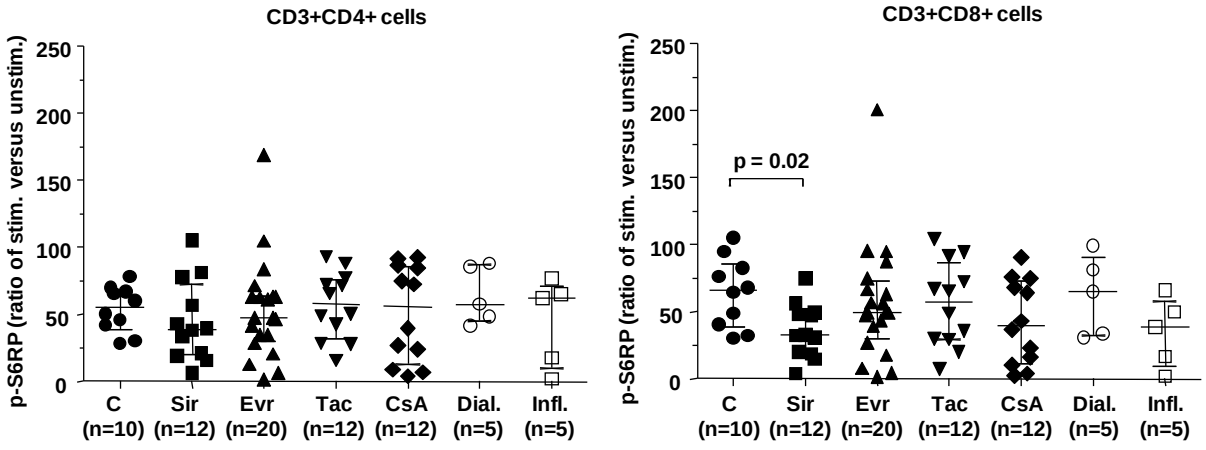


Figure 10: Level of phosphorylation of S6RP (ratio of stimulated versus unstimulated) in CD3+CD4+ cells and CD3+CD8+ cells in different patient groups (horizontal lines show median and inter-quartile range). C = controls; Sir = sirolimus; CsA = cyclosporine A; Evr = everolimus; Infl. = Inflammation (CRP>10mg/L); Tac = tacrolimus; p-S6RP = phosphorylated S6 ribosomal protein. This Figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

In comparison to the control group, the median p-S6RP was lower in both cell subsets in trough samples from patients who were under therapy with Evr or Sir. Statistical analyses showed a significant decrease in phosphorylation of S6RP only in CD3+CD8+ cells in the samples with Sir when compared to the control group (p=0.02). The differences between the other patient groups and the control group in both cell subsets were not significant (p>0.05).

4.2.4 Within-run im precision

The assay imprecision was ≤17% in 2 blood samples containing Evr in both CD3+CD4+ and CD3+CD8+ cells. Using 2 blood samples from healthy controls without use of immunosuppressant, the imprecision was ≤27% in both cell subsets (Table 6). The level of phosphorylation of p-S6RP is represented as the ratio of stimulated versus non-stimulated)

Table 6: Within-run precision of the phosphoflow assay of S6RP in CD4+ (a) and CD8+ (b) cells.

a) Imprecision of phosphoflow assay of p-S6RP in CD3+CD4+ cells

	P-S6RP (mean)	**SD**	**CV%**
Healthy control (n=6)	73	19.3	26,4
Healthy control (n=6)	63	14.1	22,2
Patient sample (Evr treated) (n=6)	59	9,5	16,0
Patient sample (Evr treated) (n=5)	75	9,4	12,5

b) Imprecision of the phosphoflow assay of p-S6RP in CD3+CD8+ cells

	P-S6RP (mean)	**SD**	**CV%**
Healthy control (n=6)	82	21,8	26,7
Healthy control (n=6)	83	15,2	18,4
Patient sample (Evr treated) (n=6)	73	12,7	17,4
Patient sample (Evr treated) (n=5)	98	15,8	16,2

CV = coefficient of variation; p-S6RP = phosphorylated S6 ribosomal protein; SD = standard deviation

4.2.5 Stability

Although some samples were stable after storage at room temperature for 24 h, other samples showed a steep fall in the level of p-S6RP phosphorylation (Figure 11). The difference between S6RP phosphorylation measured within 24 h after storage of the samples at room temperature (n=7) showed significant differences with (e.g. decrease of 67% or increase of 32%). Based on these results, all other experiments were performed within 4 h of blood collection.

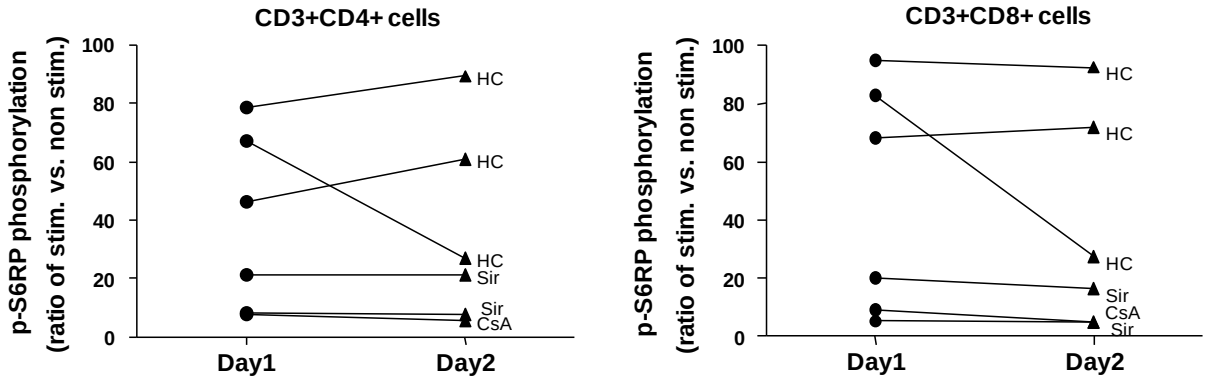


Figure 11: Change in p-S6RP phosphorylation in CD3+CD4+ cells and CD3+CD8+ cells after storage of the samples for 24 hours at room temperature. HC= healthy control; Sir = sirolimus-treated patient; CsA = cyclosporine A-treated patient; p-S6RP = phosphorylated S6 ribosomal protein. This figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

4.2.6 Method comparison

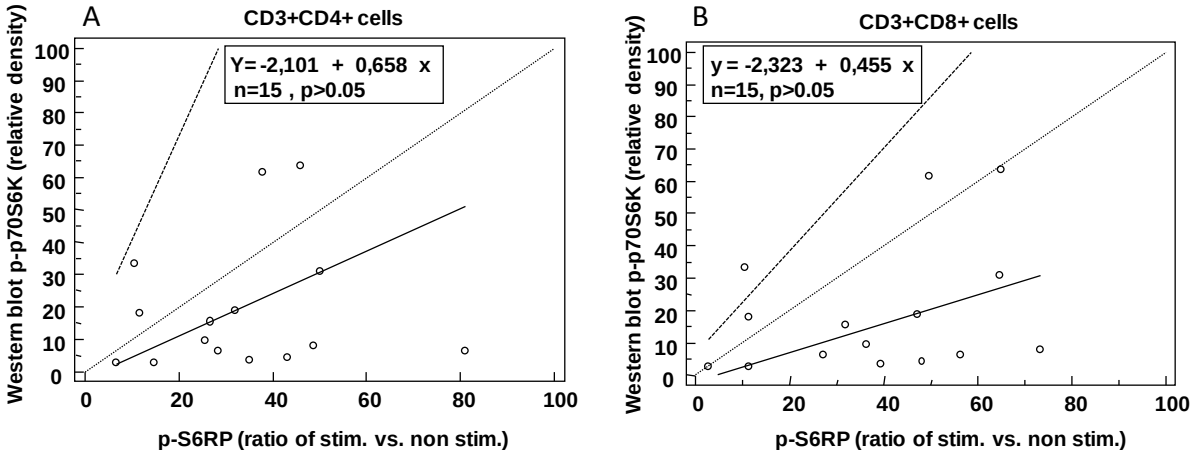


Figure 12: Comparison of western blot (p-p70S6K) and phosphoflow (p-S6RP) results in CD3+CD4+ cells (A) and CD3+CD8+ cells (B). Scatter diagram with regression line, confidence interval for the regression line and the identity line. The phosphoflow assay and the western blot were performed as given in Material and Methods. This Figure was obtained from the published work of our group (Abdel-Kahaar et al., 2016)

There was no significant correlation ($p<0.05$) between the level of p-S6RP measured by phosphoflow and the level of p-p70S6K measured by western blot in both cell subsets. Passing & Bablok regression analysis showed no agreement between the two different methods to assess the PD effect of mTORIs (Figure 12).

4.3 Effect of pharmacogenetics on Tac pharmacokinetics in the early period after renal transplantation

4.3.1 Demographic characteristics of the study cohort

A total of 121 renal transplant recipients were enrolled in this retrospective study. The demographic data of the study population are described in Table 7.

Table 7: Demographic characteristics of the study cohort:

N	121
Sex of patients (male/female)	77 (64%)/44(36%)
Age of patients (years, median/range)	55 (15 – 77)
Weight (kg, median/range)	76 (42 – 118)
Transplantation before dialysis (pre-emptive)	10 (8%)
Re-transplantation	22 (18%)
Living/deceased donor	52(43%)/69(57%)
Induction therapy	
• Basiliximab	104 (86%)
• Thymoglobulin	17 (14%)
Age of donors (years, median/range)	56 (19 – 88)
ABO incompatibility	15 (12%)
HLA mismatches (A, B, DR, median/range)	3 (0 – 6)
Panel reactive antibodies > 50%	20 (17%)
Cold ischemia time (min, median/range)	467 (39 – 2113)
Warm ischemia time (min, median/range)	45 (21 – 86)
Underlying disease	
– Glomerulonephritis	27 (22%)
– Polycystic kidneys, adult type (dominant)	24 (20%)
– Interstitial nephritis/pyelonephritis	7 (6%)
– Etiology uncertain	36 (30%)
– Others	27 (22%)

4.3.2 Frequency of the studied SNPs in the study cohort

The frequencies of the different alleles of the SNPs are shown in Table 8. Most of patients who carry the *CYP3A4*22* allele were also *CYP3A5* non-expressers, however there was no linkage disequilibrium between the two SNPs observed (D' =1, r^2=0.004). In a similar way, most of the patients who carry the *CYP3A4*1B* were *CYP3A5* expressers but they show high

degree of LD (D' =0.92, r^2=0.69). The clustering of *CYP3A4*1B* and *CYP3A4*22* carriers according to *CYP3A5* status is shown in Table 9.

