Pharmacodynamic monitoring of calcineurin inhibitor therapy in renal allograft recipients

Master Thesis in Pharmacology by

Lene Pham

School of Pharmacy
Faculty of Mathematics and Natural Sciences
University of Oslo

Department of Medical Biochemistry and
Department of Pharmacology, Rikshospitalet
Oslo University Hospital

15. November 2012

Master Thesis in Pharmacology

Pharmacodynamic monitoring of calcineurin inhibitor therapy in renal allograft recipients

Lene Pham

Department of Pharmaceutical Biosciences
School of Pharmacy
Faculty of Mathematics and Natural Sciences
University of Oslo

and

Department of Medical Biochemistry and
Department of Pharmacology, Rikshospitalet
Oslo University Hospital

15. November 2012

Supervisors:

Sara Bremer, Department of Medical Biochemistry,
Rikshospitalet, Oslo University Hospital

Stein Bergan, Department of Pharmaceutical Biosciences, School of
Pharmacy, University of Oslo and Department of Pharmacology,
Rikshospitalet, Oslo University Hospital

Year: 2012

Title: Pharmacodynamic monitoring of calcineurin inhibitor therapy in renal allograft recipients

Written by: Lene Pham

Printed by: Reprosentralen, University of Oslo

Acknowledgements

The present work was performed at the Department of Medical Biochemistry at Oslo University Hospital, Rikshospitalet in the time period August 2011 to October 2012.

This project would have not been possible without the support of many people.

First of all, I would like to express my sincere gratitude to my supervisors Sara Bremer and Stein Bergan for your guidance and encouragement throughout the project. It was my greatest privilege to work along with such skilled and dedicated persons. I am very grateful for your editorial assistance, critical reading and helpful comments on the thesis. Without your help, it would have been difficult to complete this work. Sara, you have made this an inspiring and exciting year. You have been continuous supportive whenever I was in need, even at times I found it difficult to maintain the motivation. Your friendly encouragement is highly appreciated. Appreciation continuous to Stein for your insightful comments and motivation when it was most required.

My sincere thanks go to Nils Tore, Christine, Helge, Ingjerd, Ragnhild, Anders and rest of Thursday's colloquium. This group had been source of friendship as well as good advice and collaboration.

I would like to thank Marit and everyone in GEN that you have been helpful and flexible in providing laboratory facilities. Great thanks to Laila and all bioengineers in SAFE for your care and attention during my stay at the department and of course for morning coffees and many delicious cakes.

I would also like to thank Pål-Dag Line and Morten Skauby at Department of Transplantation Medicine for the cooperation and technical assistance with providing samples for the pilot study.

Finally, my deepest gratitude from my deep heart goes to my beloved family, thank you for your endless love, patient, understanding and encouragement throughout my master thesis. I cannot find the appropriate words that could describe my appreciation for your spiritual support and faith in my ability to obtain my goals in this long journey. I also thank my friends who kept me smiling during tough times in the master thesis pursuit. Thank you!

I will remember the time at Rikshospitalet as exciting, challenging, fun and informative. The master thesis has given me insight into an exciting research field. It has been a year I will never forget.

Oslo, 2012

Lene Pham

TABLE OF CONTENTS

Abstract

Background and objectives:

Calcineurin inhibitors (CNIs) are widely used in organ transplantation (Tx). Due to the narrow therapeutic window, regular drug monitoring is necessary to balance sufficient efficacy with minimal toxicity. Until now, the monitoring has mainly been based on measurements of drug concentrations. However, despite concentration guided dosing of CNIs, patients still suffer from acute or chronic rejection and CNI related toxicity during CNI therapy. Pharmacokinetic (PK) measurements do not necessarily reflect the CNI effect on immune cells and pharmacodynamic (PD) monitoring of the nuclear factor of activated T cells (NFAT)-regulated gene expression has been suggested as a promising supplementary tool for individualizing CNI therapy.

The objective of the present project was to develop and validate an assay to examine NFAT-regulated genes as PD biomarkers of CNI response in blood samples from renal transplant recipients.

Materials and methods:

Heparinized peripheral whole blood samples with and without addition of different immunosuppressive drugs were stimulated with phorbol 12-myristate13-acetate (PMA) and ionomycin for up to 72 hours at 37°C. Samples from renal allograft recipient were drawn before and 1.5 hours after tacrolimus (Tac) dosing and stimulated with PMA and ionomycin for 3 hours at 37°C. Following cell lysis, total RNA was isolated with the automated MagNA Pure LC instrument and reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche). The expression of NFAT-regulated genes, interleukin 2 (*IL2*), interferon-γ (*IFNG*) and colony-stimulating factor 2 (granulocyte-monocyte) (*CSF2*) was determined by real-time PCR on the LightCycler® 480 instrument (Roche). The calculations included normalization to the expression of three reference genes, 5-aminolevulinate synthase 1 (*ALAS1*), beta-2-microglobulin (*B2M*) and ribosomal protein L13A (*RPL13A*), and PCR efficiency correction.

Results:

SYBR Green I analysis, gel electrophoresis and Sanger sequencing demonstrated selective amplification of the target gene sequences. Standard curves were linear over a range corresponding to starting concentrations of 10^7 to 10^2 cDNA templates per reaction. The between and within assay coefficient of variability (CV) was ≤ 19.8% and ≤ 14.8%, respectively. The maximum response to PMA and ionomycin was observed after 3 hours incubation, resulting in 120 000, 20 000 and 8000 fold increases of *IL2, IFNG* and *CSF2* expression, respectively. *Ex vivo* exposure to different immunosuppressive drugs and concentrations, demonstrated that inhibition of NFAT-regulated gene expression seemed to be relatively CNI specific and inversely correlated to CNI concentration. There was a large variability of NFAT-regulated gene expression between patients before Tx. About one week after Tx, 4 of 5 patients demonstrated residual gene expression ≤ 13%. Three of these patients demonstrated Tac C_0 levels within the therapeutic target range.

Conclusions:

Several studies support the potential of NFAT-regulated gene expression as a PD tool for further individualization and improvement of CNI therapy. A reverse transcription (RT) and real-time PCR assay was developed and validated to determine *IL2, IFNG* and *CSF2* gene expressions in whole blood samples.

The preliminary results of the present study support the use of NFAT-regulated gene expression as PD biomarker of Tac response. However, the value of monitoring NFAT-regulated gene expression during Tac therapy needs to be determined in prospective intervention trials with larger patient cohorts.

Abbreviations

ALAS1	5-aminolevulinate synthase 1
Allo-Tx	Allotransplantation
AUC	Area-under-the-blood-concentration-*versus*-time-curve
$AUC_{0\text{-}12h}$	AUC from 0 to 12 hours
APC	Antigen-presenting cell
B_2M	Beta-2-microglobulin
C_0	Trough concentration (before intake of a drug)
$C_{1.5}$	Concentration at 1.5 hours post intake of a drug
C_2	Concentration at 2 hours post intake of a drug
cDNA	Complementary DNA
C_{max}	Concentration at maximum
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CsA	Cyclosporine A
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
Cp	Crossing point
CV	Coefficient of variation
CYP	Cytochrome P450
ddNTP	Dideoxynucleotide triphosphate
DSA	Donor specific antibody
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F1	Forward primer
FKBP	FK506-binding protein
FL1	Forward probe

FRET	Fluorescence resonance energy transfer
GC	Guanine-Cytosine
HLA	Human leukocyte antigen
IFNG	Interferon gamma
IL2	Interleukin 2
IL2R	Interleukin 2 receptor
IMPDH1	IMP (inosine 5'-monophosphate) dehydrogenase 1
IMPDH2	IMP (inosine 5'-monophosphate) dehydrogenase 2
LBB	Lysis/Binding Buffer
LC1	Reverse probe
Mg^{2+}	Magnesium
MHC	Major histocompatibility complex
MS2-RNA	RNA of bacteriophage MS2
mTOR	Mechanistic target of rapamycin
NFAT	Nuclear factor of activated T cells
PD	Pharmacodynamic
PHA	Phytohemagglutinin
PK	Pharmacokinetic
PMA	Phorbol 12-myristate 13-acetate
R1	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
RPL13A	Ribosomal protein L13A
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative PCR
Tac	Tacrolimus
TDM	Therapeutic drug monitoring

TE-buffer	Tris-EDTA (ethylenediaminetetraacetic acid) buffer
TNF-α	Tumor necrosis factor-alpha
Tx	Transplantation
UV	Ultraviolet
qPCR	Quantitative PCR

(Gene names are written in italic in this master thesis)

1 Introduction

1.1 Transplantation

Allotransplantation (Allo-Tx, frequently referred to only as transplantation) involves the transfer of cells, tissues or organs between individuals of the same species. The procedure allows the replacement of an irreversibly damaged organ. Today, organ transplantation (Tx) has become a vitally important treatment for patients with end-stage organ failure. The most frequently transplanted organs in Norway are kidneys, liver, heart, lungs and pancreas (Figure 1).

Oslo University Hospital, Rikshospitalet, has the national function of all organ transplants in Norway, while 28 other hospitals in the country can identify potential donors.

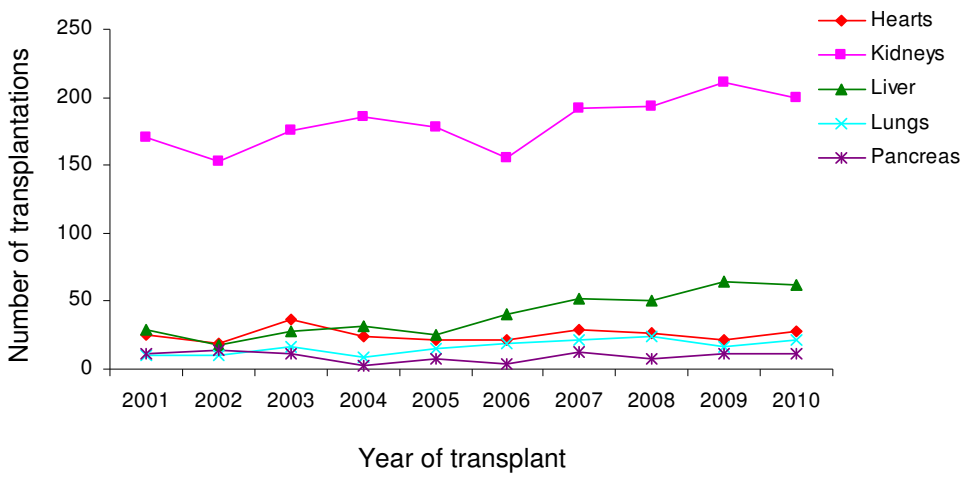


Figure 1: Number of transplants per year from 2001 to 2010. (Data from the annual report for organ donation and transplantation January 1 – September 30 2010 at Oslo University Hospital, Rikshospitalet).

1.1.1 Renal transplantation (Tx)

The world's first successful renal Tx with a graft from a living related donor was performed in 1954 in Boston, USA. Two years later, the first Tx in Norway of a kidney from a living unrelated donor was performed. Despite blood-group (ABO) and tissue (human leukocyte antigen) incompatibility, the patient lived for 30 days after Tx. [1] Over the years, there have been important breakthroughs in surgical skills, tissue typing and immunosuppressive drugs. Consequently, the number of transplantations and the patient survival rates have increased [2].

Renal Tx is an option for patients with irreversible chronic end-stage renal failure (according to The Kidney Disease Outcomes Quality Initiative: stage 5). Several studies have reported increased survival among renal allograft recipients compared to patients in dialysis [3,4]. Thus, renal Tx is the treatment of choice for many patients with end-stage renal disease [5-7]. The most common causes of chronic renal failure among all age groups are diabetes, chronic glomerulonephritis, polycystic kidney disease, systemic lupus erythematosus and interstitial nephritis [8].

1.2 Immune response

1.2.1 Allorecognition

The powerful immune responses that occur against tissue or the transplanted organ are related to the genetic differences between donor and recipient.

The main barrier to allo-Tx is recognition of the major histocompatibility complex (MHC) molecules expressed on donor tissue. In humans, the MHC is referred to as the human leukocyte antigen (HLA) system and the gene complex is located on chromosome 6. HLA genes are divided into four classes based on their functions and structures. Diversity of antigen presentation is mediated by class I and II, while class III and IV includes components of the complement system, cytokines and heat shock proteins.

The HLA class I molecules are expressed on almost all nucleated cells of the body and present antigens of intracellular origin to $CD8^+$ cytotoxic T cells. This represents a pivotal

defense function against cells that are damaged or infected with viruses. Class II molecules are constitutively expressed on antigen-presenting cells (APCs) such as dendritic cells, B cells and macrophages. These molecules can present foreign peptides derived from exogenous antigens to $CD4^+$ helper T cells.

HLA class I molecules consist of an alpha chain, encoded by HLA genes (*HLA-A*, *HLA-B* and *HLA-C*), and beta-2-microglobulin *(B2M),* while the class II molecules are heterodimers of alpha and beta chains, which are both encoded by HLA genes (*HLA-DP*, *HLA-DQ* and *HLA-DR* loci). The HLA genes are highly polymorphic and each individual express a unique set of HLA molecules [9]. Several studies have shown that a good match between donor and recipients HLA molecules is significant for the outcome after Tx. Especially, matching at the *HLA-DR* loci has been reported to have significant impact on graft survival [10,11]. The HLA class I and II molecules represent the most important antigenic differences between individuals and are also known as Tx antigens [12,13].

ABO blood-group antigens are expressed on the erythrocytes and on most epithelial and endothelial cells. Previously, ABO incompatibility was considered an absolute contraindication for renal Tx. Following the implementation of pre transplant desensitization protocols, including removal of preexisting ABO antibodies, Tx can now be performed across ABO blood group barriers [14].

1.2.2 Immune activation

T cell activation is normally triggered by the recognition of a foreign peptide/HLA complex by the T cell receptor complex, followed by the interaction between T cell membrane receptors and APC membrane ligands (adhesion and costimulatory molecules). This induces intracellular signaling pathways and initiates the transcription of several nuclear factors of activated T cells (NFAT)-regulated genes, which are essential for the proliferation and differentiation of T cells. One of the key mediators in this context is the cytokine interleukin 2 (*IL2).*

1.2.3 Allograft rejection

Following allo-Tx, an immune response can be initiated by different immunologic recognition mechanisms.

1. In the direct pathway of antigen presentation, the HLA molecules on APCs of the donor are directly recognized by the T cells of the recipient.

2. The indirect pathway of antigen presentation involves recipient APCs presenting processed HLA peptides derived from donor cells [15,16].

3. The semi-direct pathway of antigen presentation refers to a combination of direct and indirect pathways [16-18].

Unless the immune system is modulated by immunosuppressants, recognition of foreign HLA molecules or ABO blood-group antigens will lead to rejection of the transplanted organ. Depending on the immunological mechanism and the time of presentation, the process is often referred to as hyperacute, acute or chronic rejection (Figure 2 Section 1.3) [14,19].

1.2.3.1 Hyperacute rejection

Hyperacute rejection occurs within minutes to hours post transplant and is caused by preexisting alloreactive antibodies of the patient mediating damage to the graft. This type of rejection is due to genetic differences between donor and recipient ABO blood-group antigens and to lesser extent, HLA molecules. Preexisting host antibodies in the blood will immediately react with antigens from the donor organ and activate the complement system leading to cell death, local coagulation and irreversible rejection of the organ. However, because donor-recipient crossmatch testing is performed pre Tx, hyperacute rejection is rarely seen [14].

1.2.3.2 Acute rejection

Acute rejection may occur at any time post-operatively, but is most frequent within the first days and up to 6 months post Tx. Acute rejection can be mediated by lymphocytes (cellular) and/or circulating antibodies (humoral). Furthermore, unlike hyperacute rejection, acute rejection may be prevented with immunosuppressive therapy [14,20].

1.2.3.3 Chronic allograft injury

Chronic allograft injury occurs several months up to years after Tx. The reaction is characterized by slow deterioration of graft function with the development of tubular atrophy and interstitial fibrosis [14,20,21].

1.3 Immunosuppressive therapy

After allo-Tx, lifelong immunosuppression is required to prevent rejection of the graft and maintain long-term allograft survival (Figure 2). The immunosuppression therapy usually consists of a combination of drugs (Figure 3) with different mechanisms of action. This provides additive or synergistic immunosuppression while minimizing the toxicity of each drug [22,23].

Since the introduction of combined azathioprine and glucocorticoid therapy approximately 50 years ago, there has been a remarkable improvement in the immunosuppressive therapy. Over the previous years, several potent immunosuppressive agents aim to diminish toxic side effects which lead to the improvement of the patients' quality of life.

Allograft recipients treated with lifelong immunosuppressive drugs have an increased risk of drug toxicity, infections and malignancies. This may have negative impact on long-term allograft and patient survival after Tx [14]. To achieve the right balance of immunosuppression, the combination of immunosuppressive agents is kept to the lowest effective doses. The initial post transplant period is associated with the highest risk of acute

allograft rejection. Thus, obtaining a sufficient degree of immunosuppression is particular important during the first weeks after Tx.

The choice of immunosuppressive regimen depends on various clinical factors and the type of organ transplant [24]. At Rikshospitalet, the standard maintenance drug regimen currently consists of a calcineurin inhibitor (CNI: cyclosporine or tacrolimus), prednisolone and mycophenolic acid. In addition, methylprednisolone (250 mg) and the interleukin 2 receptor (IL2R) monoclonal antibody basiliximab are given intravenously on the day of surgery and at day 4 post Tx (basiliximab only). In renal transplant recipients, the CNI of choice is tacrolimus (Tac), except in patients with impaired glucose tolerance who will receive cyclosporine (CsA). Furthermore, the CNIs are frequently replaced or administered in reduced doses in combination with a mechanistic target of rapamycin (mTOR) inhibitor, sirolimus or everolimus, in patients with an increased risk of CNI toxicity or malignancies [25].

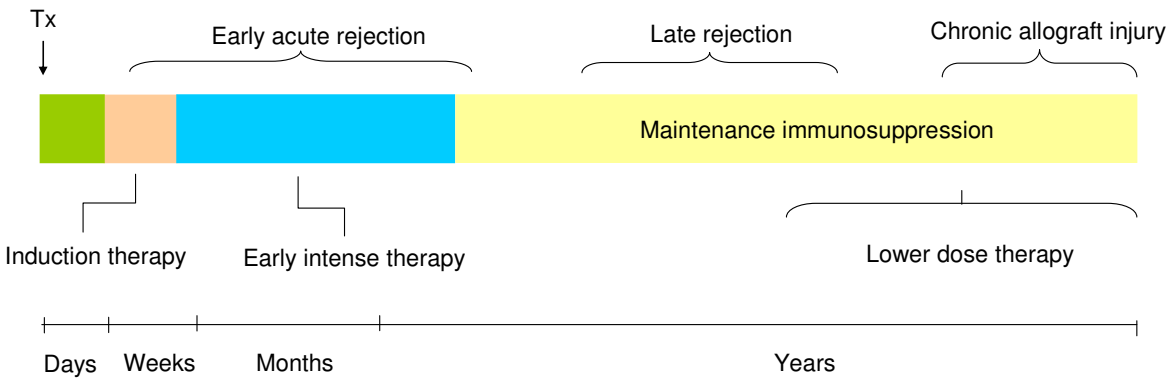


Figure 2: Risk of rejection and immunosuppressive dose regimens during different phases of post transplantation (Tx).

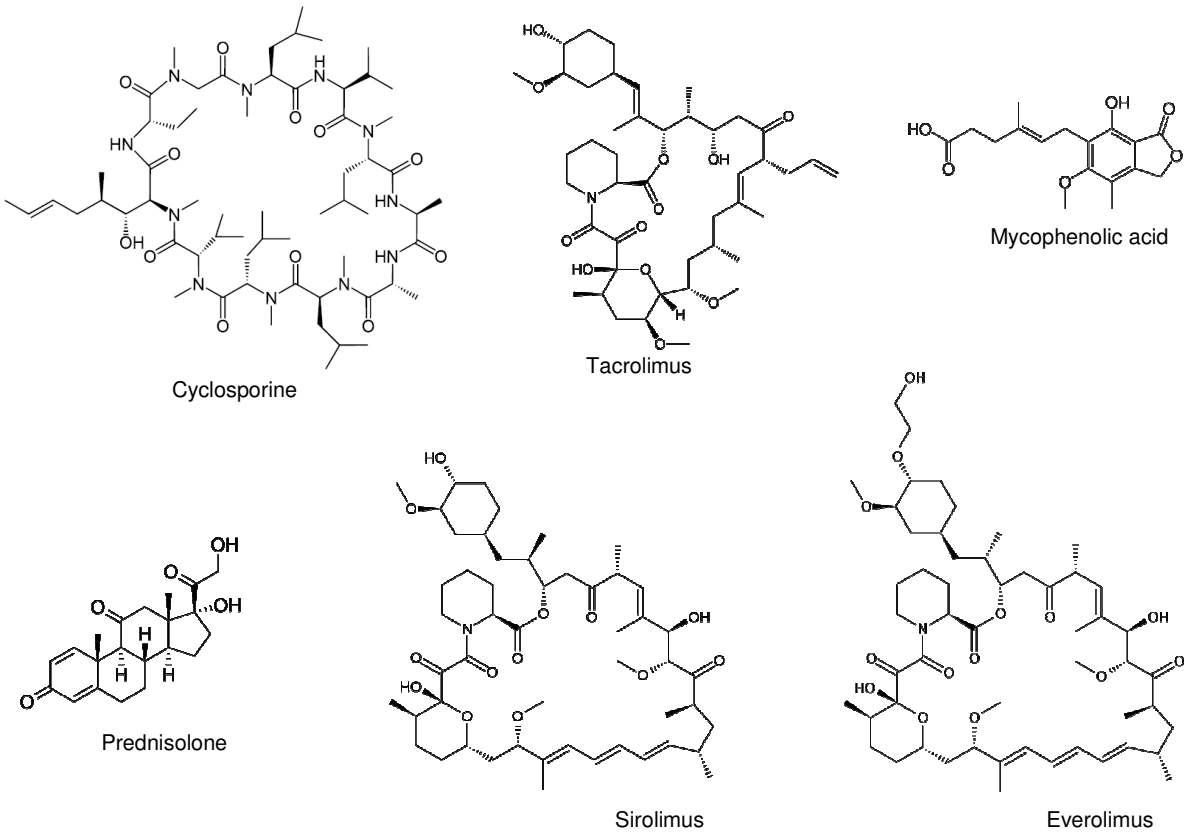


Figure 3: Molecular structure of immunosuppressive drugs used after solid organ transplantation.

1.3.1 Calcineurin inhibitors (CNIs)

The two CNIs, CsA and Tac, are not structurally related (Figure 3) but have similar mechanisms of action.

The principal action of CNIs is the inhibition of the phosphatase calcineurin, which is a key component of T cell activation. CsA and Tac bind to the immunophilins cyclophilin and FK-506-binding protein (FKBP), respectively, in the cytoplasm (Figure 4). These complexes block the phosphatase activity of calcineurin and inhibit the dephosphorylation of the transcription factor NFAT. This prevents the translocation of the transcription factor into the nucleus and thereby inhibits the transcription of several proinflammatory cytokine genes including *IL2*, *IL3, IL5,* colony stimulating factor 2 (granulocyte-monocyte) (*CSF2*),

interferon gamma (*IFNG*) and tumor necrosis factor-alpha (*TNF-α*). The NFAT-regulated cytokines play an important role in the initiation of immune responses and inhibition of these cytokines prevents T cell activation and proliferation (Figure 4).

Both CsA and Tac have an excellent efficacy profile with low acute rejection rates [26]. Several studies have reported similar patient and graft survival after renal Tx with CsA and Tac based immunosuppression [27,28]. However, Tac has been associated with a reduced incidence of acute rejection episodes [29-36].

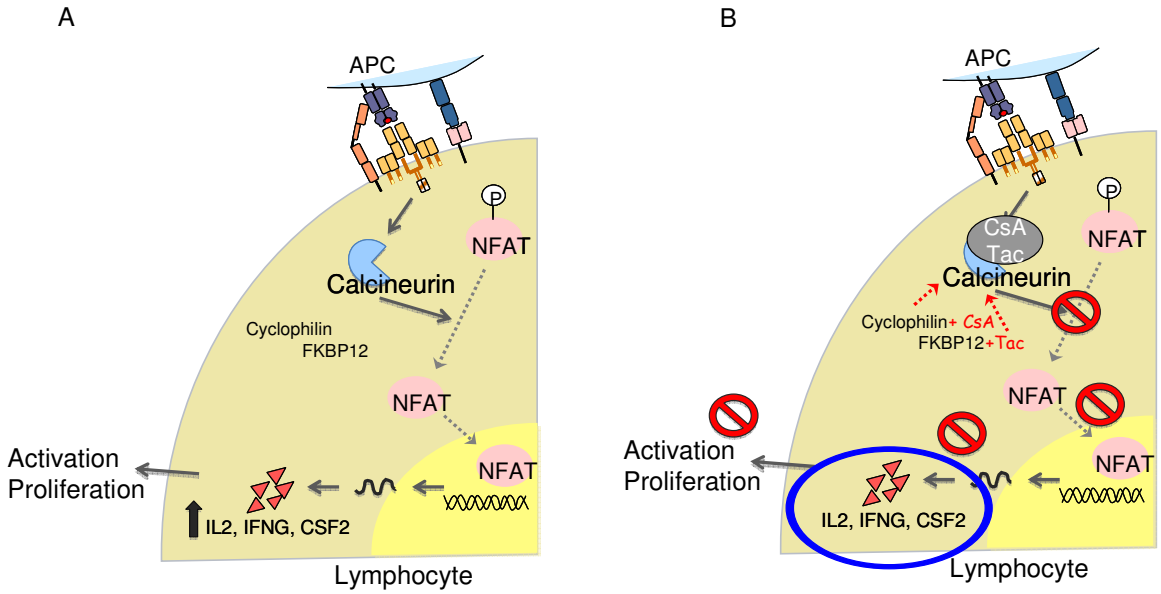


Figure 4: The effect of calcineurin inhibitors in lymphocytes. A: Stimulation of the T cell receptor and costimulatory receptors results in increased calcineurin activity, dephosphorylation of the nuclear factor of activated T cells (NFAT), increased transcription of cytokines and finally lymphocyte activation and proliferation. **B:** After binding to immunophilins, cyclosporine (CsA) and tacrolimus (Tac) inhibit calcineurin activity and finally the transcription of NFAT-regulated cytokines and cell proliferation.

APC; Antigen-presenting cell, *CSF2*; colony stimulating factor 2 (granulocyte-monocyte), FKBP12; FK-506-binding protein 12, *IFNG*; interferon-gamma, *IL2*; interleukin 2

1.3.1.1 Cyclosporine (CsA)

Since the introduction into clinical practice in 1978, CsA has revolutionized the field of solid organ Tx and has developed into the standard drug for preventing allograft rejection. Both patient and graft survival rates have significantly improved [37]. CsA is a lipophilic metabolite extracted from soil fungi like *Tolypocladium inflatum* and *Cylindrocarpon lucidum*. The mechanism of action is described in Section 1.3.1.

CsA exhibits a wide inter- and intraindividual variability in pharmacokinetic (PK) profiles. Following oral administration, absorption of CsA is slow. The poor and variable bioavailability of the first CsA formulation, Sandimmune, led to the development of CsA microemulsion (CsA-ME: Neoral), presenting reduced variability in gastrointestinal absorption and 30% higher bioavailability [38-41].

CsA is metabolized predominantly by cytochrome P450 (CYP), 3A4 enzymes, in the liver and intestine and is a substrate of the drug transporter P-glycoprotein (ABCB1). The primary pathway of metabolites elimination is through biliary excretion [42,43]. The mean elimination half-life ranges from 6 up to 20 hours. CsA is subject to numerous drug (*e.g.* azole antifungals and some macrolide anibiotics) and food (*e.g.* grapefruit) interactions that may increase or decrease CsA concentration in whole blood.

Adverse effects associated with CsA include nephrotoxicity, hypertension, neurotoxicity, gum hypertrophy, hirsutism, post Tx diabetes mellitus, hyperlipidemia and hemolytic-uremic syndrome. The incidence of nephrotoxicity and diabetes has been related to drug exposure [35,44]. Although CsA and Tac share similar adverse effect profiles, the incidence of hypertension, dyslipidemia, gingival hyperplasia and hirsutism is reported to be higher with CsA [45].

1.3.1.2 Tacrolimus (Tac)

CsA has been diminished over the last years while the use of Tac is increasing [35,46-48]. Tac is a macrolide immunosuppressant first isolated from *Streptomyces tsukubaensis* in 1984 [49]. Although similar mechanisms of action (Section 1.3.1), Tac is 10 – 100 times more potent than CsA on a mg by mg basis [50]. Since 1994 Tac has been used clinically to prevent

allograft rejection in renal patients. Currently, oral Tac is available as immediate-release, twice-daily formulations (Prograf, Tacni, Modigraf) and a prolonged-release, once-daily formulation (Advagraf). Although the clinical efficiency is reported to be comparable, pharmacokinetics differ between the immediate- and prolonged-release formulations [43,51].

As for CsA, the oral bioavailability of Tac is poor and highly variable, ranging 5 – 93% (mean 25%) [52,53]. Following oral administration of the immediate-release formulations, peak whole blood concentrations usually occur after 1 – 2 hours. The absorption is reduced in the presence of food. In blood, Tac is extensively distributed to erythrocytes and is approximately 99% protein-bound. Similar to CsA, Tac is a substrate of the drug efflux transporter P-glycoprotein (ABCB1) and is metabolized by CYP3A4 and CYP3A5 enzymes in the liver and intestine [54]. The metabolites are predominantly eliminated by biliary excretion and the elimination half-life of Tac is variable and ranges from 3.5 up to 40.5 hours [43,55]. As reported for CsA, Tac is also subject to range of drug and food interactions such as erythromycin, St. Johns Wort or grapefruit (Section 1.3.1.1).

Nonimmune adverse effects of Tac are similar to those reported for CsA. However, Tac has been associated with a higher rate of new-onset diabetes mellitus and post Tx neurological side effects such as tremor, headache and sleep disturbances [35,56]. The side effects appear to be dose dependent and are usually reversible. However, the effect on renal function may persist.

1.3.2 Glucocorticoids

Glucocorticoids were the first immunosuppressive drugs used in Tx and they are still a part of first-line treatment for both prevention and treatment of acute allograft rejection [57].

Glucocorticoids are non-specific agents with immunosuppressive effect on a wide variety of cells. At the cellular level, the majority of immunosuppressive effects are either directly or indirectly related to transcriptional effects on a variety of cytokines. Consequently, glucocorticoids inhibit a broad range of immune responses mediated by T cells, B cells and phagocytes.

Glucocorticoids have numerous well-known side effects such as impaired wound healing, increased risk of infection, hypertension, dyslipidemia, and glucose intolerance. These side effects contribute to the high prevalence of cardiovascular disease in transplant recipients [58].

1.3.3 Purine analogues and proliferation inhibitors

Since the 1990s, mycophenolic acid has progressively replaced azathioprine in standard immunosuppressive protocols because of fewer side effects and a reduced risk of rejection [40,59-61]. Mycophenolic acid, a noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), prevents DNA synthesis by blocking *de novo* purine synthesis in T and B cells and thereby inhibiting cell proliferation and function.

Mycophenolic acid inhibits bone marrow function and may cause gastrointestinal adverse effects. Other side effects are associated with overimmunosuppression, especially a higher incidence and severity of cytomegalovirus (CMV) infections. However, mycophenolic acid is not considered to be nephrotoxic [58,62].

1.3.4 Interleukin 2 receptor antibody

Basiliximab (Simulect) is a chimeric monoclonal antibody used for induction therapy. It is administered intravenously the day of Tx and 4 days post Tx. The drug binds to the CD25 antigen on T cells and thereby prevents the binding of IL2 to its receptor (IL2R). Consequently, activation of T cells is inhibited [63]. The use of basiliximab has been reported to reduce the incidence of acute rejection in renal transplant recipients [64,65].