Table 8: Genotype and allele frequencies of the studied SNPs in the study cohort:

SNP	Allele frequency (%)		Genotype frequency (n, %)	
ABCB1 1236 C>T	C	57.4%	CC	43 (35.5)
rs1128503	T	42.6%	CT	53 (43.8)
			TT	25 (20.7)
ABCB1 2677 G>T/A	G	57.85%	GG	41 (33.9)
rs2032582	T	40.5%	GT/GA	58 (47.9)
	A	1.65%	TT/TA	22 (18.2)
ABCB1 3435 C>T	C	51.4%	CC	35 (28.9)
rs1045642	T	48.6%	TC	54 (44.6)
			TT	32 (26.4)
CYP3A5*3	C	91.1%	CC	103 (85.1)
rs776746	T	8.9%	TC	17 (14.1)
			TT	1 (0.8)
CYP3A4*1B	T	95.5%	TT	110 (90.9)
rs2740574	C	4.5%	CT	11 (9.1)
CYP3A4*22	G	95%	GG	109 (90.1)
rs35599367	A	5%	GA	12 (9.9)

Table 9: Clustering of *CYP3A4*1B* and *CYP3A4*22* carriers according to *CYP3A5* status:

CYP3A5	*CYP3A4*1B*		*CYP3A4*22*	
	*1/*1	*1/*1B	*1/*1	*1/*22
Non-expressers	102	1	92	11
Expressers	8	10	17	1

4.3.3 Effect of genotypes on the dose-adjusted concentration of Tac

As the Tac trough levels (C0) were not available every day for all patients, we could not assess the C0/dosage ratio for the different genotypes on a daily basis. Instead, we used the available trough levels to measure the area under the time-concentration curve (AUC) as a rough estimation of the exposure to Tac. In addition, we compared the firstly-measured Tac C0 levels between the various genotypes. The daily dosage of Tac was available for almost all patients in our cohort until the patient discharged from the hospital or until the 16th day after transplantation. Table 10 indicates the number of patients with available Tac dosages at each day until day 16 after transplantation.

Table 10: Number of patients with available Tac dosages at each day of the follow-up period:

Day	***N***	**Day**	***N***
Day1	*121*	Day 9	*120*
Day2	*121*	Day 10	*112*
Day3	*121*	Day 11	*104*
Day4	*121*	Day 12	*93*
Day5	*121*	Day 13	*88*
Day6	*121*	Day 14	*80*
Day7	*120*	Day 15	*64*
Day8	*120*	Day 16	*56*

The AUC of dosage and weight-adjusted Tac concentrations was significantly higher in patients who are homozygous for the variant allele *CYP3A5*3*. This effect was persistent in the first two weeks after transplantation. Patients who carry the *CYP3A4*22* variant allele showed also a significantly higher AUC of dosage and weight-adjusted Tac concentrations when considering the two week period (days 1-16) as well as the second week (days 8-14) but not the first week (days 1-7) after transplantation. SNPs of the *ABCB1* genes (individual SNPs and the T-T-T haplotype) and the *CYP3A4*1B* allele did not show a significant effect on the AUC of dosage and weight-adjusted Tac concentrations in this period (Table 11; Figures 13, 14, 15, 16).

Table 11: Statistical significance of the correlation between genetic variants and the AUC of Tac levels (dosage and weight adjusted)

Genetic variant	Statistical Test	Unadjusted p-value		
		AUC of Tac level/dosage/weight		
		Day 1-16	Day 1-7	Day 8-14
ABCB1 1236 C>T	Kruskal-Wallis	0.65	0.516	0.432
ABCB1 2677 T>G/A	Mann–Whitney U test (A carriers vs others)	0.138	0.574	0.0601
ABCB1 3435 C>T	Kruskal-Wallis	0.488	0.85	0.172
ABCB1 haplotype T-T-T versus non T-T-T	Mann–Whitney U test	0.823	0.605	0.933
*CYP3A5*3*	Mann–Whitney U test (CC vs CT&TT)	**0.0014**	**0.0018**	**0.0025**
*CYP3A4*1B*	Mann–Whitney U test	0.167	0.105	0.386
*CYP3A4*22*	Mann–Whitney U test	**0.045**	0.284	**0.008**

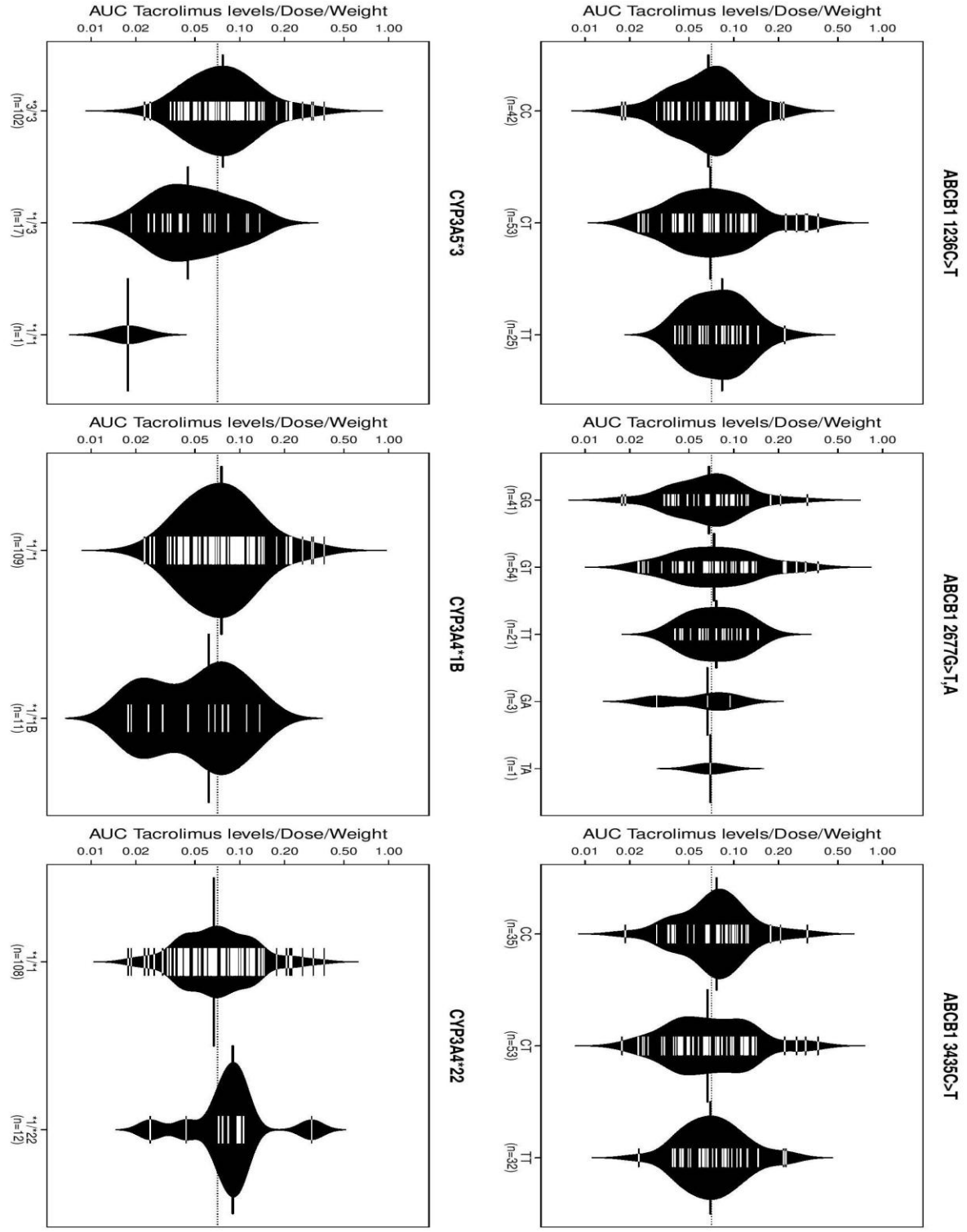


Figure 13: Beanplots illustrating the AUC of dosage&weight-adjusted Tac levels versus time in the first week after transplantation (AUC_{1-7}) for each of the investigated genetic variants. The individual measurements are shown as lines. The median for each group is shown by a thick black line; the overall median in a plot is shown as a dotted line.

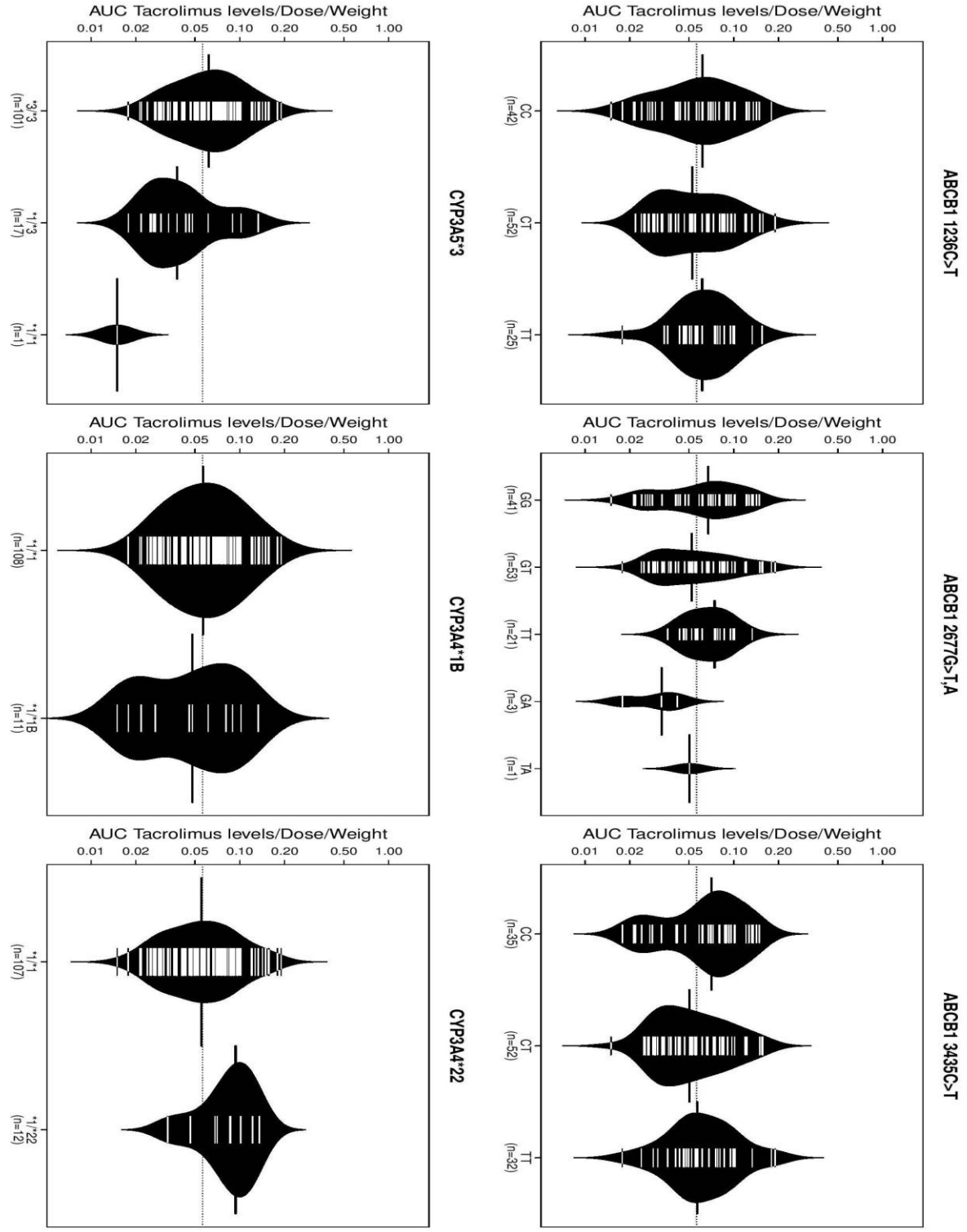


Figure 14: Beanplots illustrating the AUC of dose&weight-adjusted Tac levels versus time in the second week after transplantation (AUC_{8-14}) for each of the investigated genetic variations. The individual measurements are shown as lines. The median for each group is shown by a thick black line; the overall median in a plot is shown as a dotted line.

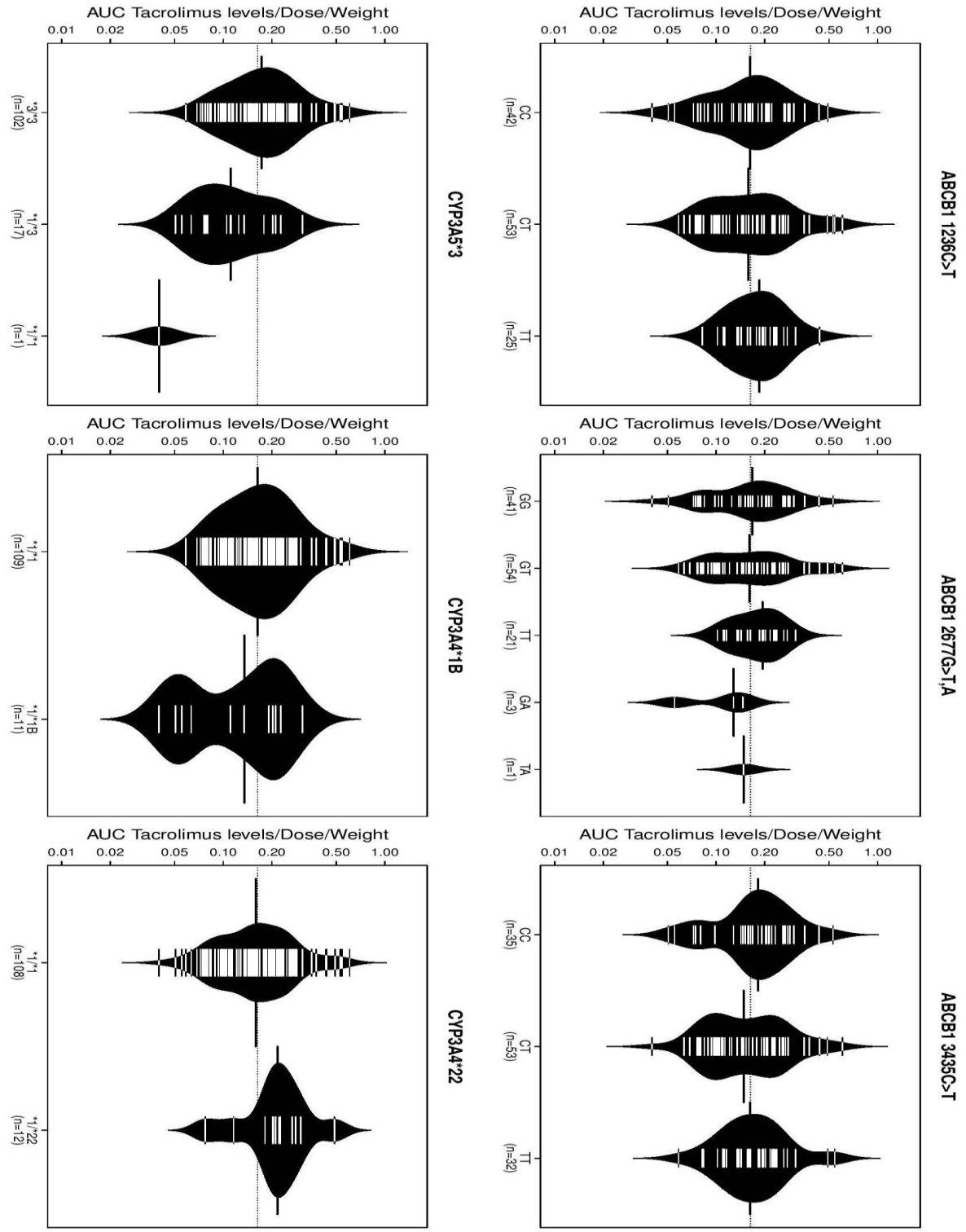


Figure 15: Beanplots illustrating the AUC of dose&weight-adjusted Tac levels versus time at days 0-16 after transplantation ($AUC_{1\text{-}16}$) for each of the investigated genetic variations. The individual measurements are shown as lines. The median for each group is shown by a thick black line; the overall median in a plot is shown as a dotted line.

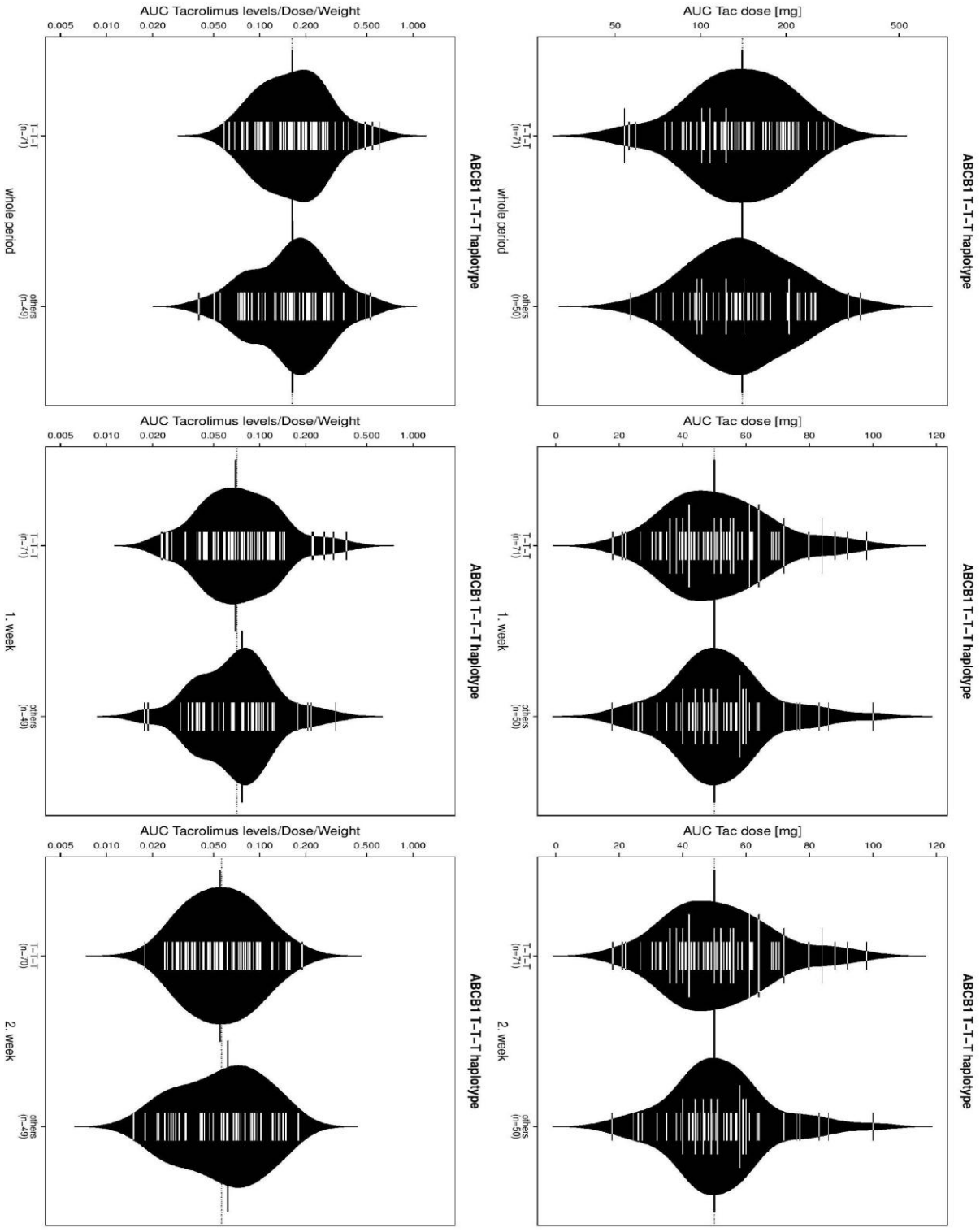


Figure 16: Beanplots illustrating the AUC of dose&weight-adjusted Tac levels versus time as well as the AUC of Tac dosage versus time during the first week, second week and at days 0-16 after transplantation (AUC_{1-7}, $AUC_{8-}14$, $AUC1_{-16}$) for the *ABCB1* haplotype (T-T-T) compared to the other genotypes. The individual measurements are shown as lines. The median for each group is shown by a thick black line; the overall median in a plot is shown as a dotted line.

4.3.4 Effect of genotypes on Tac-dose requirements

The results are illustrated in figure 17. None of the selected *ABCB1* SNPs showed a significant effect on the Tac dosage requirement in the first 2 weeks after transplantation. Patients who carry the *CYP3A5*3/*3* genotype showed a significantly lower dose requirement (mg/kg/day) compared to those who carry at least one functional allele (*CYP3A5*1*) considering a 2 week period after transplantation. There was also a trend of significance toward a decreased dose requirement in patients who carry *CYP3A4*22*, again considering a 2 week period after transplantation; the difference reached statistical significance only at day 10 (p= 0.0258). A similar trend in the opposite direction was shown for patients who carry the *CYP3A4*1B* requiring higher dosages of Tac; only at day 12, 15 and 16 data reached statistical significance keeping in mind that Tac dosages were missing for some patients at day 15 and 16 who already were discharged from hospital.