1.3.5 Mechanistic target of rapamycin (mTOR) inhibitors

Sirolimus, or rapamycin, is a macrolide antibiotic with a similar molecular structure to Tac. Sirolimus binds to the same immunophillin as Tac. Unlike the Tac complex, the sirolimus complex inhibits mTOR, a protein kinase essential for cell cycle progression and

proliferation. Thus, inhibition of mTOR prevents T cell proliferation. More recently, everolimus, a synthetic derivate of sirolimus, was introduced. Sirolimus and everolimus share the same molecular mechanism of action, but differ in pharmacokinetic characteristics [66].

Compared to CNIs, mTOR inhibitors are less effective in preventing rejection episodes. However, mTOR inhibitors may be beneficial in the context of CNI minimization or withdrawal and to reduce the risk of post Tx malignancies [67-70].

The side effect profiles of sirolimus and everolimus are similar and include dose-dependent bone marrow toxicity, impaired wound healing, acne, mouth ulcers, abdominal pain, hyperlipidaemia, thrombocytopaenia and anaemia [58].

1.4 Individualization of CNI therapy

Both CsA and Tac are characterized by a narrow therapeutic range and large inter- and intraindividual variability with respect to both PKs and pharmacodynamics (PD). This emphasizes the need for therapeutic drug monitoring (TDM) to optimize the immunosuppressive effect while minimizing toxicity.

1.4.1 Therapeutic drug monitoring (TDM)

The conventional TDM of CNIs involves the measurement of drug concentrations in whole blood (Figure 5). Individualization of CNI therapy based on concentration measurements is known to reduce the incidence of rejection episodes, CNI-specific toxicity and infections related to overimmunosuppression.

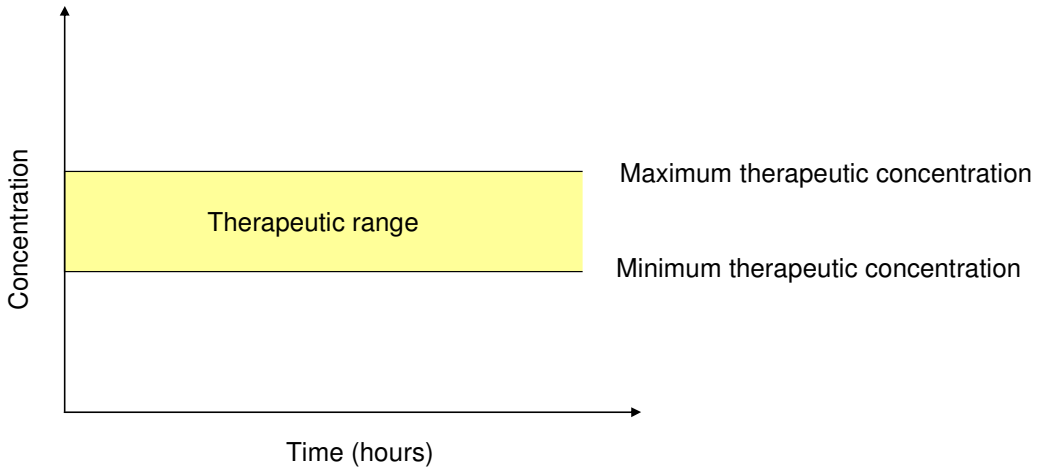


Figure 5: Therapeutic Drug Monitoring (TDM). Individualization of drug therapy based on drug concentration measurements may improve clinical outcomes, especially of drugs with a narrow therapeutic range.

The area-under-the-blood-concentration-*versus*-time-curve (AUC) from 0 to 12 hours ($AUC_{0\text{-}12h}$) is considered an optimal estimate of total CNI exposure since CNI AUC is associated with clinical endpoints. However, $AUC_{0\text{-}12}$ measurements are impractical for clinical routine [71]. Currently, CNI therapy is usually monitored by measurement of trough concentrations (C_0). However, C_0 is not strictly correlated to $AUC_{0\text{-}12h}$ levels [72] and does not reflect the biologic effect of CNIs in an individual patient. Several studies have reported that C_0 levels did not differentiate patients at risk of acute rejection or nephrotoxicity [73-75]. Compared to conventional monitoring, measurement of post dose concentrations has been reported to improve clinical outcomes during the first months of CsA therapy. Measurements of CNI concentrations at 1.5 ($C_{1.5}$) or 2 (C_2) hours post dose have been shown to be good predictors of Tac and CsA exposure, respectively [30,76,77]. Furthermore, measurement or estimation of the $AUC_{0\text{-}12h}$ by using two or three concentrations at the beginning of dose interval may better predict the clinical effect to CNIs [78].

1.4.2 Clinical outcomes of CNI therapy

Over the last couple of decades, immunosuppressive therapy in Tx have improved significantly. The introduction of CsA and Tac has contributed to remarkable improvements of short-term outcomes. Currently, the 1 year patient and graft survival rates in renal Tx are approximately 90% [27].

Importantly, the long-term allograft survival rates (>1 year post Tx) have not improved in parallel with the short-term outcomes. Morbidity and mortality remain much higher than in the general population, mainly due to the long-term complications [79,80]. Considering long-term outcomes, 36 – 50% of renal allografts are lost due to patient death related to cardiovascular diseases, infections or malignancies. The leading cause of late graft loss among surviving recipients is chronic allograft nephropathy with tubular atrophy and interstitial fibrosis, a common complication of long-term CNI therapy [81-84]. This suggests a potential for improving long-term outcomes after Tx by reducing adverse effects related to CNIs and to overimmunosuppression more generally.

1.4.3 Pharmacodynamic monitoring of CNIs

PD is the study of drug effects in the body. Compared to PK monitoring, measurement of molecular or cellular responses may provide a more direct estimation of clinical response of a drug. So far, PD monitoring of immunosuppressive drugs has not been implemented in clinical routine practice in Tx. However, PD monitoring of CNIs is currently investigated in many clinical studies [31,80].

The PD effects of CNIs can be assessed with either direct (specific markers) or indirect (global markers) biomarkers [85]. A number of studies have investigated potential associations between clinical outcomes and PD biomarkers like intracellular IL2 synthesis [86], intracellular calcineurin activity [87] or the expression of intracellular adenosine triphosphate production in $CD4^+$ cells after phytohemaglutinin (PHA) stimulation [88,89]. So far, the most promising strategy seems to be the measurement of NFAT-regulated gene expression [77,80]. Several studies in different patient cohorts have reported an association between NFAT-regulated gene expression levels and clinical outcomes like acute rejection episodes, infections and malignancy after Tx [56,90-92]. This supports a potential

for monitoring NFAT-regulated gene expression, in combination with PK monitoring, to individualize and improve CNI therapy in solid organ recipients.

1.5 Immune activation assay

In resting lymphocytes, the expression levels of NFAT-regulated genes in whole blood are very low. *Ex vivo* activation of lymphocytes imitates the immune activation of lymphocytes *in vivo*, *e.g.* in response to a foreign organ, and thereby provides an estimate of the immune activation potential of an individual.

PHA, phorbol 12-myristate 13-acetate (PMA) and ionomycin are common stimulants utilized in a variety of immunologic assays. PHA results in cell agglutination and activates T cells by interacting with T cell receptor complex [93,94]. The combination of PMA and ionomycin activates protein kinase C and increases intracellular calcium levels, which in turn results in T cell activation [95].

1.6 Gene expression analysis

1.6.1 RNA isolation

The quality and quantity of starting nucleic acids is critical for gene expression analysis. Whole blood contains relatively small amounts of mRNA and high levels of ribonucleases (RNase). Thus, special precautions are required before, during and after RNA isolation to avoid RNA degradation by RNases or unintended regulation of gene expression. RNA isolation relies on good laboratory technique and RNase-free techniques. Furthermore, RNA isolation protocols include strong denaturants to inhibit endogenous RNases. Several different techniques and protocols are available for isolation of RNA including organic extraction methods, spin basket formats, magnetic particle methods, and direct lysis methods. The

optimal technique may differ depending on the type of sample material, sample volume and number of samples.

1.6.2 Reverse transcription PCR

The number of individual mRNA molecules in a sample is very low and quantification of mRNA levels depends on the use of sensitive methods. Reverse transcription (RT) followed by real-time PCR constitutes a sensitive analysis method which is increasingly used for gene expression measurements. The procedure starts with the conversion of RNA into cDNA, which in turn serves as template in amplification reactions. The procedure can be performed as one- or two-step processes. In one-step RT, the RT and PCR reactions are performed in the same tube, which limits sample handling and thereby reduces the risk for pipetting errors and contamination. The two-step procedure allows separate optimization of the RT and PCR steps, which in turn may improve the sensitivity and precision of the assay. Furthermore, a two-step approach is useful for detecting multiple mRNAs from a single RNA sample.

Different strategies can be used to prime the cDNA synthesis:

1. Oligo(dT) primers bind to the poly(A)tail of mRNA molecules. This strategy may favour synthesis of full-length cDNA, however, cDNA synthesis efficiency may differ throughout the length of the mRNA.

2. Random hexamer primers bind to many sites throughout the length of an RNA. The priming strategy is independent on the presence of a poly(A)tail (*e.g.* tRNA, rRNA).

3. Sequence-specific primers bind to specific target sequences and are always used in one-step RT-PCR.

1.6.3 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) involves monitoring of amplification products in each PCR cycle using fluorescent dyes.

1.6.3.1 PCR

PCR has evolved far beyond simple amplification to an extremely useful technique, which is implemented in a variety of research fields and diagnostic assays.

Using PCR, specific DNA sequences can be copied or "amplified" many thousands-to-millions fold. In addition to a template sequence, the necessary reagents are specific primers, DNA polymerase, Mg^{2+} and nucleotides. The DNA polymerase requires a small area of double-stranded DNA and Mg^{2+} as a cofactor to initiate DNA replication. Thus, the amplified sequence depends on the sequence of the primers.

The PCR technique is based on thermal cycling. A PCR program usually consists of 20 – 50 cycles, where each cycle comprises (2–) 3 different temperature steps.

1. Denaturation at 94 – 98°C: During this step, double-stranded DNA will denature to single-stranded DNA.

2. Annealing at 50 – 65°C: Primers hybridize to the sense and antisense strands of the DNA template. The annealing temperature is a critical parameter affecting the efficacy and specificity of the PCR. The melting temperature of the primers depends on the length and base composition of the oligonucleotides.

3. Extension/elongation at 72°C: The elongation temperature is the ideal working temperature for the DNA polymerase. The most frequently used is the heat stable bacterial DNA polymerase Taq (Thermus aquaticus). Starting at the primer, DNA polymerase generates complementary copies by incorporating nucleotides that are complementary to the target template.

The next cycle of PCR is then ready to start over.

Theoretically, the amount of product is doubled in each cycle of PCR as illustrated in Figure 6.

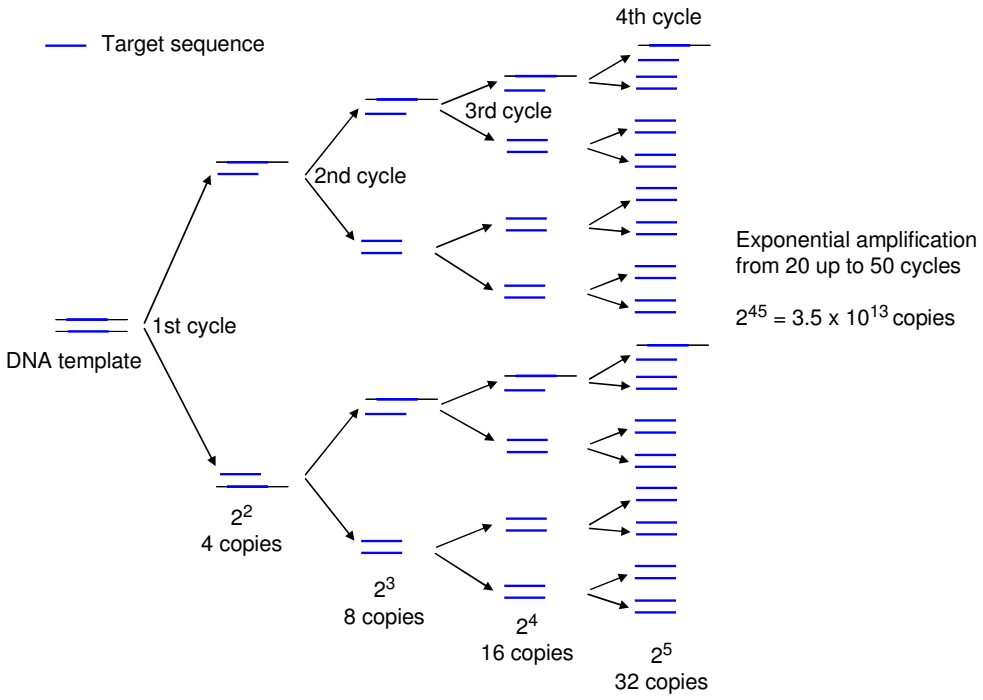


Figure 6: Exponential amplification of a specific DNA sequence by PCR. After n cycles, the number of DNA copies is 2^n.

1.6.3.2 Detection formats

The fluorescent signal generated during real-time PCR is directly proportional to the amount of PCR product present. Several different fluorescent reporters are available for real-time PCR, and each has specific assay design requirements. The detection formats can be divided into sequence-independent and sequence-specific.

Sequence-independent detection formats

Sequence-independent detection formats include intercalating dyes such as SYBR Green I, and detect all double-stranded DNA. The format cannot discriminate between specific PCR products, non-specific products and primer dimers. SYBR Green I emits very little

fluorescence when it is free in solution. However, once bound to double-stranded DNA, the fluorescence intensity increases about 100 fold (Figure 7) [96,97]. The sequence-independent detection formats are inexpensive, easy to use and allow sensitive detection of different target sequences. The ability to bind to all dsDNA molecules can be exploited to detect non-specific products or primer dimers by performing a melt curve analysis (see Section 1.6.7.1 for further details).

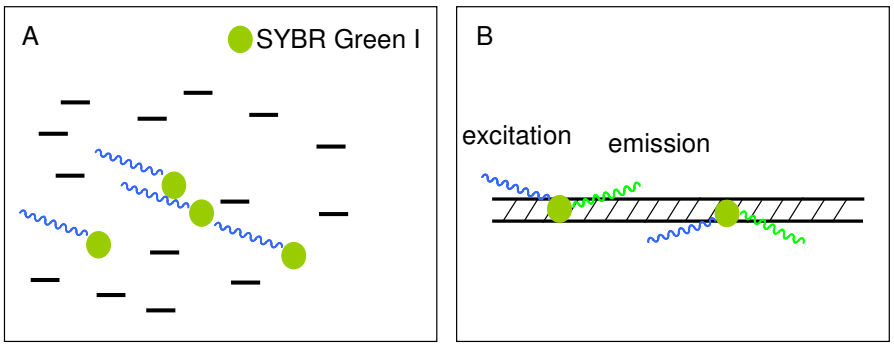


Figure 7: SYBR Green I-based detection. A: SYBR Green I fluorescence emission is low when free in solution with denatured DNA and primers. **B**: Binding of SYBR Green I to double-stranded DNA generates much higher fluorescence (100 fold).

Sequence-specific detection formats

Sequence-specific detection formats imply that the fluorescence signal will only increase if the probe is bound to a specific DNA sequence. The formats include fluorophore-labeled probes like hydrolysis probes, simpleProbe and hybridization probes.

Hydrolysis (TaqMan) probes are oligonucleotides with a fluorescence reporter and a quencher dye attached. During PCR, the probe is cleaved by the 5´→ 3´-nuclease activity of the DNA polymerase, separating the reporter and quencher dyes. This generates a fluorescent signal that increases with each cycle, proportional to the rate of probe cleavage.

SimpleProbe probes are oligonucleotides labeled with a single fluorophore. Once bound to the complementary target sequence, the fluorescence increases due to a conformational change.