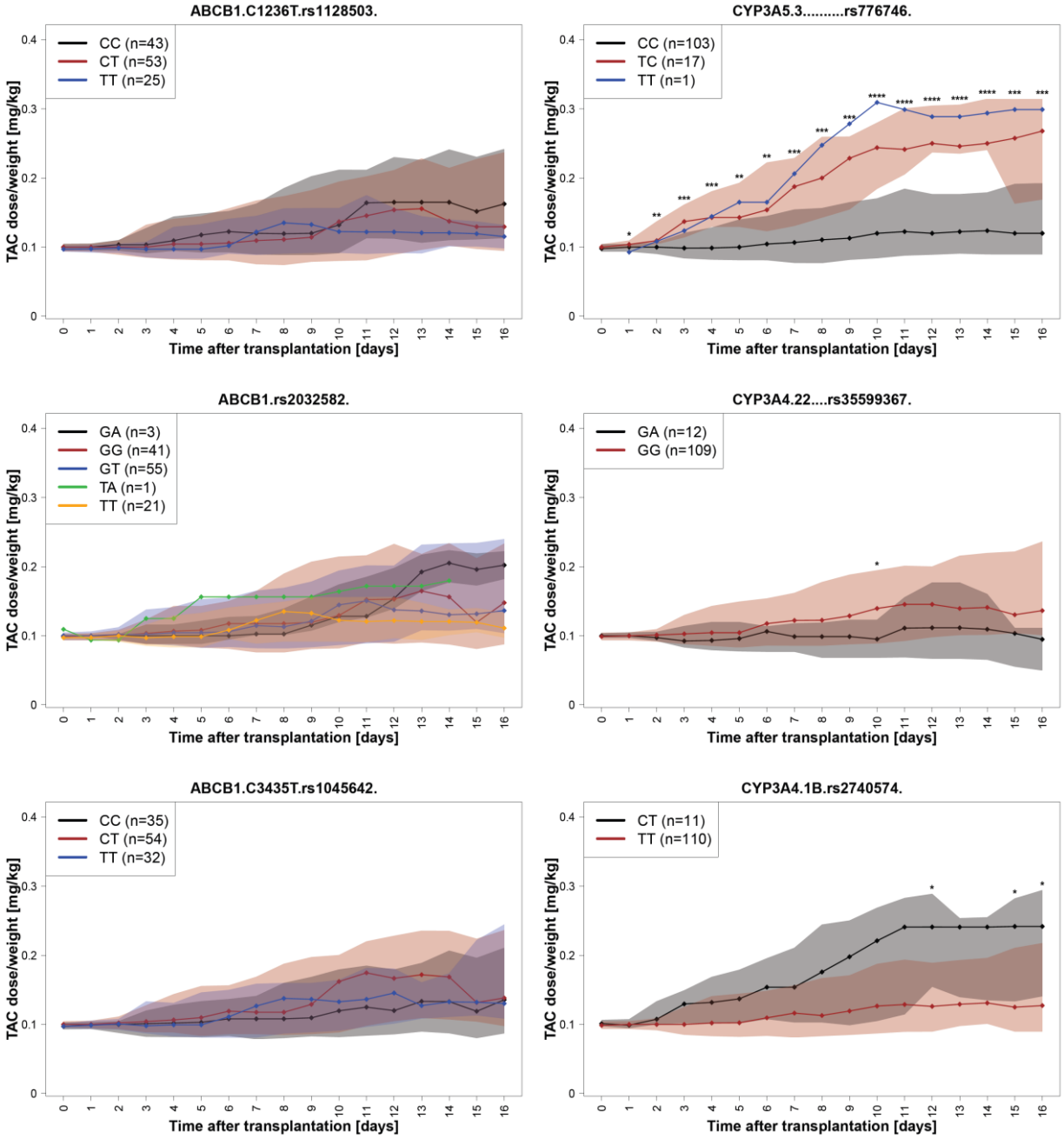


Figure 17: Tac dose per body-weight (mg/kg/day) at days 0-16 after transplantation for all investigated genetic variants. The different genotype groups are marked by different colors. Diamonds represent medians of Tac dose/weight at the different days; shaded areas are defined by 25% and 75% quantiles. Tac= tacrolimus

4.3.5 Association between the different SNPs and clinical outcomes

Delayed graft function (DGF; defined as the need for dialysis in the first week after surgery) was observed in 30 patients (25%). Acute rejection was observed in 21 patients (17%) of the study population. *ABCB1* 3435 C>T polymorphism was found to be associated with increased risk of DGF (P = 0.02). No other significant associations between the investigated SNPs and acute rejection or DGF were found. The estimated glomerular filtration rate (eGFR) measured at discharge of patients was not significantly different between the genotypes studied (Table 12).

Table 12: Association between the studied SNPs und clinical events in the early post-transplant period

Genetic variant	Unadjusted p-value		
	eGFR MDRD at discharge (ml/min)	**Delayed graft function (DGF)**	**Acute rejection**
***ABCB1* 1236**	0.811	0.707	0.905
***ABCB1* 2677 T >G/A**	0.907	0.571	0.538
***ABCB1* 3435 C>T**	0.198	**0.023**	0.533
CYP3A5*3	0.076	0.147	0.515
CYP3A4*1B	0.891	0.463	1
CYP3A4*22	0.384	0.728	1

eGFR MDRD = estimated glomerular filtration rate by *The Modification of Diet in Renal Disease study equation*

5 Discussion

"If it were not for the great variability among individuals, medicine might as well be a science, not an art" Sir William Osler, 1892

5.1 The use of biomarkers to complement therapeutic drug monitoring of immunosuppressive therapy

The field of transplantation medicine has witnessed a great success in the last few decades. This success is attributed in large part to the use of highly efficient immunosuppressive protocols **(Lechler et al., 2005)**. CNIs and mTORIs represent important components of all immunosuppressive protocols. The use of these agents requires careful monitoring because of their narrow therapeutic window (i.e. having little difference between toxic and therapeutic doses) and their unpredictable pharmacokinetics (**Halloran, 2004)**.

Currently, the PK monitoring (conventional TDM) is used to guide the therapy with these drugs where the concentration of the drug in the blood is used to adjust the dose. This approach is very valuable to prevent acute fluctuation of the drug level in the blood and consequently may contribute to avoid acute toxicity and/or rejection **(Budde and Glander, 2005)**. However, this approach is highly limited to determine the optimal starting dosage for each individual patient since it can be applied only after the drug therapy has been started. Moreover, the PK approach is of less value to optimize the dosage of immunosuppressants in the stable phase after renal transplantation. The ultimate goal of the conventional TDM is to achieve a blood concentration of the drug within a pre-defined therapeutic range. This range, however, has not been established yet for the stable phase after transplantation where the risk of rejection varies among individuals and is decreasing over time **(Olbricht, 2012)**.

It became increasingly clear in recent years that the success in the early phase of renal transplantation does not continue regarding long-term function and patient survival. Two factors are in particular responsible for this. Firstly, chronic rejection is so far uncontrollable and secondly long term side effects of immunosuppression cannot be predicted, such as nephrotoxicity, development of diabetes mellitus and cardiovascular diseases and the incidence of opportunistic infections & malignant neoplasms **(Lechler et al., 2005)**. In order

to improve the long-term results in the future, many transplant centers adopt a new strategy through tapering the dosage of the immunosuppressive drug to minimize the toxicity profile without increasing the risk for short-term graft survival. However, this strategy requires an individual approach since different patients have a different risk profile. At present time, there is a lack of indicators which allow a reliable assessment of the immune status of the individual patients **(Olbricht, 2012)**.

In line with this background, optimization of immunosuppressive drug therapy through the PK approach alone is insufficient and should be complemented with a PD and PGx approaches by the use of appropriate and validated biomarkers **(Wieland et al., 2012)**. In general, biomarkers that can be used to manage the immunosuppressive therapy after transplantation fall into 2 categories: a) drug specific biomarkers which reflect the specific pharmacological effect of an immunosuppressive agent and b) drug non-specific biomarkers which reflect the overall effect of drugs influencing the immune function **(Shipkova, 2016)** (Figure 18).

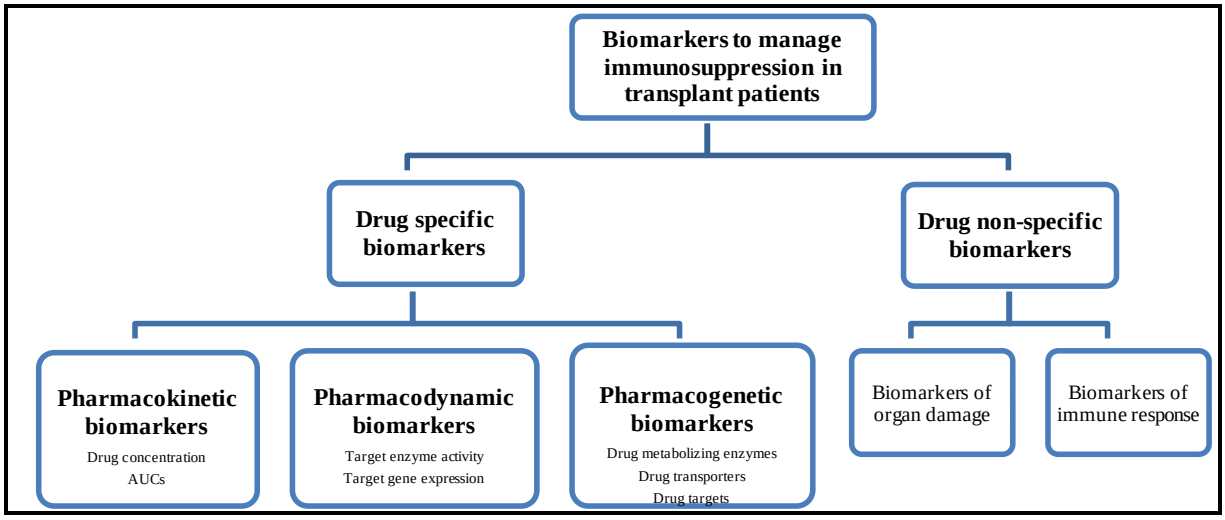


Figure 18: Types of biomarkers to manage therapy with immunosuppressive drugs after transplantation (Adapted from Shipkova, 2016)

Several biomarkers have been proposed to track the general effect of immunosuppressive therapy on immune cells (drug non-specific biomarkers). The activation and proliferation of T lymphocytes (which play a central role in the rejection reaction and in the defense against infection) are frequently monitored in *ex vivo* models in which the cells are stimulated by

mitogens. T cell activation may be monitored by e.g. the expression of surface molecules on CD3+, CD4+ or CD8+ cells, measuring intracellular ATP concentration in CD4+ cells, tracking cytokine production by activated T-cells (ELISPOT assay; enzyme-linked immunosorbent spot assay) or through measuring the level of soluble CD30 (sCD30) which is produced and released in blood by activated T lymphocytes. Lymphocyte proliferation may be traced by monitoring DNA synthesis or the expression of genes which play a role in the regulation of the cell cycle such as PCNA (proliferating cell nuclear antigen) **(Shipkova and Wieland, 2012)**. Another approach to assess the status of the immune system comprises the determination whether tolerance has been developed, which would allow minimization of immunosuppression. An indicator of a possible tolerance are regulatory T-cells (Tregs), which can be identified by typical surface markers using flow cytometry (CD4+ CD25+ CD127low/–) or by means of demethylated transcription factor FOXP3 protein **(Su et al., 2012)**. Only two of these biomarkers were approved to be used clinically and include Immuknow™ which is a functional immune assay to measures the increase in ATP production by activated T-lymphocytes and the AlloMap® which measure specific gene expression patterns to determine the risk of acute rejection in heart transplant recipients **(Wieland et al., 2012)**.

This work focused on drug-specific biomarkers (PD and PGx) which will complement the classical PK approach for the individualization of immunosuppressive therapy. Specific PD biomarkers include measuring target enzyme activity or target gene expression while PGx biomarkers comprise genetic variants of candidate genes which could impact drug disposition or response.

Different biomarkers could have different roles in optimizing immunosuppressive therapy after transplantation. The potential of each biomarker category can be seen in Figure 19. The PGx profile of the patient would be helpful in drug selection and in determining the starting dose of the drug. PD biomarkers, on the other hand, would help to select for the right dose over time **(Urtasun et al., 2008)**.

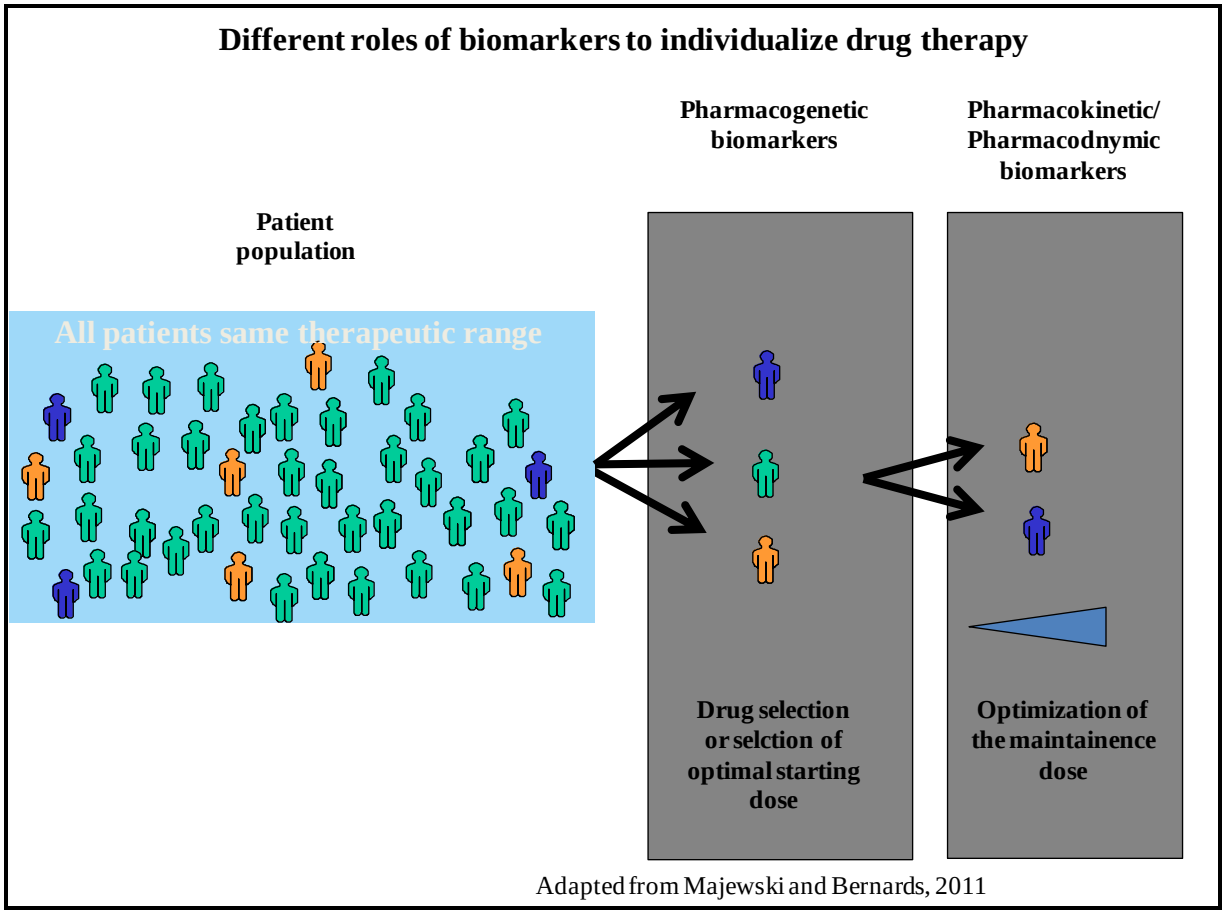


Figure 19: The potential roles of different biomarkers to individualize immunosuppressive drug therapy

5.2 Validation of biomarker assays

For a biomarker to be appropriate to be implemented into routine clinical practice, certain criteria must be fulfilled. The biomarker a) should be non-invasive, easily measurable and cost-effective; b) a short turn-around time should exist (the result should be available within 24 h); c) a reliable assay with data from clinical trials should be available and d) the assay should demonstrate satisfactory analytical performance. The availability of an optimized assay kit that is commercially supplied is also of a great advantage when it comes to the routine diagnostics, as it prevents the impairment of the assay performance by confounding factors **(Shipkova et al., 2016)**.

Despite the great number of biomarker studies that have been published in recent years, most of these biomarker assays did not find their way into clinical practice because they are, with few exceptions, neither analytically validated according to international guidelines nor suitable due to their complicated nature for routine use. Furthermore many laboratory-

developed techniques have not been cross validated between laboratories and the reproducibility of results between laboratories is questionable **(Shipkova et al., 2016)**.

In the era of personalized medicine and with the expected increase in the use of biomarkers as a crucial component of the strategy of personalized medicine, the health authorities start to provide guidelines and regulations for validation of biomarkers. According to the FDA, **a valid biomarker assay** is 'a biomarker measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results' **(FDA: Guidance for Industry Pharmacogenomic Data Submissions, 2005)**.

To foster the implementation of new biomarkers into routine practice, more effort needs to be exerted for the analytical validation of promising candidate biomarkers according to international guidelines. The validation of biomarker assays must include the entire path starting with selecting the proper sample matrix, pre-analytical aspects and analytical performance. However, the level of validation depends on the intended use of the assay and also on the assay characteristics and this is called "fit-for-purpose" validation. In general, the performance characteristics that should be covered through validation of biomarker assays include one or more of the following parameters: linearity, specificity, precision, accuracy, analytical sensitivity (limit of detection (LOD) or LOQ) robustness and stability (**Landeck et al., 2016)**.

A next step after the validation of the assay in one center is the harmonization and standardization of the results across laboratories. Different laboratories adopt different approaches for sample processing and data analyses of biomarker assays. This results in discrepancies of the generated results which consequently raises the question about the clinical validity of such assays. Consequently, harmonization of the results of biomarker assays across laboratories is an essential pre-requisite before their clinical implementation on a wide scale **(Britten et al., 2008)**.

The discrepancy of results generated from different laboratories is also a major challenge in case of PGx biomarkers. While some laboratories report significant effects of a certain SNP or haplotype on the pharmacokinetics or pharmacodynamics of drugs, other laboratories

report non-significant or sometimes contradictory results. To overcome such a problem, large multicenter trials are needed where uniform phenotypic conditions have to be applied in all participating centers (study population, treatment strategy , …etc) (**de Jonge and Kuypers, 2008)**.

For a more effective approach toward the implementation of the PGx biomarker in the clinical settings, the dynamic relationship between the individual's genotype and phenotype should be also considered. The effect of genetic variation on the drug's response is changing and depends on several factors. From this perspective, the development of algorithms to incorporate the PGx data with other covariates together would have a higher predictive value than the mere consideration of a single marker **(Schwab and Schaeffeler, 2012)**. A successful implementation of this approach can be seen by the anticoagulant drug warfarin where genotyping data of CYP2C9/VKORC1 were combined with other factors e.g. age, sex, body mass index … etc. to generate an equation which can be used for dose calculation in clinical routine **(Schwab and Schaeffeler, 2011)**.

In this work, two PD biomarker assays were analytically evaluated; 1) NFAT-regulated gene expression assay which is developed to monitor the therapy with CNIs. This assay was developed in Heidelberg by Giese et al **(Giese et al., 2004)**. However, data about the performance of this assay in an independent laboratory as well as data about interlaboratory performance were lacking. Thus this assay was validated in the laboratory of Klinikum Stuttgart and a small harmonization trial with kidney center in Heidelberg was conducted as well; 2) Phosphoflow assay of S6RP: a protocol to measure the level of phosphorylation of S6RP using the new technique of phosphoflow was set up and the analytical performance of the assay was investigated. This assay was intended to be used as a PD biomarker of the mTORIs.

In addition to these two PD assays, a retrospective PGx study was also conducted to assess the prevalence of seven SNPs in functional relevant candidate genes (CYPs and ABCB1) using a cohort of 121 renal transplant recipients in Stuttgart and to elucidate the impact of the selected SNPs on Tac pharmacokinetics in the early period after transplantation.

5.3 NFAT-regulated gene expression assay

Consistent with their extensive use as immunosuppressive agents after organ transplantation, the effectiveness of CNIs on immune functions has been thoroughly studied to find out suitable PD biomarkers of their response. The change in the expression of cytokine genes under the influence of CNI is a potentially useful PD biomarker and the quantitative analysis of this change using RT-PCR has proposed as a tool for this purpose **(Giese, 2001)**.

Through their inhibitory effect on the transcription factor NFAT, three genes were found to be chiefly downregulated by CNIs; IL-2, INF-γ and GM-CSF. An assay protocol to measure the level of transcription of these genes before and after CsA or Tac intake was set up based on RT-PCR. The ratio between expression before and after drug intake expressed as RGE can be used as readout of the effect of CNI.

The correlation between the RGE and clinical events after transplantation, particularly infection and malignancy, has been demonstrated in different patient cohorts. Sommerer et al. have also proved the ability to taper the dosage of CsA in stable transplant patients without risking the graft function based on RGE **(Sommerer et al., 2008)**. Data on the analytical performance of this assay in two independent laboratories were, however, lacking.

In this study, the guidelines and recommendations for validation of methods that depend on quantitative Real-Time PCR **(Bustin et al., 2009; Broeders et al., 2014; Sanders et al., 2014)** were applied to test the performance characteristics of a PD assay based on analysis of gene expression to monitor therapy with CNI. To test the analytical performance of the assay, drug free blood samples which were supplemented with Tac *in vitro* were used. To assess the comparability of the NFAT-regulated gene expression assay between laboratories, samples of transplant patients under CsA therapy were collected and analyzed in Heidelberg where the assay has been established and set up for many years and sent to Stuttgart to be re-analyzed within 24 hours. Results of the *in vitro* experiments showed that the NFAT-regulated gene expression assay is reproducible at acceptable limits both within-series as well as between-series after supplementation with Tac. Previously published results showed that using RT-PCR to measure cytokine gene expression is sensitive and precise with a between-run precision <20% as reported by Giese et al. **(Giese, 2001; 2003)**, which was confirmed when

using this assay in the Stuttgart lab. The performance data have agreed also with published guidelines for validation of qualitative real-time PCR methods **(Broeders et al., 2014)**.

The samples were stable after 24h of storage at room temperature which is valuable in clinical situations. Inter-laboratory comparison using blood samples from kidney graft recipients showed an acceptable bias between both labs. Using a Bland-Altman plot to investigate method agreement the mean difference between both laboratories was only 4.5 %. However a trend for a difference has been noted which was not constant over the observed range of RGE. As it can be seen from the Passing-Bablok regression analysis at lower RGE the results were higher in Stuttgart, whereas at higher RGE results were lower compared to Heidelberg. According to the experiences in Heidelberg, 10 copies/µl can be easily achieved on the LightCycler instrument for all three genes (T. Giese, personal communication) whereas in Stuttgart the LOQ was between 100-200 copies per reaction which corresponds to 20-40 copies/µl in our qPCR protocol. This may explain the discrepant results particularly at the low end of the RGE observed in the inter-laboratory comparison experiment. This trend could not be further verified or ruled out in this small comparison study due to the limited number of samples. Although this shift between low and high RGE had no major impact on the overall satisfactory method congruence, this emphasizes the requirement to carefully validate or verify the performance of sophisticated biomarker assays before implementing them in a clinical setting.