Hybridization probes are a pair of sequence-specific oligonucleotides that hybridizes side by side on the PCR product. The 3' end of the upstream probe is labeled with fluorescein, which acts as a fluorescence resonance energy transfer (FRET) donor, while the 5' end of the downstream probe is labeled with an acceptor dye. When both probes are bound to their complementary target sequence the dyes will be in close proximity to each other, allowing FRET from the donor to the acceptor fluorophore (Figure 8). Finally, the acceptor fluorophore emits fluorescent light with a different wavelength.

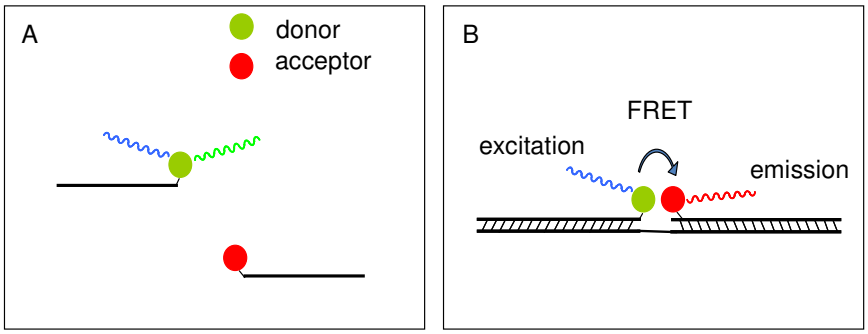


Figure 8: Hybridization probe-based detection. A: Hybridization probes include a donor probe labeled with fluorescein in the 3´end and an acceptor probe labeled with an acceptor dye in the 5´end. The donor dye is excited by blue light and emits green fluorescent light. **B**: Binding of the hybridization probes to their specific complementary regions results in fluorescence resonance energy transfer (FRET) from the donor to the acceptor probe and finally emission of fluorescence with a different wavelength.

1.6.3.3 Primer and probe design

Successful qPCR requires good primer design. Primers should bind selectively to the target sequence. For adequate selectivity, the primers should ideally comprise 18 – 30 base pairs and have GC content in the range of 40 – 60%. There are several software tools available for oligonucleotide design and evaluation of primer and probe sequences (Table 4). Primer and probe design is further described in Section 4.4.

1.6.4 Relative mRNA quantification

Gene expression levels may be determined by absolute or relative real-time PCR quantification strategies (Figure 17 Section 4.6). Absolute quantification depends on calibration curves to relate PCR signals to input copy number. In contrast, relative quantification determines the change in target mRNA expression levels relative to the level of reference mRNA, *e.g.* mRNA of one or more endogenous "housekeeping" genes. The calculation of relative gene expression is based on the cycle number where the fluorescence rises above background (begins its exponential phase), defined as the crossing point (Cp) (Figure 9). The Cp is inversely proportional to the initial cDNA template concentration.

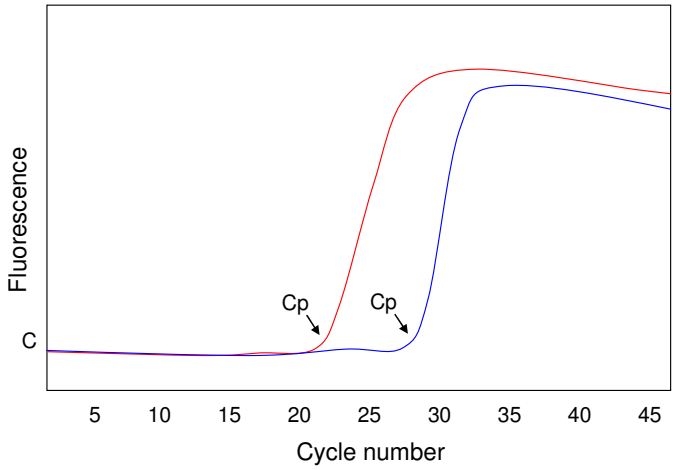


Figure 9: Amplification curves. The data generated by real-time fluorescence monitoring during PCR result in amplification plots. The crossing point (Cp) is the cycle number where the fluorescence rises above background. The figure illustrates the amplification curves of two samples with different initial template concentrations. A higher Cp value represents a lower initial concentration.

1.6.4.1 Reference genes

Reliable quantification of relative gene expression levels depends on normalization to a fixed reference. The reference genes should correct for sample-to-sample variation, differences in initial amount, isolation yields, RNA/DNA degradation, RNA/DNA quality, pipetting and cDNA synthesis efficiency. Furthermore, the reference genes may also identify the presence of PCR inhibitors. Reference genes are usually genes encoding endogenous products that are

essential for normal cell function. Several studies have documented that there is no single reference gene with constant expression levels across different sample materials and experimental conditions [98-100]. Thus, it is generally recommended to normalize the expression of target genes to a reference gene index based on several reference genes [101]. Furthermore, to reduce the probability of co-regulation, the reference genes should preferably belong to different functional classes.

1.6.4.2 PCR-efficiency correction

In theory, the number of PCR products doubles at each cycle if the PCR-efficacy is 100% (E = 2, Equation 1A). However, in practice, the efficiency is often less than 100% (Equation 1B) because of PCR inhibitors like heparin, hemoglobin, myoglobin, lipids, polysaccharides, amount and quality of RNA/DNA, non-specific products, cycle conditions and PCR reagents and equipment [102-104]. The samples may be affected to different extent. Various strategies have been established to normalize these variations (see methods).

A

$$N_{Cp} = N_0 \times 2^{Cp}$$

B

$$N_{Cp} = N_0 \times E^{Cp}$$

Equation 1: The basic equation describing PCR. A: PCR-efficiency is assumed to be 100%. **B**: The actual PCR-efficiency is included in the calculation.

Cp; crossing point, N_{Cp}; number of molecules at Cp, N_0; initial number of molecules, n; number of PCR cycles performed, E; PCR-efficiency

---

Different approaches have been reported to estimate the actual efficiency of the real-time PCR. The standard curve approach is based on the amplification of serial dilutions of cDNA and construction of standard curves by plotting Cp values against the log of initial template concentrations (Section 4.5.4). Based on the slope of the standard curve, PCR-efficiency is estimated according to Equation 2, Section 4.5.4. A slope of -3,337 indicates a PCR-efficiency of 100% (E = 2).

1.6.5 Optimization of PCR conditions and assay validation

In addition to the selection of suitable primers and probes, it is necessary to optimize and validate the final assays.

1.6.5.1 SYBR Green I analysis

Melting curve analysis with SYBR Green I is a fast and sensitive method to evaluate the selectivity of the primers.

Intercalating dyes like SYBR Green I allows real-time PCR-products to be directly evaluated by melting curve analysis. Initially, double-stranded DNA amplicons give high levels of fluorescence. As the temperature gradually increases double-stranded DNA melts into single-stranded DNA. A typical plot of the dissociation curve is shown in Figure 10A. In Figure 10B, the intensity of fluorescence is plotted against temperature and then the -ΔF/-ΔT (change in fluorescence/change in temperature, y-axis) is plotted against temperature (x-axis). The melting temperature of a PCR product depends on the length and GC content of the DNA. Non-specific PCR-products and primer dimers usually present melting temperatures significantly different from the specific product and are easily detected.

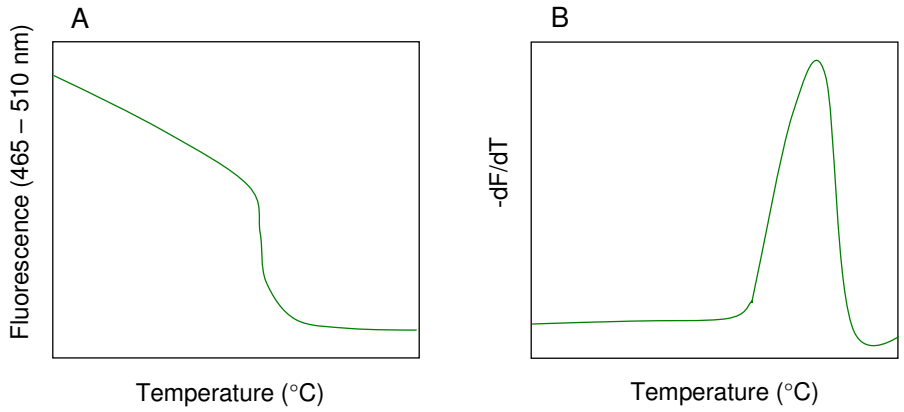


Figure 10: SYBR Green I-based melting curve analysis. A: Raw melting curve profiles using SYBR Green I as a fluorescent dye. **B**: Negative first derivative melting curve plots using SYBR Green I as a fluorescent dye.

1.6.5.2 Agarose gel electrophoresis

Gel electrophoresis is a technique used to separate the products based on molecular size and charge. As DNA is negatively charged due to phosphate groups in the backbone of DNA, it will migrate towards the positive electrode (anode).

The electrophoretic distance of DNA molecules through agarose gels is also dependent on their size, the composition of the buffer, the length of the electrophoresis, the properties of the gel, and the amount of current applied. The number of base pairs of a PCR product determines the size of the molecule. When traveling horizontally through an agarose gel, large molecules will migrate slower through the pores of the gel than small molecules. Following gel electrophoresis, DNA bands can be visualized in gels using intercalating dyes, such as ethidium bromide. Finally, the bands of the PCR-products are compared with the bands of a molecular weight marker including DNA fragments of known size.

1.6.5.3 DNA sequencing

The actual sequence of PCR-products may be determined by DNA sequencing. Currently, several sequencing strategies are available. The chain terminator method or Sanger dideoxy method relies on base-specific chain termination after incorporation of dideoxynucleotides (ddNTPs). At each step of chain extension, the DNA polymerase adds either a deoxynucleotide or the corresponding ddNTP. The ddNTPs are nucleotide base analogs that lack the 3′-hydroxyl group required for the formation of phosphodiester bonds. The four ddNTP (ddA, ddC, ddG and ddT) are labeled with different fluorescent molecules. This results in the formation of products of various lengths. Finally, the products are separated by capillary electrophoresis and characterized by fluorescence measurements.

2 Goals of the project

Following organ Tx, lifelong immunosuppressive therapy is needed to prevent rejection of the transplanted organ. Currently, CNIs represent the cornerstone in most immunosuppressive regimens. Despite contributing to remarkable improvements in short-term outcomes, CNIs may have negative impact on long-term outcomes post Tx. Due to the narrow therapeutic range and PK variability of CNIs, dosing is usually guided by PK measurements. Furthermore, PD monitoring may provide a more direct estimation of the CNI response and thereby allow further optimization of CNI therapy.

The main goal of the project is to investigate NFAT-regulated gene expression as a potential PD biomarker of CNI response in renal transplant recipients.

The sub-goals are:

1. To establish and validate a real-time PCR assay for the quantification of NFAT-regulated gene expression in whole blood samples.

2. To assess the effect of *ex vivo* exposure to different immunosuppressive drugs and concentrations on NFAT-regulated gene expression.

3. To investigate the NFAT-regulated gene expression in clinical samples from renal allograft recipients treated with Tac.

3 Materials

The reagents and equipment used in this project are listed in Table 1 and Table 2, respectively. In addition, centrifuge, vortex mixer, pipettes and filter tips were used.

Table 1: Reagents

Reagents	**Vendor/ Producer**	**Cat. No.**
BigDye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, California, USA	4 336 917
Bromophenol blue	Sigma-Aldrich Norway AS, Oslo, Norway	B-6131
Dithiothreitol (DTT), 10 g	Roche Diagnostics GmbH, Mannheim, Germany	10 708 984001
DNA-molecular Weight marker V	Roche Diagnostics GmbH, Mannheim, Germany	10 821 705 001
DNA ZAP ™ I and II	Ambion/Applied Biosystems, Austin, TX, USA	AM9890
ExoSAP-IT	Amersham Biosystems, Foster City, California, USA	US 77705
Ionomycin calcium salt	Sigma-Aldrich Norway AS, Oslo, Norway	I 3909
Lectin from Phasesous vulgaris	Sigma-Aldrich Norway AS, Oslo, Norway	L 4144
LightCycler® 480 SYBR Green I Master	Roche Diagnostics GmbH, Mannheim, Germany	4 707 516 001
LightCycler® 480 Probes Master	Roche Diagnostics GmbH, Mannheim, Germany	4 707 494 001
Lysis/Binding Buffer with guanidinium thiocyanate	Roche Diagnostics GmbH, Mannheim, Germany	3 542 394 001
MagNA Pure LC DNA Isolation Kit-High Performance kit	Roche Applied Science, Penzberg, Germany	3 542 394 001
MS2 RNA	Roche Diagnostics GmbH, Mannheim, Germany	10 165 948 001
Primers and hybridization probes (sequences are given in Table 5)	TIB® MOLBIOL, Berlin, Germany (synthesis according to user's design)	
Phorbol 12-myristate 13-acetate	Sigma-Aldrich Norway AS, Oslo, Norway	P 1585
Red Blood Cell Lysis Buffer, 100 mL	Roche Diagnostics GmbH, Mannheim, Germany	11 814 389 001

Reliant Gel system 4% Nusieve 3:1 Plus Agarose, 8 well	Lonza BioScience Rockland, ME, USA	54927
RPMI 1640 medium with L-glutamine	Sigma-Aldrich Norway AS, Oslo, Norway	R 8758
TE-buffer 1X, Molecular Biology Grade	Promega Corporation, Madison, WI, USA	V 6231
Transcriptor First Strand cDNA Synthesis Kit	Roche Diagnostics GmbH, Mannheim, Germany	4 897 030 001
TBE-Buffer 10X	Bio-Rad Laboratories, Inc., Hercules, California, USA	161-0733

Table 2: Equipment

Equipment	Vendor/ Producer	Cat. No.
ABI PRISM® 3100 Genetic Analyzer	Carlsbad, California, USA	Laboratory supplier
Applied Biosystems 2720 Thermal Cycler	Applied Biosystems, Foster City, California, USA	Laboratory supplier
Biorad Molecular Imager GelDoc ™ XR	Bio-Rad Laboratories, Inc., Hercules, California, USA	Laboratory supplier
Sodium-Heparin tubes, 6 mL	Greiner Bio One, GmbH Bad Haller, Kremsmunster Austria	456051
LightCycler® 480 instrument	Roche Applied Science, Mannheim, Germany	Laboratory supplier
LightCycler® 480 Multiwell Plate 96	Roche Applied Science, Mannheim, Germany	4 729 692 001
LightCycler® 480 Sealing Foil	Roche Applied Science, Mannheim, Germany	4 729 757 001
MagNA Pure LC ™ 2.0 instrument	Roche Applied Science, Mannheim, Germany	12 236 931 001
MagNA Pure LC Cartridge Seal	Roche Applied Science, Mannheim, Germany	03 118 827 001
MagNA Pure LC Cooling Block, LC Centrifuge Adapters	Roche Applied Science, Mannheim, Germany	12 190 664 001
MagNA Pure LC Cooling Block, 96 well PCR Plate	Roche Applied Science, Mannheim, Germany	12 189 674 001

MagNA Pure LC Greasing Set	Roche Applied Science, Mannheim, Germany	3 561 402 001
MagNA Pure LC Processing Cartridges	Roche Applied Science, Mannheim, Germany	3 004 147 001
MagNA Pure LC Reactions Tips large	Roche Applied Science, Mannheim, Germany	03 004 171 001
MagNA Pure LC Reactions Tips small	Roche Applied Science, Mannheim, Germany	03 004 180 001
MagNA Pure LC Reactions Tub 20 medium	Roche Applied Science, Mannheim, Germany	03 004 058 001
MagNA Pure LC Reactions Tub 30 medium	Roche Applied Science, Mannheim, Germany	3 045 501
MagNA Pure LC Reactions Tub large	Roche Applied Science, Mannheim, Germany	03 004 040 001
MagNA Pure LC Sample Cartridges	Roche Applied Science, Mannheim, Germany	3 004 112 001
MagNA Pure LC Tip Stands	Roche Applied Science, Mannheim, Germany	3 004 155 001
MagNA Pure LC Tub Lids small medium	Roche Applied Science, Mannheim, Germany	03 004 082 001
MagNA Pure LC Tub Lids large	Roche Applied Science, Mannheim, Germany	03 004 074 001
MagNA Pure LC Waste Bag	Roche Applied Science, Mannheim, Germany	3 004 201 001
Microtubes 1.5	Sarstedt, AC, Numbrecht Germany	S 044 523
Multiply®-Pro cup 0,2 ml	AC, Numbrecht Germany	S 044080 43/14
Nanodrop® ND-1000 Spectrophotometer	Saveen Werner, Thermo Fisher Scientific, Wilmington, USA	ND 1000
Tube-strip Picofuge®	Stratagene, Medcompare Diagnostics, California, USA	Laboratory supplier

3.1 Blood samples for assay development and validation

Assay development was based on blood samples from three healthy volunteers. Whole blood was drawn in heparinized blood tubes on repeated occasions. The assay validation also included pre dose and 1.5 hours post dose samples from a renal transplant recipient treated with Tac. The following sample processing steps are described in Section 4.1.