Taken together the data shows for the first time that the relatively complex assay can be applied in different laboratories leading to comparable results. These results were obtained despite the modification of a critical step of the assay protocol which is RNA isolation. The laboratory in Stuttgart used another approach to isolate RNA to accommodate the assay procedures to its own instruments. An important factor for this positive result is certainly a centralized primer production and a well-developed and described assay procedure which is provided to the customers in the package insert of the primers. The comparability of results between laboratories could be further improved by manufacturing standards and stable quality control material for the intra-laboratory control of accuracy and precision as well as for providing samples for a proficiency testing scheme which can be used for external quality control.

The possibility of obtaining comparable results of biomarker assays among different laboratories by adoption of uniform procedures in the field of transplantation medicine has been proven by Ashoor et al. They have cross-validated an IFN-γ Elispot assay for measuring the T-cell alloreactivity in renal transplant recipients and showed that the implementation of standard operating procedure (SOP) regarding sample processing and data analyses has remarkably improved the reproducibility of the assay with an interlaboratory CV of about 30% **(Ashoor et al., 2013)**. This activity was done under the umbrella of the CTOT (Clinical Trials in Organ Transplantation) consortium which is an association of multiple transplant centers in North America deeply engaged in transplant-related biomarker development and standardization. Other successful examples of biomarker assay harmonization by the CTOT consortium are the establishment of a SOP for gene expression profiling **(Keslar et., 2013)** and the standardization of solid phase multiplex-bead arrays for detection of HLA-antibodies **(Reed et al., 2013)**.

As a further extension of the efforts to promote the harmonization of biomarker immune-monitoring, the transplantation community launched recently a project called "Global Virtual Laboratory (GVL)". The objective of this project is the establishment and the universal dissemination of assay protocols which allow reliable monitoring of the alloimmune response **(Geissler et al., 2015)**.

In a future perspective, the NFAT-regulated gene expression assay has a potential to be used in multicenter trials involving local laboratories. Under these conditions it is feasible that in an acceptable time frame clinical data from multicenter trials with different grafts and immunosuppressive regimens will be available to estimate the true diagnostic sensitivity and specificity of the assay as well as the specific cut off values for CsA and Tac to predict clinical events. In addition, the NFAT-regulated RGE assay fulfills criteria which make it likely that the assay may reach routine application. However, one drawback is the requirement to collect two samples per patient one before and one after drug intake. This procedure may be suitable for an in-patient application but is inconvenient for an outpatient setting.

5.4 Phosphoflow assay of S6RP

The response to mTORIs entails many changes at the molecular level. The p70 ribosomal protein S6 kinase (p70S6K) and its target the S6 ribosomal protein (S6RP) are consecutive

molecules in the signaling cascade downstream to mTOR (Figure 3). Inhibition of the kinase activity of mTOR by the mTORIs resulted in decreased level of phosphorylation of these molecules which suggest the level of phosphorylation as a potential biomarker for mTOR inhibiting drugs.

Hartmann et al. were able to measure the level of phosphorylation of p70S6K by western blot and they observed a significant decrease in the level of phosphorylation of p70S6K in patients treated with Sir when compared with patients under non-mTORI immunosuppression or with healthy controls. The level of phosphorylation was also correlated with the incidence of acute rejection **(Hartmann et al., 2005)**. The use of Western blot is, however, suitable for research purposes but not for routine use.

Flow cytometry is a powerful analytical platform and it is superior to many of the conventional methods since it is sensitive, rapid and high-throughput **(Krutzik et al., 2004)**. The development of antibodies against phosphoproteins together with protocols for fixation and permeabilization of the cells has enabled the utilization of flow cytometry for detection of phosphoproteins inside the cells; this is called phosphoflow **(Krutzik and Nolan, 2003)**.

In this work, the level of phosphorylation of S6RP was measured through a phosphoflow protocol employing a commercial kit for fixation and permeabilization of leucocytes in whole blood samples (PerFix-p Kit, Beckman Coulter, Inc.) and anti-human p-S6RP (S235/S236) as a phosphoantibody. The assay performance was tested both *in vitro* where blood samples were spiked with different immunosuppressive agents as well as with clinical patient samples. In addition three volunteers took a single dose of Evr and the level of p-S6RP and the drug concentration in blood were measured 1h and 3h thereafter. A method comparison with western blot assay employing an anti p-p70S6K was also conducted

In vitro experiments showed that p-S6RP reflects the effect of Evr in CD4+ as well as CD8+ cells. This effect has been verified *in vivo* with 3 healthy volunteers took a single dose of Evr. It was shown that the lowest level of p-S6RP correspond to the peak concentration of Evr in blood (Figure 8). However, on testing the specificity of the assay *in vitro* through spiking blood samples with large concentrations of non-mTORI immunosuppressants, a significant effect of CsA to decrease S6RP in CD8+ cells has been observed (Figure 9). A similar observation has been also reported by other research groups **(Hoerning et al., 2015; Wang**

and Fan, 2015). This observation may reflect the complexity of the signaling network that involves activation of mTOR which could be modulated by several other signaling pathways **(Abdel-Kahaar et al., 2016)**.

Applying the assay to measure S6RP in blood samples from different patient groups showed that the level of phosphorylation of S6RP is decreased in both CD4+ and CD8+ cells in Sir and Evr treated patients groups. However, this effect was statistically significant only with Sir in CD8+ cells. This effect may be attributed to the differences in the physiochemical properties between Sir and Evr (which may lead to differences in their pharmacokinetics or pharmacodynamics) on one side and differences between CD4+ and CD8+ cells regarding their different profiles of signaling cascades on the other side **(Abdel-Kahaar et al., 2016)**.

While Sir is a naturally occurring macrolide, Evr was produced by adding a hydroxyethyl group at position 40 of Sir. Owing to this chemical modification, the PK properties of the 2 drugs vary considerably. Compared to Sir, Evr is more polar and has a much shorter elimination half life (28 h compared to 62 h in mean) **(Klawitter et al., 2015)**.

"The role of mTOR signaling in regulation of proliferation and differentiation of T cells varies between CD4+ and CD8+ cells and may entail diverse mechanisms. Likewise, pharmacological inhibition of mTOR signaling may induce different responses in both cell subsets. High dose of rapamycin inhibits both complexes of mTOR (mTORC1 and mTORC2) in CD4+ cells but not in effector CD8+ cells where mTORC1 only is inhibited. Analysis of the functional markers of PBMC in liver transplant patients who receive Evr by polychromatic flow cytometry showed that CD4+ and CD8+ cells express different panels of markers upon activation with a superantigen which is another evidence on the differential response of CD4+ and CD8+ cells to mTOR inhibition" **(Abdel-Kahaar et al., 2016)**.

While the assay concept was verified both *in vitro* and *in vivo,* the results of inter-method comparison of the same samples were disappointing. Parallel measurements of the same samples with western blot revealed lack of agreement between results from both methods. However, it should be noted that western blot analysis was performed with anti-p-S6K (Thr389) phosphoantibody while our phosphoflow protocol employs an anti-p-S6RP (Ser235/236).

In summary, the phosphoflow assay of p-S6RP as a PD biomarker of mTORIs provides several advantages including the possibility to work with whole blood samples and its satisfactory analytical performance. However the assay turns to be non-specific to the effect of mTORIs which questions its fitness for purpose. Whether further optimization of the assay protocol would improve the diagnostic utility, still needs to be determined.

5.5 Effect of pharmacogenetics on Tac pharmacokinetics in the early period after renal transplantation

The early period post-transplant is very critical since it is associated with the highest incidence of graft dysfunction or rejection which was found to be attributed to non-optimal exposure to Tac **(Staatz et al., 2001; Kuypers et al., 2010a)**. There is also no role for TDM to determine the initial dosing of Tac. Therefore, the presence of indicators of patient's response to drugs would be of major help in dose optimization regarding this critical time **(Abboudi and MacPhee, 2012)**. It is well known that for some examples genetic variation can account for up 95% of interindividual variability in drug effects and disposition. Most of these variations are related to drug metabolizing enzymes or transporting proteins **(Evans and McLeod, 2003)**. In this study, the influences of seven SNPs in *CYP3A4/5* and *ABCB1* on Tac PK in the early period after transplantation were investigated. The studied SNPs include: *CYP3A5*3, CYP3A4*22, CYP3A4*1B,* and four SNPs in the *ABCB1* gene (3435T>C, 2677T>G/A and 1236T>C).

The significant role of the *CYP3A5*3* on the PK of Tac in the early period post-transplantation was confirmed. The frequency of *3/*3 genotype (non-expressers) in the study cohort was about 85%; these patients showed an increased AUC compared to *CYP3A5* expressers. This effect was persistent when considering the first week ($AUC_{1\text{-}7\ days}$), the second week ($AUC_{8\text{-}14\ days}$) or the whole follow-up period ($AUC_{1\text{-}16\ days}$). In addition, non-expressers required lower doses of Tac compared to expressers and the difference was statistically significant at all time points. After exclusion of re-transplant patients and patients who were on Tac therapy before the transplantation for any reason, the firstly-measured C0 of Tac in de novo renal transplant recipients (n=77) was significantly lower in expressers versus non-expressers (P = 0.00045). These results were in line with previously published results **(Zhang X et al., 2005; Roy JN et al., 2006 and Gervasini G et al., 2012)**.

Recently, a newly discovered SNP in intron 6 of the *CYP3A4* gene (*CYP3A4*22*, rs35599367) was found to be associated with a higher Tac C0 and a lower Tac dose requirement compared to homozygous wild type subjects early after transplantation **(Elens et al., 2013a and Pallet et al., 2015)**. However, the effect of this SNP was not unequivocal. Some research groups reported an effect of this SNP on Tac dose requirement **(Gijsen et al., 2013)** while others did not find any effect of this SNP **(Tavira et al., 2013)**.

In our study cohort of 121 renal transplant recipients, the frequency of the *CYP3A4*22* carriers was about 10%. Patients who carry this allele required lower weight-adjusted doses of Tac than non-carriers, whereas the difference reached statistical significance only at day 10 post transplantation. The firstly-measured Tac C0 level in de novo transplant recipients (n=77) was higher in *CYP3A4*22* carriers (ranging from 4.6 to 24.4, median 9.7mg) versus non-carriers (ranging from 2.5 to 28, median 7.6mg), however the difference was not statistically significant. Although we observed a statistically-significant difference between the 2 genotypes regarding the AUC in the second week post-transplant ($AUC_{8\text{-}14\ days}$, p= 0.0078), this result should be interpreted with caution because many patients were discharged from the hospital after the 10^{th} day post-operative (see Table 10) and only limited every day Tac C0 levels were available for patients who stayed longer.

The *CYP3A4*1B* was described to be an increased-in-function allele as its presence has been linked to increased gene expression **(Amirimani et al., 2003)**. The results of the study cohort support this hypothesis as patients who carry this polymorphism required higher dosages of Tac during the first two weeks post-transplant. However almost all patients who carry *CYP3A4*1B* (10 out of 11) were also *CYP3A5* expressers and a high degree of LD was observed between the two SNPs. A similar finding has been reported by Gervasini et al. in a cohort of 103 renal transplant recipients **(Gervasini et al., 2012)**.