3.2 MarkIt pilot study

Five renal transplant recipients were enrolled in the MarkIt pilot study at Oslo University Hospital, Rikshospitalet, from February to March 2012. The study is still ongoing and the planned enrollment is a total of 30 patients. Inclusion criteria were renal Tx with a graft from a living donor and recipient age above 18 years. Patients were excluded if there were any medical limitations of blood sampling. The patients received immunosuppression according to the relevant protocol for renal Tx. Standard quadruple therapy consists of basiliximab, Tac, glucocorticoids and mycophenolic acid. The study was approved by the Regional Committee for Medical and Health Research Ethics in Norway. Informed consent was obtained from all study participants and they were free to withdraw from the study at any time.

3.2.1 Samples from renal transplant recipients

For the measurement of NFAT-regulated gene expression, whole blood was drawn in a heparinized tube (6 mL) on the following occasions:

1. Once at 0 – 4 days before Tx and initiation of immunosuppressive therapy
2. Pre dose and at 1.5 hours after Tac dosing on one day within 6 – 9 days post transplant
3. Pre dose and at 1.5 hours after Tac dosing on one day within 5 – 7 weeks post transplant

4. Additionally, blood samples will be drawn pre and post dosing approximately 1 year post transplant.

In parallel with the NFAT-regulated gene expression materials, whole blood was also sampled in ethylenediaminetetraacetic acid (EDTA) tubes (Vacuette®, Greiner Bio One) and PAXgene™ Blood RNA tubes (PreAnalytix®, Qiagen) for the measurement of drug concentrations and assessment of potential mRNA biomarkers in other ongoing projects.

4 Methods

Good laboratory practice is essential in order to achieve reliable results of gene expression (Figure 11). RNA is susceptible to degradation by RNases. To prevent contamination and establish an RNase-free environment, the instruments were decontaminated with UV-light, 70% ethanol and DNA Zap^{TM} before and after use. Pre-PCR, PCR and post-PCR operations were physically separated to prevent contamination with PCR products. Furthermore, protective disposable gloves and laboratory coats were worn at all times when handling reagents and sample materials. Reagents were of PCR quality and disposable plastic equipments were sterile and RNase-free. To avoid or minimize unintentional gene expression regulation *ex vivo*, RNA was preferably stabilized as soon as possible after blood collection.

For quality controls, one negative control for each target gene was included per LightCycler 96-well PCR plates. Negative controls contained all PCR reagents except the cDNA template which was replaced by water. This allowed detection of potential contamination of the PCR reagents.

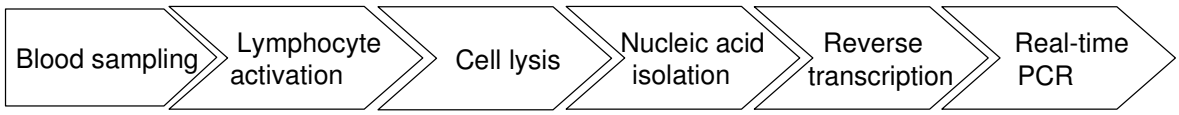


Figure 11: Overview of the steps of nuclear factor of activated T cells (NFAT)-regulated gene expression assay.

4.1 Lymphocyte activation and sample preparation

Lymphocytes were activated *ex vivo* within 2 hours after blood sampling (Section 3.1 and 3.2.1). Pre and post dose samples were activated simultaneously.

Three different lymphocyte activation principles and different sample preparation procedures were compared by real-time PCR analysis of *IL2*, *IFNG* and *CSF2* gene expression levels.

The lymphocyte activation media consisted of complete RPMI 1640 medium with PHA (10 mg/L) alone, PMA (100 µg/L) in combination with ionomycin (5 mg/L) or all three agents together (PHA + PMA + ionomycin). The mitogen concentrations were based on previous studies [105]. Each of the three activation media, as well as a control medium without mitogens, was added to equal volumes of whole blood in 5 mL, Falcon/BD tubes. The mixture was incubated at 37°C for 0, 3, 24, 48 and 72 hours.

Two different procedures were compared for sample preparation after *ex vivo* incubation of whole blood with mitogens. A two-step approach involved removal of red blood cells using the Red Blood Cell Lysis Buffer before lysis of leukocytes by addition of Lysis/Binding-buffer (LBB) with 1% (w/v) dithiothreitol (DTT). A simplified procedure involved cell lysis in a single step by adding LBB directly to the whole blood cell suspension.

After optimization, the sample preparation of the final assay involved incubation of 150 µL whole blood with 150 µL activation medium containing PMA and ionomycin in Falcon/BD 5 mL tubes. In addition, unstimulated controls were prepared by incubation of whole blood with medium without mitogens. The tubes were gently mixed and incubated for 3 hours at 37°C, 5% CO_2 and 95% humidity. After incubation, 200 µL cell suspension was transferred to 1.5 mL Sarstedt microtubes, lysed with 700 µL LBB with 1% (w/v) DTT by vortexing for 30 sec. The tubes were then frozen at −70°C until further preparations.

4.2 Isolation of total RNA

After thawing, cell lysates were mixed and transferred into MagNA Pure Sample Cartridges. Total RNA was isolated on the MagNA Pure LC™ instrument, using MagNA Pure LC RNA Isolation Kit-High Performance kit and the RNA HP external lysis protocol according to the manufacturer's instructions. The total RNA isolation procedure included cell lysis with LBB and digestion of remaining proteins with Proteinase K. Released RNA was bound to the silica

surface of Magnetic Glass Particles due to the chaotropic salt conditions, isopropanol and the high ionic strength of the LBB. DNA was removed by DNase treatment and furthermore, followed by additional washing steps. The RNA was magnetically separated from the residual sample materials and finally, purified total RNA was eluted from the silica particles at 70°C.

The initial sample and elution volumes were set to 800 µL and 50 µL, respectively. Total RNA was stored at –70°C until further preparations.

4.3 Two-step reverse transcription and quantitative PCR

Gene expression of *IL2, IFNG* and *CSF2* was analyzed using RT-qPCR on the LightCycler® 480 instrument. An overview of the assay workflow is illustrated in Figure 12.

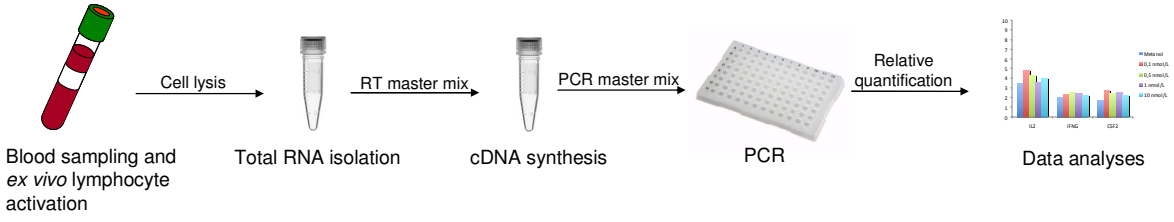


Figure 12: Workflow of nuclear factor of activated T cells (NFAT)-regulated gene expression assay.

RT; reverse transcription

4.3.1 Reverse transcription (RT)

The RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's protocol. The cDNA synthesis was primed by a combination of random hexamer and anchored-oligo(dT)$_{18}$ primers. MS2 RNA (final

concentration of 10 µg/mL) was included as carrier RNA to stabilize RNA template and reduce the binding of RNA to microtubes and pipette tips. The RT reaction was performed in a final volume of 20 µL per reaction in 0.2 mL thin-walled PCR tubes using a thermal block cycler with a heated lid. The RT procedure, including thermal cycler program and reaction components, is summarized in Table 3.

Table 3: Reverse transcription procedure

Step	**Temperature (°C)**	**Time (minutes)**	**Reagents (volume/reaction)**	**Comments**
1	65	5	Total RNA (5 µL) Water (PCR-grade) with MS2 RNA (5 µL) Random hexamer primers (1 µL) Anchored-oligo(dT)$_{18}$primer (2 µL)	Denaturation of RNA secondary structures After 5 min incubation, PCR tubes were immediately placed on ice to prevent reformation of RNA secondary structures. This allows primer binding to the RNA templates
2	25	10	Master mix of: Transcriptor Reverse Transcriptase Reaction Buffer (4 µL) Protector RNase inhibitor (0.5 µL) Deoxynucleotide Mix (2 µL) Transcriptor Reverse Transcriptase (0.5 µL)	Binding of primers and initiation of cDNA synthesis
3	Gradual increase of the temperature from 25 to 55°C. Slope +30, 0.3°C/sec			
4	55	30		cDNA synthesis
5	60	5		cDNA synthesis at increased temperature to overcome potential RNA secondary structure
6	85	5		Inactivation of the Transcriptor Reverse Transcriptase enzyme
7	4	“Forever”		The reaction mixture is cooled to 4°C
8	End			

The products from two parallel cDNA synthesis reactions, 20 µL each, were mixed and diluted with PCR-grade water containing MS2 RNA to a final volume of 150 µL. (40 µL cDNA + 110 µL water with MS2 RNA). This provided suitable template concentrations and enough cDNA to perform approximately 30 PCR reactions. cDNA was stored at −20°C until PCR analysis.

A calibrator sample was prepared by generating a pool of cDNA from activated lymphocytes (Sections 4.1, 4.2 and 4.3.1), aliquoted (1000 and 150 µL aliquots) and stored at −70°C and −20°C (Figure 13).

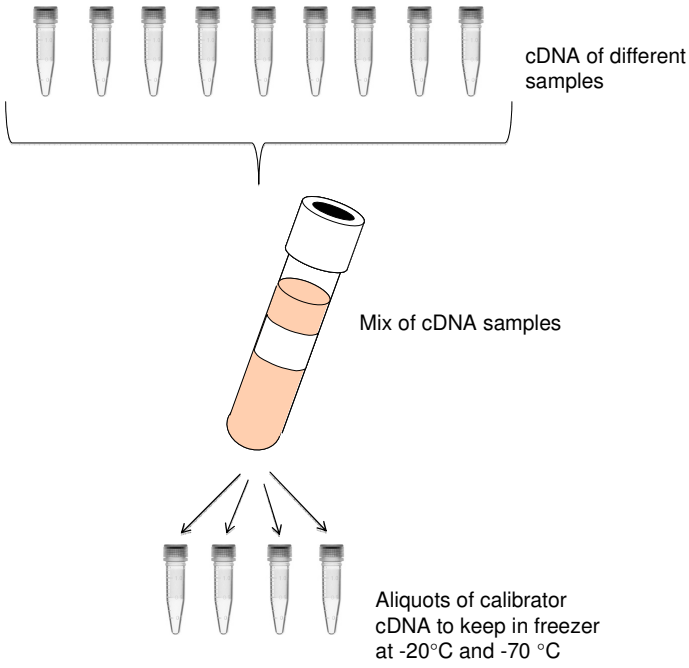


Figure 13: Preparation of cDNA calibrator. cDNA were pooled from samples of three healthy individuals to obtain average expression level of the samples and the pool was used as calibrator.

4.3.2 Real-time PCR

Real-time PCR was performed in 96-well plates on the LightCycler® 480 instrument. In addition to the samples, each PCR run included a calibrator sample (positive control) and at least one negative control sample. Sequences of target genes (*IL2*, *IFNG* and *CSF2)* and reference genes, 5-aminolevulinate synthase 1 (*ALAS1)*, *B2M* and ribosomal protein L13A (*RPL13A)* were amplified from the same cDNA pool in separated reactions. Each reaction was conducted in triplicate (initial experiments under optimization) or duplicate (MarkIt pilot study) to adjust for random variations. The median Cp values based on the two or three replicate analyses were used for further calculation of the relative quantification for each gene to be evaluated. The reaction mixture consisted of 5 µL cDNA (sample, calibrator or negative control), Probes Master 2X conc., gene specific primer pairs (0.5 µM of each for both target and reference genes) and hybridization probes (0.15 µM of each for target genes and 0.2 µM of each for the reference genes) to a final volume of 20 µL. The cDNA and master mixes of reagents were transferred into LightCycler 96-well PCR plates using the MagNA Pure LC™ instrument. After pipetting, the plate was covered with adhesive optical sealing foil, centrifuged briefly and then placed into LightCycler® 480 instrument.

The real-time PCR protocol on the LightCycler® 480 instrument consisted of the following programs (Figure 14):

Program 1: Denaturation	95°C for 10 min
Program 2: Amplification	95°C for 10 sec, 57°C for 10 sec and 72°C for 10 sec; 45 cycles
Program 3: Melt curve analysis	99°C for 5 min, 45°C for 60 sec, gradual increase up to 70°C
Program 4: Cooling	40°C for 30 sec

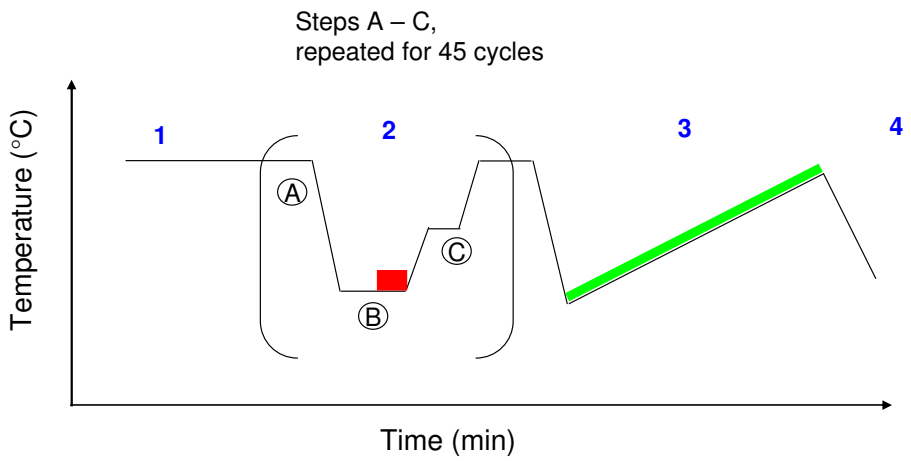


Figure 14: Programs on the LightCycler® 480 instrument. 1: denaturation, 2: amplification, 3: melting analysis and 4: cooling. The amplification programs consists of steps A – C, repeated for 45 cycles. A: denaturation, B: annealing, C: extention/elongation. Fluorescence is measured at the end of each annealing step (2B; red box) and continuously during melting in program 3 (green line), providing data for relative quantification and melting curve analysis.

4.4 qPCR development, optimization and validation

In this project, the qPCR development, optimization and validation focused on the amplification and detection of the target genes *IL2*, *IFNG* and *CSF2*. The quantification of the reference genes, *ALAS1*, *B2M* and *RPL13A,* has been optimized and validated in previous studies [106,107].

4.4.1 Primer and probes

The previously established reference gene real-time PCR protocol was utilized further as the assay was suitable for amplification of target genes (Section 4.3.2). This implies restrictions to the melting temperatures of primers and probes and the length of target gene amplicons. In

general, sequence regions with considerable homology with other oligonucleotide sequences or known sequence variations was avoided as binding sites for primers and probes. Primers and probes were designed using the tools listed in Table 4, as well as manual evaluation of sequences.