The majority of the previous studies failed to find an independent effect of *ABCB1* individual SNPs or haplotypes on Tac PK. Still, there were few studies which showed differences in PK of Tac among different *ABCB1* genotypes; however these results were not conclusive and could not be replicated (see Table 3, reviewed in **Wolking et al., 2015**). In line with these studies, no effect of *ABCB1* individual SNPs or haplotypes on Tac PK in the present study cohort was found.

The incidence of DGF was higher among patients who carry the *ABCB1* 3435 C>T polymorphism and the difference was statistically significant (P= 0.02). This correlation was noticed despite the lack of a significant effect of this polymorphism on Tac exposure or dose requirements in the study cohort which may reflect a toxic effect of increased intracellular Tac rather than the whole Tac blood level. Similar results have been reported by Cattaneo et al. where they found that carriers of the T variant allele of either the *ABCB1* 3435 C>T or the 2677 G>T/A polymorphisms had a three-fold risk for DGF in a cohort of CsA-treated renal transplant recipients **(Cattaneo et al., 2009)**.

This study is among the relatively few studies to investigate the impact of genetic variation especially the new *CYP3A4*22* SNP on the PK of Tac early after transplantation. The most consistent effect in the cohort under study seems to be attributed to *CYP3A5*3* and to a lesser extent to *CYP3A34*22* while the impact of other SNPs is uncertain. Yet this study has some limitations. The most significant limitation of this study is the missing Tac C0 at each time point which precluded the analysis of the differences between various genotypes in PK parameters on a daily basis. A second limitation is the sample size which is relatively small. This small sample size implies difficulties in analyzing SNPs with low frequencies. For instance we identified only 12 patients who carry the *CYP3A4*22* polymorphism and almost all of them are *CYP3A5* non-expressers, thus we were unable to investigate the impact of *CYP3A4*22* among the *CYP3A5* expressers.

6 Summary

The development of pharmacodynamic and pharmacogenetic biomarkers to complement the currently used pharmacokinetic approach to optimize immunosuppressive therapy after organ transplantation remains an ongoing need in the field of transplantation medicine. Several biomarker studies have been published in the last few years however, only few biomarker assays found their way to clinical practice. This is due to lack of validation, cross validation and standardization of potential biomarker assays.

This work entailed the analytical validation of two pharmacodynamic (PD) biomarker assays. These include "NFAT-regulated gene expression assay" to monitor therapy with calcineurin inhibitors and "phosphoflow assay of S6RP" to monitor therapy with mTOR inhibitors. Additionally, a retrospective pharmacogenetic study to investigate the frequency and the impact of seven SNPs in the genes of *CYP450* and *ABAB1* on tacrolimus pharmacokinetics early after transplantation in a cohort of 121 renal transplant recipients was also conducted.

It was proven that the "NFAT regulated gene expression assay" can be set up with satisfactory analytical performance in a routine molecular biology laboratory. In a small inter-laboratory comparison, it was noted that the results generated from both laboratories are well correlated (r=0.951) but showed an inconsistent bias depending on the magnitude of residual gene expression (RGE, the readout of the assay).This observation denotes the need for careful validation of the biomarker assays as well as the harmonization of their results across laboratories before their clinical implementation.

Measurement of phosphorylation status of S6RP through a phosphoflow protocol based on a commercial kit and utilizing whole blood as sample matrix has been proved to be non-sophisticated and performs analytically well. However, specificity and stability of the assay as well as the disagreement with western blot (based on anti-phospho p70S6 kinase) questions its fitness for purpose as a PD biomarker of therapy with mTORIs.

In a retrospective pharmacogenetic study, the effect of the *CYP3A5*3* polymorphism on Tac pharmacokinetics early after transplantation was validated in a cohort of 121 renal transplant recipients in Stuttgart. Most of the individuals in the study cohort (85%) were *CYP3A5* non-expressers (*3/*3) and showed increased exposure to Tac as well as decreased dose requirements in the first two weeks after transplantation. The frequency of the newly

discovered *CYP3A4*22* SNP was about 10% in the study cohort which coincides with previously published results. Patients who carry this variant allele showed increased exposure as well as decreased dosage requirement of Tac.

In conclusion, this work proved the possibility to establish biomarker assays for individualizing immunosuppression with a satisfactory analytical performance in routine clinical laboratory especially when optimized assay protocols are applied and particularly when these are in the form of test kits. The reproducibility of the results across laboratories requires, however, careful harmonization/standardization of the assay conditions. The determination of the *CYP3A5*3* as well as *CYP3A4*22* polymorphisms may help to optimize the initial Tac dose,.

Future perspectives:

To facilitate the implementation of biomarker assays in the routine diagnostics in the future, standardization of the pre-analytical aspects as well as the analytical procedures are required. The clinical usefulness of the assay should be then assessed in prospective clinical trials involving many centers.

7 Zusammenfassung

Die Nierentransplantation (NTx) ist eine Standardtherapie für viele Patienten im Endstadium einer Nierenerkrankung, da sie zu einer niedrigeren Sterblichkeitsrate sowie verbesserter Lebensqualität im Vergleich zu Dialyseverfahren führt. Das Gebiet der Transplantationsmedizin hat große Fortschritte in den letzten Jahrzehnten gemacht. Dieser Erfolg ist zu einem großen Teil auf die Anwendung effektiver immunsuppressiver Therapien zurückzuführen. Die Calcineurin-Inhibitoren (Ciclosporin und Tacrolimus) und mTOR-Inhibitoren (Sirolimus und Everolimus) sind wichtige Komponenten der immunsuppressiven Therapie. Die Verwendung dieser Medikamente erfordert jedoch eine sorgfältige Überwachung der Patienten aufgrund ihres engen therapeutischen Bereichs und ihrer schlecht vorhersagbaren Pharmakokinetik.

Augenblicklich wird die Therapie mit diesen Medikamenten durch ein pharmakokinetisches Therapeutisches Drug Monitoring (TDM) gesteuert, bei dem die Dosis anhand der Konzentration des Medikaments im Blut eingestellt wird. Dies ist sehr gut geeignet um akute Schwankung der Medikamentenkonzentration im Blut zu kontrollieren und folglich eine akute Toxizität oder Abstoßungsreaktion zu vermeiden. Das TDM kann jedoch nicht dazu verwendet werden, um die optimale Anfangsdosis für jeden einzelnen Patienten zu bestimmen, da es erst nach Beginn der Arzneimitteltherapie erfolgt. Es ist auch weniger gut für die Anpassung der Dosis der Immunsuppressiva in der stabile Phase nach der Transplantation geeignet, um Langzeitnebenwirkungen zu minimieren.

Vor diesem Hintergrund wird deutlich, dass eine Optimierung der immunsuppressiven Therapien allein durch den pharmakokinetischen Ansatz nicht ausreichend ist, sondern dies mit pharmakodynamischen und/oder pharmakogenetischen Ansätzen unter Verwendung geeigneter Biomarker ergänzt werden sollte. Während pharmakodynamische Biomarker mit pharmakologischen Effekten der Medikamente auf Zielmoleküle assoziiert sind, konzentrieren sich pharmakogenetische Untersuchungen hauptsächlich auf arzneimittelmetabolisierende Enzyme (z.B. Cytochrom P-450 Polymorphismen) und Arzneimitteltransporterproteine (z.B. Mutationen in Genen der Familie der ATP-Bindungs-Kassetten-Transporter) und damit in der Folge auf pharmakokinetische Parameter.

Trotz der großen Anzahl von Biomarker-Studien, die in den letzten Jahren veröffentlicht wurden, fanden die meisten Biomarker-Assays ihren Weg in die klinische Praxis nicht, weil sie, mit wenigen Ausnahmen, weder nach internationalen Leitlinien analytisch validiert noch wegen ihres komplizierten Aufbaus für den Routineeinsatz geeignet sind. Außerdem wurde die Reproduzierbarkeit der Assays zwischen unterschiedlichen Labors meistens nicht getestet. Weitere Studien sind daher erforderlich, um die Routinetauglichkeit der Biomarker-Assays mit dem Potential für eine klinische Anwendung zu testen.

Diese Arbeit umfasst die analytische Validierung von zwei pharmakodynamischen Biomarker-Assays: die „NFAT-regulierte Genexpression“ zur Überwachung der Therapie mit Calcineurin-Inhibitoren und den „Phosphoflow Assay von S6RP“ zur Überwachung der Therapie mit mTOR-Inhibitoren. Zusätzlich wurde eine retrospektive pharmakogenetische Studie durchgeführt, in der die Häufigkeit und die Auswirkungen von sieben Einzelnukleotid-Polymorphismen (SNPs) in Kandidatengenen (CYP450 Enzyme und P-Glykoprotein) für Tacrolimus (Tac) in der ersten Phase nach der Transplantation in einer Kohorte von 121 Patienten nach Nierentransplantation untersucht wurden.

Bestimmung der NFAT-regulierten Gen-Expression: Eine quantitative Analyse der NFAT-regulierten Gene (IL-2, INF-γ und GM-CSF) wurde als neuer pharmakodynamischer Biomarker für die Überwachung der Therapie mit Calcineurininhibitoren untersucht. Die Verfügbarkeit eines kommerziellen Kits (Search LC, Heidelberg) und ein gut etabliertes Testprotokoll machen diesen Biomarker-Assay zum vielversprechenden Kandidaten für die klinische Routinediagnostik. Voraussetzungen für die Umsetzung des Assays in der Routinepraxis sind allerdings die analytische Robustheit und vergleichbare Ergebnisse zwischen verschiedenen Laboratorien. Daher wurde das ursprüngliche Protokoll vom Institut für Immunologie in Heidelberg im Institut für Laboratoriumsmedizin des Klinikum Stuttgarts etabliert und verifiziert und eine Vergleichsstudie zwischen beiden Laboren durchgeführt.

Für die analytische Validierung des Assays wurden Vollblutproben von gesunden Probanden mit Tac *in vitro* inkubiert. Linearität, Präzision, die Bestimmungsgrenze sowie die Stabilität der Proben wurden untersucht. Für den Interlaborvergleich wurden Proben von Patienten unter Ciclosporintherapie zuerst in Heidelberg analysiert und dann in Stuttgart innerhalb von 24 Stunden re-analysiert.

Tac verringerte die Expression der NFAT-regulierter Gene *in vitro* in einer konzentrationsabhängigen Weise mit einer „Residual Gene Expression“ (RGE, verbleibende Genexpression) von 15%, 47%, 71% und 89% jeweils für die Konzentrationen von 50 ug/L, 25 ug/L, 12.5 ug/L und 6.25 ug/L Tac. Die Intra- und Inter-Assay-Variationskoeffizienten (CV%) bei drei verschiedenen Tac Konzentrationen (jeweils n = 6) lagen bei <17%. Die Bestimmungsgrenze lag jeweils bei 100 cDNA-Kopien per Reaktion für die IL-2-, INF-γ- und GM-CSF-Genexpression. Wiederholungsmessungen derselben Probe nach 24 Stunden (Stabilität) ergaben einen Unterschied in der RGE von ≤ 19% (n = 3). Obwohl der Interlabor-Vergleich von Patientenproben gut (r = 0.951) korrelierte, zeigte die statistische Analyse einen inkonsistenten Unterschied in Abhängigkeit von der Größe der RGE.

Daraus kann geschlossen werden, dass die Bestimmung der NFAT-regulierten Gen-Expression mit einer zufriedenstellenden analytischen Zuverlässigkeit in einem molekularbiologischen Routinelabor etabliert werden kann und vergleichbare Ergebnisse zwischen den Laboratorien zeigt. Jedoch erfordert die klinische Anwendung dieses Assays in der Routinediagnostik als pharmakodynamischen Biomarker für die Therapie mit CNIs eine Harmonisierung zwischen Laboratorien.

Messung der Phosphorylierung des S6RP durch Phospho-Flow: Das S6 ribosomal Protein (S6RP) wird als nachgelagerter Effekt der mTOR-Aktivierung phosphoryliert. Die mTOR-Inhibitoren Sirolimus und Everolimus unterdrücken die Phosphorylierung des S6RP in Lymphozyten. Daher kann der Phosphorylierungszustand von S6RP (p-S6RP) als Biomarker für die mTOR Hemmung durch Sirolimus und Everolimus verwendet werden. In dieser Arbeit wurde die Anwendbarkeit eines durchflusszytometrischen Phospho-Flow-Assays für die Messung des p-S6RP getestet.

Ein kommerzieller Kit (PerFix-p, Beckman-Coulter) wurde für die Fixierung und Permeabilisierung der Zellen in Vollblutproben nach initialer Stimulation mit PMA (150 µg/L, 6 min, 37°C) eingesetzt. p-S6RP wurde separat in CD3+ CD4+ und CD3+ CD8+ T-Zellen unter Verwendung eines anti-Phospho-Ser 235/236 Antikörpers mit Hilfe der Durchflusszytometrie untersucht. Der Assay wurde sowohl *in vivo* als auch *in vitro* getestet. Spezifizität, Linearität, Unpräzision in der Serie und Stabilität wurden untersucht. Das Ausmaß der S6RP-Phophsporyliserung wurde in Proben verschiedener Patientengruppen

gemessen und mit einer Kontrollgruppe verglichen. Ein Vergleich mit einer Western-Blot-Analyse der phospho-p70S6 Kinase (Thr389), die als Referenzmethode angesehen werden kann, wurde darüber hinaus durchgeführt.

Everolimus verringerte p-S6RP *in vitro* konzentrationsabhängig (bis 27.4 µg/L). Dieser Effekt wurde auch *in vivo* nach einer Einzeldosis von Everolimus bei gesunden Probanden bestätigt (n = 3). Allerdings wurde die Phosphorylierung des S6RP auch gehemmt, wenn das Vollblut *in vitro* mit 500 µg/L Ciclosporin versetzt wurde. Der Variationskoeffizient in der Serie des Assays betrug <18% bei Transplantationspatienten und <27% bei gesunden Kontrollen für beide T-Zell-Populationen. Phospho-S6RP war von begrenzter Stabilität (<24 Stunden). Patienten, die mit mTOR-Inhibitoren behandelt wurden, zeigten niedrigere p-S6RP in beiden T-Zellpopulationen. Dies war jedoch nur für CD3+ CD8+ T-Zellen von Sirolimus-behandelten Patienten statistisch signifkant (p = 0.02). Es wurde keine signifikante Korrelation zwischen dem Phosphoflow und der Western-Blot-Analyse festgestellt.

Aus diesen Ergebnissen lässt sich schließen, dass der Phosphoflow Assay von p-S6RP zwar mehrere Vorteile bietet, wie z.B. die Verwendung von Vollblut und eine befriedigende analytische Leistung. Allerdings deuten die Ergebnisse bezüglich Probenstabilität, Spezifität und Vergleichbarkeit zur Referenzmethode darauf hin, dass eine Verwendung für klinische Zwecke als pharmakodynamischer Biomarker für die bessere Steuerung der Therapie mit mTORIs zweifelhaft ist. Ob sich dies durch eine Weiterentwicklung und Optimierung des Assays verbessern lässt, bleibt zu prüfen.

Effekt der Pharmakogenetik auf die Pharmakokinetik von Tac in der frühen Phase nach der Nierentransplantation: Genetische Varianten können in einem hohen Ausmaß eine große interindividuelle Variabilität der Arzneimittelkonzentrationen in vivo bedingen mit Konsequenzen für die Arzneimittelwirkung. Die meisten dieser genetischen Varianten betreffen arzneimittelmetabolisierende oder -transportierende Proteine. In dieser Arbeit wurde der Einfluss von sieben SNPs in den Kandidatengenen *CYP3A4* und *CYP3A5* sowie dem *ABCB1* Gen (kodiert für P-Glykoprotein) auf die Pharmakokinetik von Tac in der frühen Phase nach der Transplantation untersucht. Die untersuchten Allele sind *CYP3A5*3, CYP3A4*22, CYP3A4 *1B* sowie die genetischen Varianten in *ABCB1* (3435T> C, 2677T> G/A und 1236T> C).

Insgesamt wurden 121 nierentransplantierte Patienten im Klinikum-Stuttgart in diese retrospektive Studie eingeschlossen. Ein entsprechendes Ethikvotum für die Studie lag vor. Die Genotypisierung wurde mittels Alleldiskriminierung mit etablierten und validierten Real-time PCR (TaqMan)-Assays am Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie in Stuttgart durchgeführt. Bei den Untersuchungen eines möglichen Effektes des *CYP3A5*3*-Polymorphismus auf die Pharmakokinetik von Tac zeigte sich zuerst, dass die meisten der Individuen in der Studienkohorte (85%) *CYP3A5* homozygote "Non-Expresser" (*3/*3) waren, die in Übereinstimmung mit der Hypothese eine erhöhte systemische Exposition von Tac sowie einen verringerten Dosisbedarf von Tac in den ersten beiden Wochen nach der Transplantation zeigten. Die Häufigkeit des kürzlich beschriebenen funktionell bedeutsamen *CYP3A4*22* Allels betrug etwa 10% in der Studiengruppe, was veröffentlichten Ergebnissen übereinstimmt. Patienten, die diese Variante Allel tragen zeigten erhöhte Exposition sowie einen verringerten Dosisbedarf von Tac. Die untersuchten genetischen Varianten in *ABCB1* zeigten keinen signifikanten Effekt auf die Pharmakokinetik von Tac.

Zusammenfassend konnte basierend auf den Daten der vorliegenden Arbeit gezeigt werden, dass pharmakodynamische Biomarker-Assays zur Individualisierung der Immunsuppression mit einer zufriedenstellenden analytischen Leistungsfähigkeit im klinischen Routinelabor etabliert werden können, vor allem, wenn optimierte Testprotokolle verwendet werden und insbesondere, wenn die Assays in Form von Testkits verfügbar sind. Insgesamt erfordert die Reproduzierbarkeit der Ergebnisse zwischen unterschiedlichen Laboratorien jedoch eine sorgfältige Harmonisierung bzw. Standardisierung der Testbedingungen. Die Bestimmung der *CYP3A5*3*- sowie *CYP3A4*22* Polymorphismus kann helfen die initiale Tac-Dosis zu optimieren.

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9 Declaration of contributions to the dissertation

The dissertation work was divided into two parts: The first part (the pharmacodynamic assays) was carried out at the laboratory of the Klinikum Stuttgart under the supervision of Prof. Eberhard Wieland while the second part (the pharmacogenetic assays) was carried out at the Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology (IKP) under the supervision of Prof. Matthias Schwab.

The following experiments have been performed:

a) After training by Dr. Hannah Rieger, I carried out all experiments of the validation of NFAT-regulated gene expression assay independently. Mr. Manabu Abe supported me with some technical assistance in the molecular biology unit at the laboratory of Klinikum Stuttgart.

b) After a period of observation with Mrs. Mariana Kabakchiev, I continued to test the analytical performance of the phosphoflow assay of S6RP; I tested the following parameters of the assay: concentration-effect relationship, specificity *in vitro* and with clinical patient samples (different patient groups), verification of the assay concept in three healthy volunteers, inter-method comparison (the phosphoflow measurements while the western blot part was carried out at the University Hospital Ulm) as well as stability of the assay. The preliminary experiments of the assay including optimization of the assay conditions were carried out at the laboratory of Klinikum Stuttgart by Mrs. Mariana Kabakchiev.

c) After a period of observation with Ms. Andrea Jarmuth, I performed the following experiments at the IKP: Isolation of the DNA from the collected blood samples, measuring the concentration of the DNA and preparing a uniform dilution of all samples and placing them in the PCR plates. For genotyping of all selected variants by real-time PCR (TaqMan) I was trained by Ms. Andrea Jarmuth.

All the work regarding extraction of patients' data from the records of Klinikum Stuttgart as well as their organization in appropriate tables was done by myself.

Statistical analysis of the pharmacodynamic part of the thesis was carried by myself under the supervision of Prof. Wieland. The statistical analysis of the pharmacogenetic data was performed by the support and supervision of Dr. Stefan Winter at IKP.

I confirm that I wrote the manuscript myself under the supervision of Prof. Wieland and Prof. Schwab. Any additional sources of information have been duly cited.

10 Publications

10.1 Publications in peer reviewed journals

Abdel-Kahaar E, Kabakchiev M, Hartmann B, Wieland E, Shipkova M (2016): Performance of a phosphoflow assay to determine phosphorylation of S6 ribosomal protein as a pharmacodynamic read out for mTOR inhibition. Clin Biochem. [Epub ahead of print]

10.2 Posters and talks

14th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology, Rotterdam Niederlande Oktober 2015:

- Presentation: Analytical validation and cross validation of a commercially available NFAT-regulated gene expression assay. Abdel-Kahaar E, Rieger H, Sommerer C, Giese T, Shipkova M, Wieland E
- Poster: Performance of a phosphoflow assay to determine phosphorylation of S6 ribosomal protein as a read out for mTOR inhibition. Abdel-Kahaar E, Kabakchiev M, Hartmann B, Wieland E, Shipkova M.

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I wish also to thank Dr. Maria Shipkova who provided me with instructions regarding the analytical aspects of the study and for coordinating the construction of the biobank and her continuous help and support. Special thanks go to Dr. Hannah Rieger for always providing me with constructive advices whenever needed.

Thanks also to Mrs. Mariana Kabakchiev for her cooperation in relation to the recruitment of the patients' samples for the study and Dr. Stefan Winter for his help regarding the statistics.

I would like to express my eternal appreciation towards my parents and family who have been there for me no matter where I am, for all unconditional support and patience. Your support gave me the strength all the way long.

Curriculum Vitae

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Work Experience

2013-	Research fellow at Central Institute for Clinical Chemistry and Laboratory Medicine, Klinikum Stuttgart and Dr. Margarete Fischer-Bosch Institute for Clinical Pharmacology, Stuttgart, Germany
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Education

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Courses and meetings

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Okt. 2014	Symposium "Organisation von Klinischen Studien an einem Klinikum", Dr. Margarete Fischer-Bosch Institut für Klinische Pharmakologie
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