Table 4: Tools for primer and probe design

Tool	**Application**
GenBank www.ncbi.nlm.nih.gov/genbank	Target gene transcript sequences were retrieved from Genbank, which provides an annotated collection of all publicly available DNA sequences.
LightCycler® Probe Design Software 2.0	Suggests potential primer and probe sequences and allows analysis of oligonucleotide sequences.
OLIGO Primer Analysis Software version 7.22	Analysis of oligonucleotide sequences. Calculations of hybridization temperatures and potentially secondary structures between or within oligonucleotides (provides more detailed information than LightCycler® Probe design).
BLAST (Basic Local Alignment Search Tool)	Analysis of homology between sequences (transcript and genomic).
Alamut, dbSNP and SNPCheck	Checks if there are reported sequence variants in the binding sites of primers and probes.

The selected primer and probe sequences are listed in Table 5. Reference gene sequences are listed in Appendix A. The oligonucleotides were synthesized by TIB MOLBIOL. Lyophilized primers and probes were dissolved in Tris-EDTA buffer to a concentration of 20 µM and 6 µM, respectively, and divided into small aliquots to avoid repeated freezing and thawing. The primer and probe solutions were stored at –20°C.

Table 5: Primer and probe sequences

Gene	Name	Sequence
IL2	IL2_F1	AAACTTTCACTTAAGACCCAG
	IL2_R1	AGTGTTGAGATGATGCTTTG
	IL2_FL1	GGGATCTGAAACAACATTCATGTGTG-Fluorescein
	IL2_LC1	LC Red 640-TATGCTGATGAGACAGCAACCATTGTAGAATTT-Phosphate
IFNG	IFNG_F1	GCAGAGCCAAATTGTCTCCT
	IFNG_R1	ATGCTCTTCGACCTCGAAAC
	IFNG_FL1	AACTGTCGCCAGCAGCTA-Fluorescein
	IFNG_LC1	LC Red 640-ACAGGGAAGCGAAAAAGGAGTCAG-Phosphate
CSF2	CSF2_F1	TTGAAAGTTTCAAAGAGAACCT
	CSF2_R1	GATGACCATCCTGAGTTTCTA
	CSF2_FL1	GACTGCTGGGAGCCAGTC-Fluorescein
	CSF2_LC1	LC Red 640-GGAGTGAGACCGGCCAGATGAG-Phosphate

Abbreviations: F1; forward primer, R1: reverse primer, FL1; forward probe, LC1; reverse probe

4.4.2 Optimization of primer concentrations

The primer concentrations were titrated to optimize PCR-efficiency while maintaining specificity. For each primer set (forward and reverse primer) of target genes, concentrations of 0.25 µM, 0.50 µM, 0.75 µM and 1.0 µM were tested in different combinations. The different primer concentrations were evaluated based on Cp values and the shape of the amplification curves.

4.5 Assay validation

Assay validation focused on the target genes *IL2*, *IFNG* and *CSF2* as the reference genes have been established and validated in previous projects. The selectivity of the primers and probes was evaluated by SYBR Green I melting curve analysis, gel electrophoresis and DNA sequencing. Standard curves were constructed to determine the PCR-efficiency, dynamic

range, sensitivity and limit of quantification of each target gene. The precision of the method were evaluated within-runs and between runs.

4.5.1 SYBR Green I melting curve analysis

Target gene amplicons were studied by SYBR Green I melting curve analysis using the LightCycler® 480 SYBR Green I Master kit according to the manufacturer's instructions. Following 45 PCR cycles, the melting program started with a denaturation step at 95°C for 5 min and reduction to 38°C before a gradual increase from 38 up to 90°C (ramping rate of 2.2°C/sec) with continuous monitoring of fluorescence (Figure 14 Section 4.3.2). The resulting melting curves were evaluated using the Tm Calling Analysis tool of the LightCycler® 480 software. The analysis also displays the melting curves as melting peaks by plotting the first negative derivative of the fluorescence curves versus temperature. This allows easier visualization of the melting temperatures of different samples.

4.5.2 Gel electrophoresis

For each target gene, 5 µL of PCR product was mixed with 1 µL gel-loading buffer 6X with bromophenol blue and loaded into the wells of a 4% NuSieve 3:1 Plus Agarose Reliant® Gel with ethidium bromide. Furthermore, a molecular weight marker, no. V (Roche) and a negative control were included in parallel. The gel was run in tris-borate-EDTA buffer at 120 volts for approximately 1 hour. Finally, DNA was visualized in UV-light using the Biorad Molecular Imager GelDoc™ XR.

4.5.3 Sanger sequencing

The PCR products of the target genes were purified with ExoSAP-IT. Each reaction contained 1 µL PCR product, 2 µL water and 1.2 µL ExoSAP-IT and the mixture was incubated on a block cycler at 37°C for 15 min followed by 15 min at 80°C. The following sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit according to

the manufacturer's instructions. Briefly, 13.8 µL Big Dye Terminator master mix including ddNTPs was added to the purified PCR product (4.2 µL) followed by 2 µL of either forward or reverse primer. The tubes were sealed with caps and centrifuged briefly and placed back in the PCR block cycler. The temperature program of the sequencing reaction is summarized in Table 6.

Following sequencing reaction, the fluorescently labeled DNA fragments were separated by capillary electrophoresis on the ABI PRISM® 3100 Genetic Analyzer instrument at the Department of Medical Genetics, Oslo University Hospital. The sequencing procedure is summarized in Figure 15.

Table 6: Sequencing temperature program

Step	Temperature	Comments
1	1.0 °/sec to 96.0 °C	Gradual increase
2	96.0 °C	For 1 min
3	1.0 °/sec to 96.0 °C	
4	96.0 °C	For 10 sec
5	1.0 °/sec to 50.0 °C	Gradual decrease
6	50.0 °C	For 5 sec
7	1.0 °/sec to 60.0 °C	Gradual increase
8	60.0 °C	For 4 min
9	Step 3 – step 8	Steps 3 to 8 are repeated 24 times
10	1.0 °/sec to 4.0 °C	Gradual decrease
11	4.0 °C	Forever – End

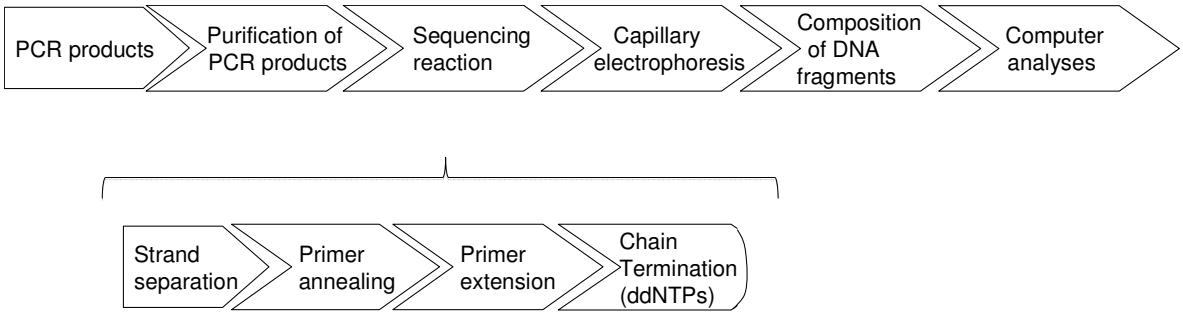


Figure 15: Overview of the sequencing workflow.

ddNTPs; dideoxynucleotides

4.5.4 Generation of standard curves

To generate standard curves, target gene sequences were initially amplified without the inclusion of probes, and PCR product concentrations were determined by measuring absorbance at 260 nm using the Nanodrop® ND-1000 Spectrophotometer. The *IL2, IFNG* and *CSF2* amplicons are 168, 290 and 154 nucleotides long, respectively, and the average molecular weight of one nucleotide is 330 g/mol. The numbers of cDNA templates were calculated according to the following equation: Number of templates/µL = (measured concentration g/µL x 6,022 x 10^{23} copies/mol) / (amplicon length in nucleotides x 330 g/mol). Target gene amplicons were serially diluted with water and MS2 RNA (10 µg/mL) to obtain dilution series ranging from 10^7 to 10^1 templates per reaction. The dilution series were amplified by real-time PCR, including four – six parallel reactions per dilution step and standard curves were constructed for each target gene by plotting the logarithm of initial copy numbers versus the corresponding Cp values (Figure 16). The average PCR-efficiency values were determined by three separate standard curves of each gene and calculated according to Equation 2.

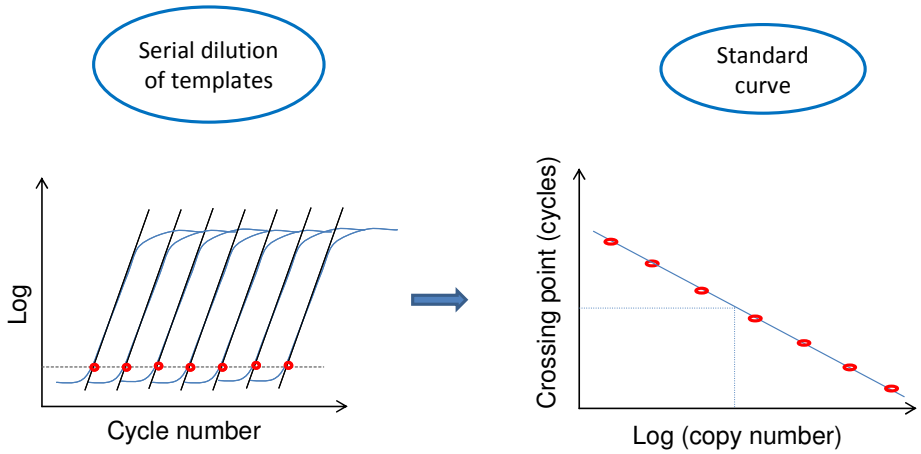


Figure 16: Construction of standard curve. Serially diluted PCR products from 10^7 to 10^1 templates/reaction were amplified and standard curves were constructed by plotting the logarithm of initial copy numbers versus the corresponding crossing point values.

$$\text{PCR-efficiency} = 10^{-1/\text{slope}}$$

Equation 2: Calculation of PCR-efficiency.

4.5.5 Assay precision

The assay precision was determined by calculating the coefficient of variation (CV) of relative concentrations and normalized ratios of gene expression. The within-run precision was calculated for the entire dynamic range based on the replicates that generated the standard curves. Furthermore, within-run precision was determined based on the replicates of actual samples with a range of expression levels. The between-run CV was calculated based on analysis of the same calibrator sample on 8 different days.

4.6 Quantification of relative gene expression

Target nucleic acids can be quantified by PCR by absolute or relative quantification strategies. The expression of the *IL2*, *IFNG* and *CSF2* target genes was determined by relative quantification, using reference genes for normalization. Furthermore, the assay included calibrator normalization and correction for differences in PCR-efficiency as illustrated in Figure 17.

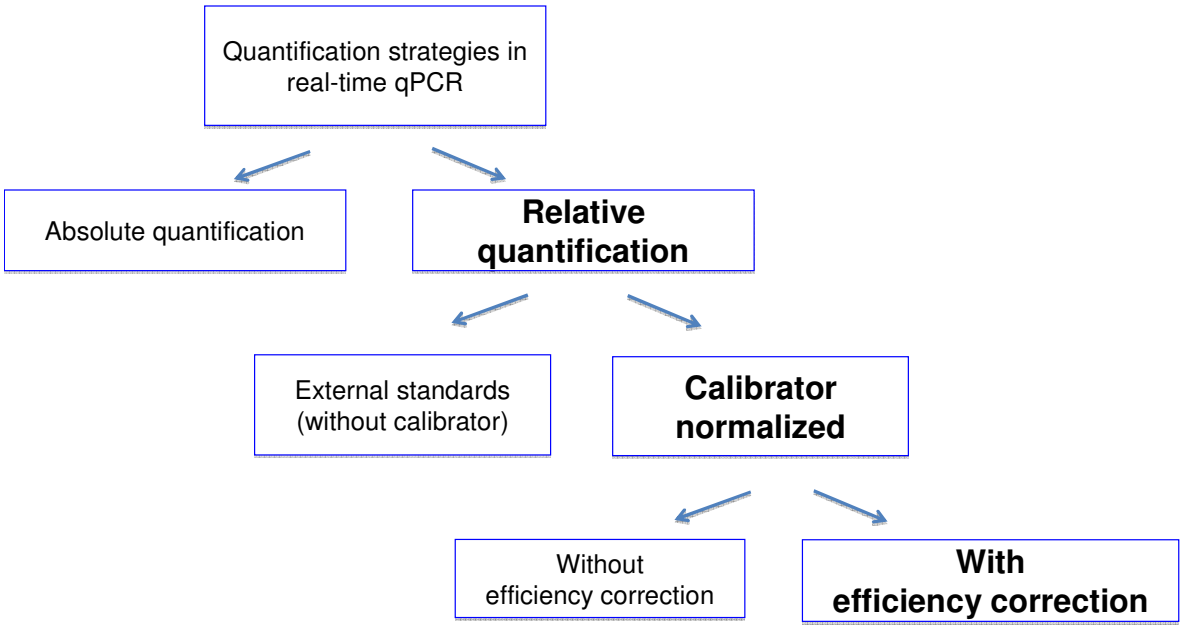


Figure 17: Quantification of target nucleic acids using either absolute or relative quantification. The relative quantification strategy of this project is highlighted in bold.

qPCR; quantitative PCR

4.6.1 Calculation of relative gene expression

The relative gene expression was determined using the LightCycler® 480 Relative Quantification Software Version 1.5. Relative concentrations of each target and reference gene were calculated based on Cp values and the gene specific average PCR-efficiency,

derived from the three standard curves constructed (Section 4.5.4). The relative concentrations of each target gene were then normalized to a geometric mean of the three reference genes (reference gene index) as given in Equation 3. Moreover, the target/reference ratio of each sample divided by the target/reference of a calibrator sample (Equation 4) provides a constant calibration point within and between runs.

$$\text{Relative expression} = \frac{[\text{IL2}] \text{ or } [\text{IFNG}] \text{ or } [\text{CSF2}]}{([\text{ALAS1}] \times [\text{B2M}] \times [\text{RP-L13A}])^{1/3}}$$

Equation 3: Calculation of relative expression. The expression of each target gene is normalized to the geometric mean expression of three reference genes**.**

ALAS1; 5-aminolevulinate synthase 1, *B2M*; Beta-2-microglobulin, *CSF2*; colony stimulating factor 2 (granulocyte-macrophage), *IFNG*; interferon gamma, *IL2*; interleukin 2, *RPL13A*; ribosomal protein L13A

$$\text{Calibrator normalized relative expression} = \frac{\text{Relative expression}_{\text{Sample}}}{\text{Relative expression}_{\text{Calibrator}}}$$

Equation 4: Relative expression with calibrator normalization. The target/reference ratio of each sample was divided by the target/reference ratio of the calibrator.

4.7 *Ex vivo* exposure to immunosuppressive drugs

The validated assay, including *ex vivo* stimulation of whole blood, RT and real-time PCR, was used to investigate the potential effect of different immunosuppressive drugs on NFAT-regulated gene expression.

CsA, Tac, mycophenolic acid and prednisolone were dissolved in methanol and added to whole blood to achieve a range of pharmacologically relevant blood concentrations. The final drug concentrations are given in Table 7.

Drug free whole blood (150 µL) from healthy volunteers was spiked with 2 µL drug solution. Immune activation of the whole blood samples was performed by the addition of 150 µL PMA and ionomycin for up to 72 hours at 37°C. Potential effects of CsA and Tac were investigated in samples from two different individuals, while mycophenolic acid and prednisolone only were added to samples from one individual. All experiments included controls without both drug and solvent and controls without drug but with methanol alone.

Further preparations were carried out in the similar way as with the other blood samples as presented in Section 4.1 – 4.3 and Figure 19 in Section 5.1.2.

Table 7: *Ex vivo* drug exposure

	Drugs	Final concentration in medium
CNIs	Tac	0.1 nmol/L
		0.5 nmol/L
		1.0 nmol/L
		10 nmol/L
		100 nmol/L
	CsA	10 nmol/L
		100 nmol/L
		1 000 nmol/L
		10 000 nmol/L
Other drugs	Mycophenolic acid	10 µmol/L
	Prednisolone	1 000 µmol/L

Abbreviations: CNIs; calcineurin inhibitors, CsA; cyclosporine A, Tac; tacrolimus

4.8 Monitoring of NFAT-regulated gene expression in Tac treated renal transplant recipients

Before initiation of the MarkIt pilot study, the applicability of the RT-qPCR assay was tested by measuring NFAT-regulated gene expression in pre dose and 1.5 hours post dose samples from a single renal transplant patient treated with Tac (the patient received other immunosuppressive drugs additionally to Tac).

4.8.1 MarkIt pilot study

Finally, the validated NFAT-regulated gene expression assay was used to investigate the PD response of Tac among 5 renal transplant recipients included in the MarkIt pilot study. Further details of the study are provided in Section 3.2.

Blood samples from patients were processed as described in Section 4.1 – 4.3 and the relative gene expression was calculated as described in Equations 3 and 4 Section 4.6.1.

Furthermore, the residual gene expression 1.5 hours after oral administration of Tac was calculated by normalizing the expression 1.5 hours post dose ($E_{1.5}$) to the expression pre dose (E_0) for each target gene, as presented in Equation 5. Finally, the NFAT-regulated gene response was determined based on the geometric mean of *IL2, IFNG* and *CSF2* residual expressions.

$$\text{Residual gene expression} = \frac{E_{1.5}}{E_0} \times 100\%$$

Equation 5: Calculation of residual gene expression. E_0 and $E_{1.5}$ represent the relative expression of target genes before and 1.5 hours after Tac dosing, respectively.

In addition to the measurements of NFAT-regulated gene expression, other projects of the MarkIt study include analyses like genotyping, metabolic activity, IMPDH activity, level of purine base and cytokine secretion.

Tac concentrations were measured in whole blood using chemoluminescent microparticle immunoassay (CMIA) on the Architect® platform (Abbott Laboratories, Abbott Park, IL, USA).

CYP3A5-genotyping was performed with DNA extraction from EDTA anti-coagulated whole blood using the MagNA Pure LC instrument and MagNA Pure LC DNA Isolation Kit I

according to the manufacturer's instructions. CYP3A5-genotyping (rs776746; NG_007938.1:g.12083G>A, A = CYP3A5*1 and G = CYP3A5*3) was performed by PCR and melt curve analysis with specific primers and hybridization probes on the LightCycler® 480 instrument. Primers were designed using LightCycler Probe Design software version 2 and hybridization probe sequences were derived from Cheung et al [108].

Demographic and clinical data of the participants in the study were collected and evaluated closely during routine examinations.

5 Results

5.1 Assay development and validation

5.1.1 Lymphocyte activation and sample preparation

The three different principles for lymphocyte activation (1: PHA, 2: PMA + ionomycin, 3: PHA + PMA + ionomycin) resulted in considerable increases in expression levels of the NFAT-regulated genes *IL2*, *IFNG* and *CSF2*. The highest expression levels were observed after 3 hours stimulation with PMA and ionomycin and the combination of PHA, PMA and ionomycin (Figure 18). Without immune activation, the expression of *IL2*, *IFNG* and *CSF2* was very low (Cp values of target genes > 35 versus reference genes 20 – 29). When whole blood was activated with PMA and ionomycin for 3 hours, the expression increased 120 000, 20 000 and 8 000 fold for *IL2*, *IFNG* and *CSF2*, respectively, compared to unstimulated cells. The up-regulation of *IL2*, *IFNG* and *CSF2* gene expression was less pronounced (100, 300 and 50 fold, respectively) after activation with PHA (Figure 18). Whole blood incubation in 48 and 72 hours was associated with pronounced hemolysis, probably caused by RNA degradation, and a considerable increase in the Cp values of both target and reference genes (median 3 cycles). Based on the results of these initial experiments, incubation of whole blood with PMA and ionomycin for 3 hours at 37°C was selected for *ex vivo* lymphocyte activation in the further studies (Figure 19 Section 5.1.2).

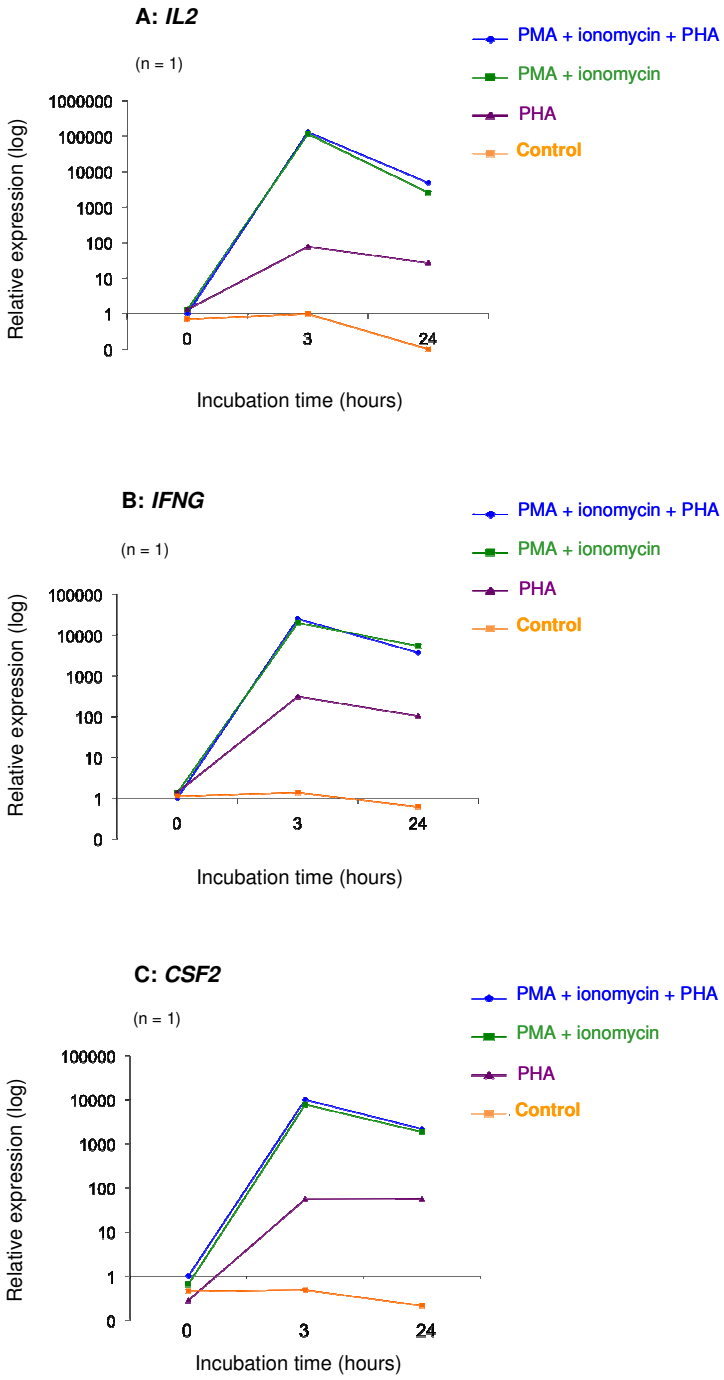


Figure 18: Gene expression of A: interleukin 2 (*IL2*), B: interferon gamma (*IFNG*) and C: colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*). *Ex vivo* activation of lymphocytes in whole blood by 0, 3 and 24 hours incubation with phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA) in combination with ionomycin or all three agents together.

5.1.2 Cell lysis procedure

Direct cell lysis by adding LBB to whole blood was easier to perform and less time-consuming compared to a two-step procedure starting with lysis and removal of erythrocytes before lysis of leukocytes. In addition, the one-step procedure resulted in generally lower Cp values (median 5 cycles for target and reference genes), which indicates better RNA yields compared to the two-step cell-lysis procedure. Thus, the one-step cell lysis procedure was chosen for further analyses (Figure 19).

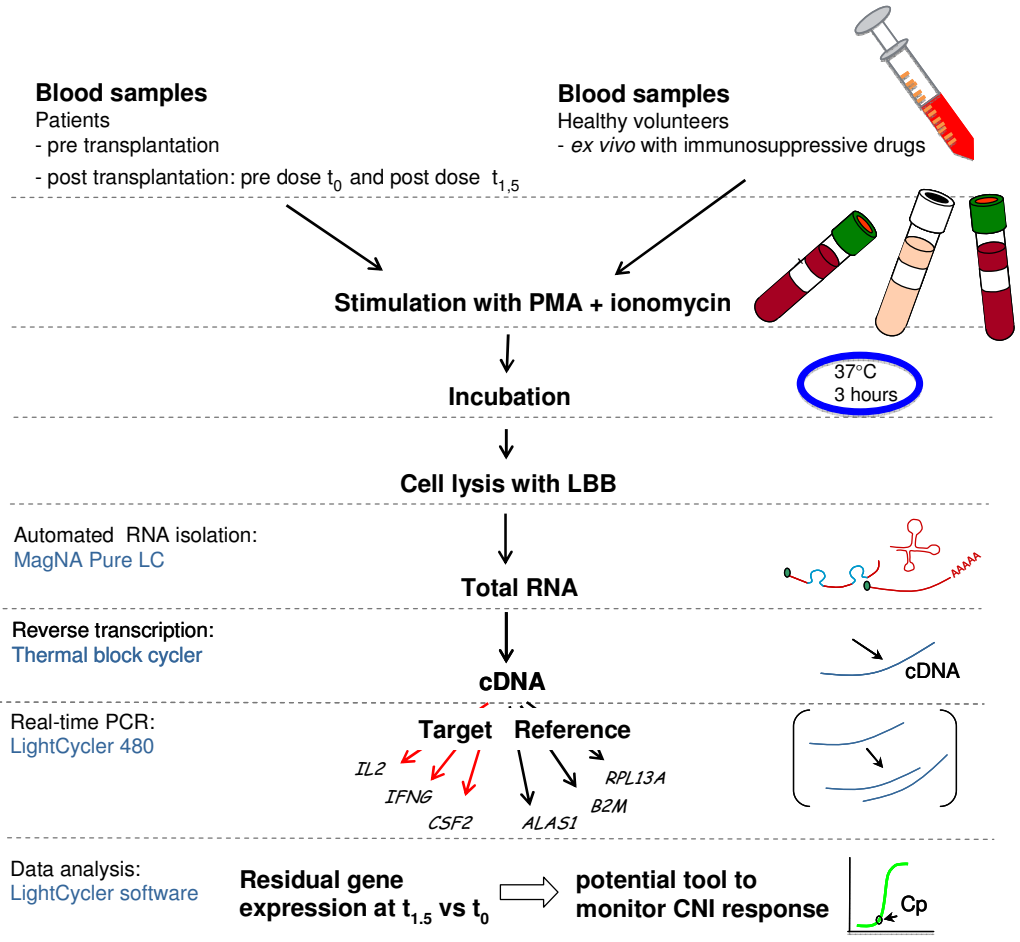


Figure 19: Assay workflow for the quantification of nuclear factor of activated T cells (NFAT)-regulated genes in whole blood samples.

ALAS1; 5-aminolevulinate synthase 1, *B2M*; beta-2-microglobulin, *CSF2*; colony stimulating factor 2 (granulocyte-macrophage), CNI; calcineurin inhibitor, Cp; crossing point, *IFNG*; interferon gamma, *IL2*; interleukin 2, LBB; Lysis/Binding Buffer, PMA; phorbol 12-myristate 13-acetate, *RPL13A*; ribosomal protein L13A

5.1.3 Primer titration

Reaction mixture containing 0.5 µM forward and reverse primer and 0.15 µM of each probe resulted in efficient amplification of *IL2*, *IFNG* and *CSF2* templates (PCR-efficiencies ranging from 1.95 to 1.97). The PCR-efficiency was further improved by primer titration. Forward and reverse primer concentrations of 0.5 µM + 0.75 µM, 0.5 µM + 0.75µM and 0.75 µM + 0.75 µM for *IL2*, *IFNG* and *CSF*2, respectively, resulted in approximately 0.3 cycle lower Cp values indicating further improvement in PCR-efficiencies (optimized PCR reagent mixtures are presented in Appendix B). Melt curve analysis with SYBR Green I confirmed the formation of one specific target gene product with primer concentrations ranging 0.5 – 0.75 µM. The final composition of the target gene PCR reagents used in this project is summarized in Table 8.

Table 8: PCR reagents for the amplification of target genes

Master mix IL2		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	3.0 µL	
Primer: IL2_F1 (20 uM)	0.5 µL	0.5 µM
Primer: IL2_R1 (20 uM)	0.5 µL	0.5 µM
Probe: IL2_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: IL2_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	

Master mix IFNG		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	3.0 µL	
Primer: IFNG_F1 (20 uM)	0.5 µL	0.5 µM
Primer: IFNG_R1 (20 uM)	0.5 µL	0.5 µM
Probe: IFNG_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: IFNG_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	

Master mix CSF2		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	3.0 µL	
Primer: CSF2_F1 (20 uM)	0.5 µL	0.5 µM
Primer: CSF2_R1 (20 uM)	0.5 µL	0.5 µM
Probe: CSF2_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: CSF2_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	

5.1.4 Primer selectivity

Melting curve analysis with SYBR Green I demonstrated single, symmetrical peaks at the expected melting temperature, approximately 80°C, 87°C and 89°C for *IL2*, *IFNG* and *CSF2* amplicons, respectively (Figure 20). The corresponding calculated melting temperature values reported by OLIGO were 80 – 84°C, 83 – 87°C and 86 – 90°C for *IL2*, *IFNG* and *CSF2*. Gel electrophoresis demonstrated single PCR products for *IL2*, *IFNG* and *CSF2* at the expected sizes of 168, 290 and 154 base pairs (Figure 21). Furthermore, Sanger sequencing confirmed amplification of the expected target gene transcript sequences (Figure 22). Altogether, these results confirmed the integrity of RNA as well as selective amplification of the expected target gene sequences.

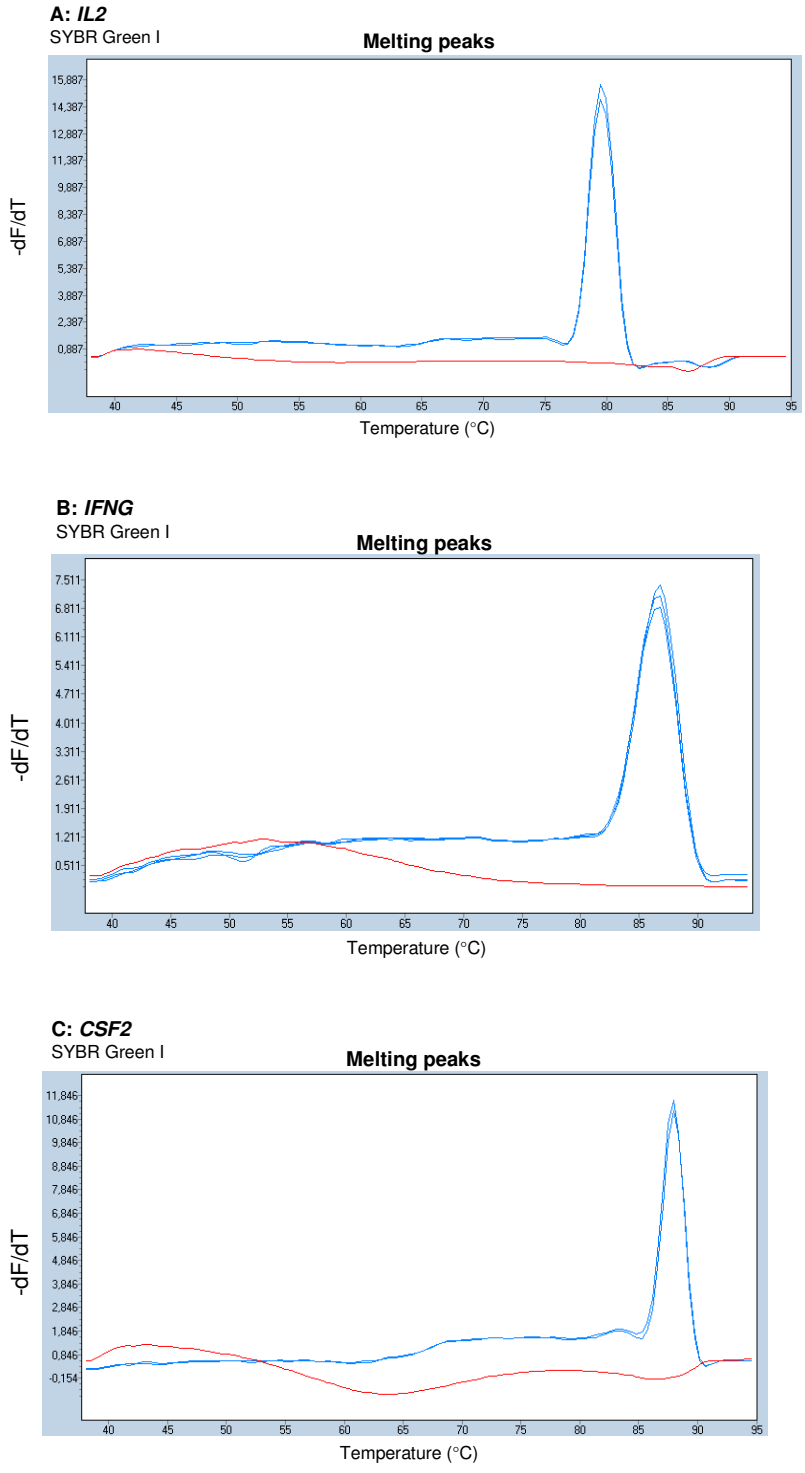


Figure 20: SYBR Green I melting curve analysis of A: interleukin 2 (*IL2*), B: interferon gamma (*IFNG*) and C: colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*) amplicons (blue lines). The red line represents the negative control. The negative first derivate of the change in fluorescence plotted as a function of temperature.

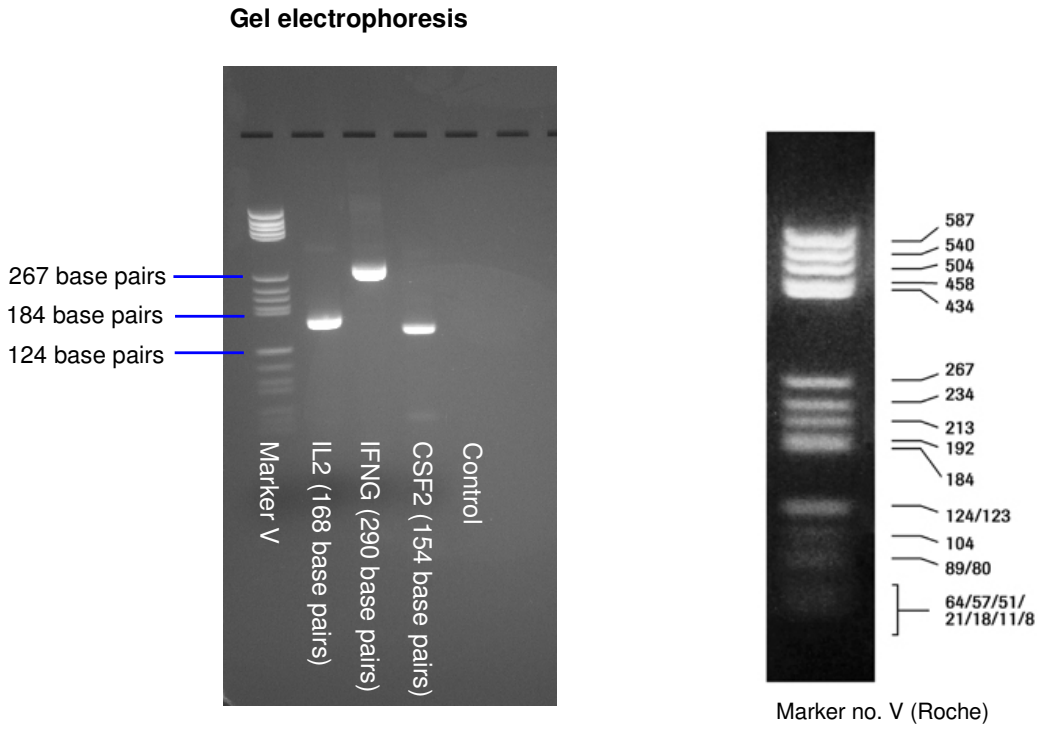
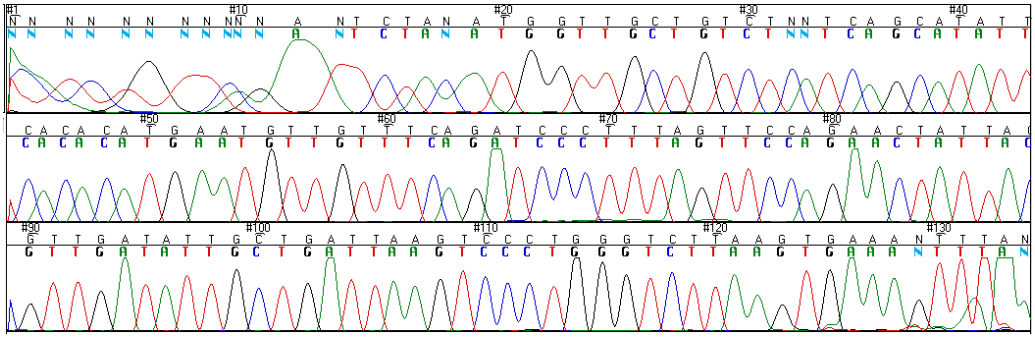
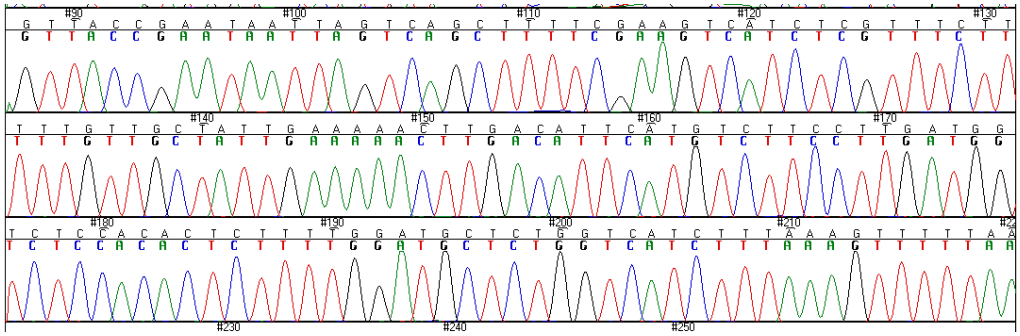


Figure 21: **Gel electrophoresis of interleukin 2 (*IL2*), interferon gamma (*IFNG*) and colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*) amplicons**. The gel includes a molecular weight marker, no. V (Roche) and a negative control sample.

A: *IL2*
Reverse primer



B: *IFNG*
Reverse primer



C: *CSF2*
Reverse primer

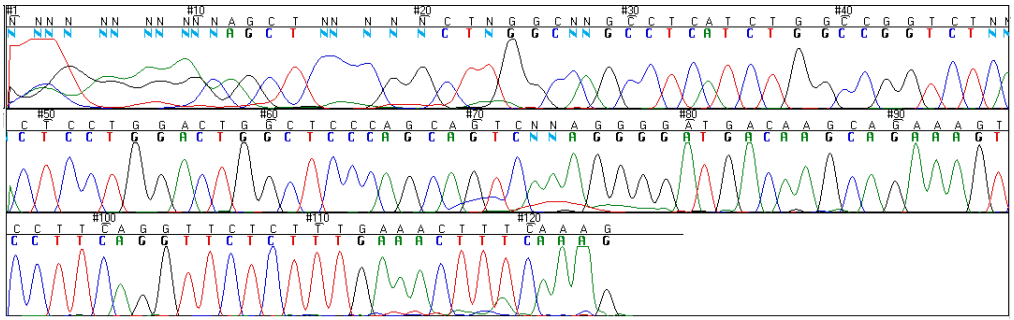


Figure 22: Sequencing of A: interleukin 2 (*IL2*), B: interferon gamma (*IFNG*) and C: colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*) PCR products (reverse primer). The sequencing results (forward and reverse primers) demonstrated amplification of the expected target sequences. Each terminal base is represented by a different colour for sequence reconstruction.

5.1.5 PCR-efficiency and linear range

Real-time PCR of *IL2*, *IFNG* and *CSF2* sequences from serially diluted cDNA template generated amplification plots as illustrated in Figure 23. Based on replicates of the standard curves, within-run CVs were ≤ 13.6 % for the target gene reactions starting with 10^7 to 10^4 templates per reaction. Furthermore, within-run CVs based on replicates of actual samples (Cp values of 31 to 36) were ≤ 13.9%, 19.8% and 16.4% for *IL2*, *IFNG* and *CSF2* reactions, respectively, starting with 10^7 to 10^4 templates per reaction. The between-run CV of target gene relative expression levels was ≤ 14.8% (Cp values below 33). Starting concentrations of 10 templates per reaction (5 double stranded molecules) did not consistently result in amplification curves, suggesting a lower limit of detection of 10^2 templates per reaction. Based on Equation 2 (Section 4.5.4), mean PCR-efficiencies of *IL2*, *IFNG* and *CSF2* were calculated to 1.96, 1.95 and 1.97, respectively. Furthermore, the standard curves were linear over a concentration range from 10^7 – 10^2 templates per reaction (Figure 24). The mean squared error of the single data points fit to the regression line of the standard curves were below 0.02 (an acceptable value should be < 0.2 according to the LightCycler Operator's manual), confirming the precision of the pipetting and real-time PCR.

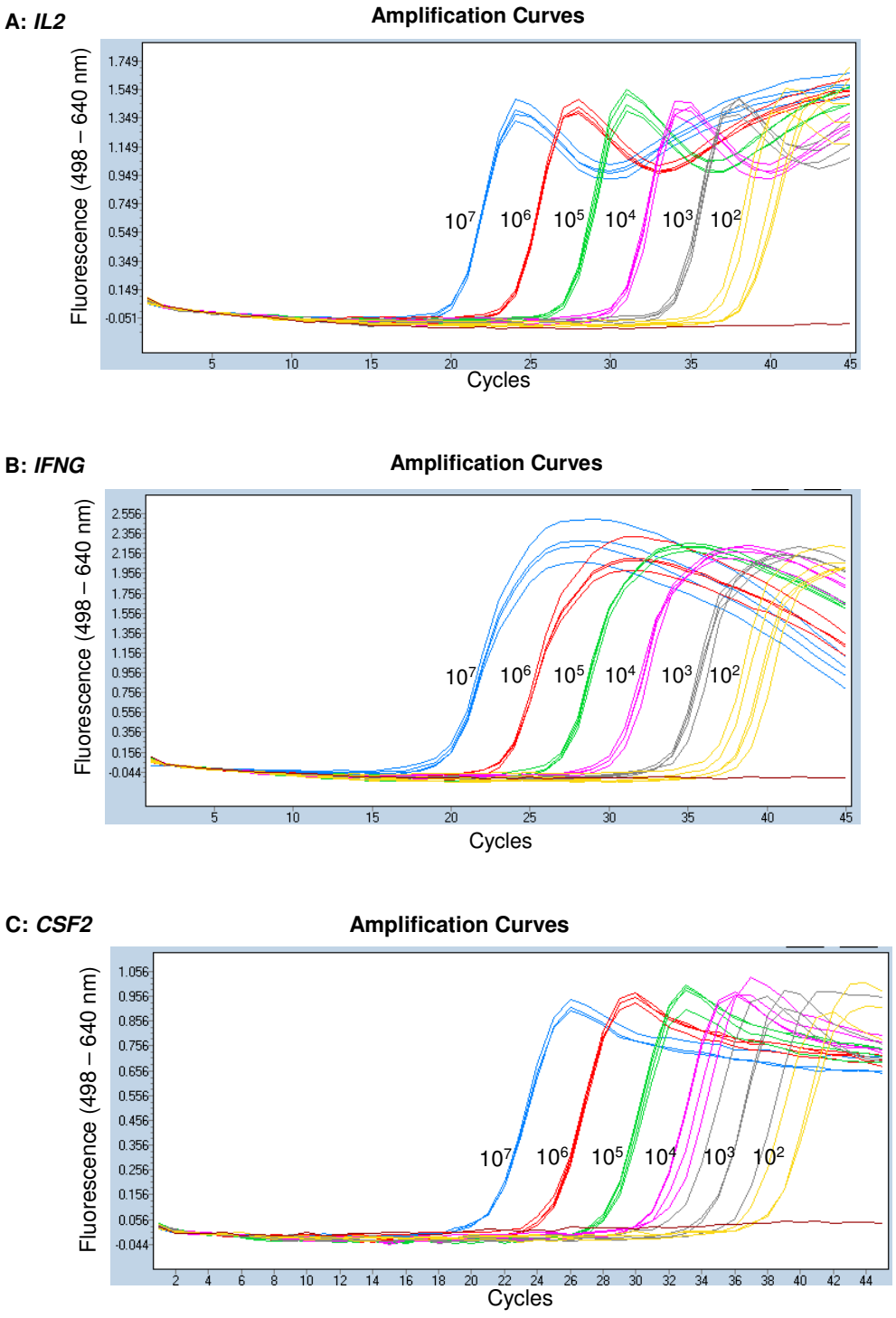
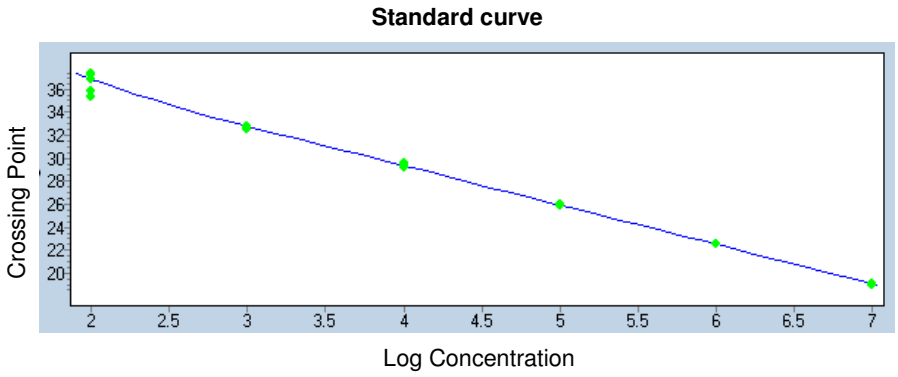


Figure 23: Amplification plots of serially diluted cDNA ranging from 10^7 – 10^2 templates/reaction for A: interleukin 2 (*IL2*), B: interferon gamma (*IFNG*) and C: colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*).

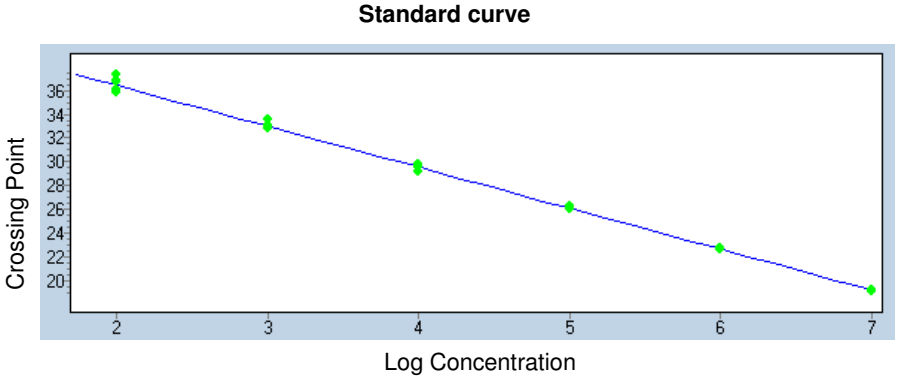
A: *IL2*

Error: 0.00719
Efficiency:1.960
Slope: -3.422
Y-Intercept: 43.59



B: *IFNG*

Error: 0.0158
Efficiency: 1.949
Slope: -3.450
Y-Intercept: 43.57



C: *CSF2*

Error: 0.0235
Efficiency: 2.002
Slope: -3.317
Y-Intercept: 43.91

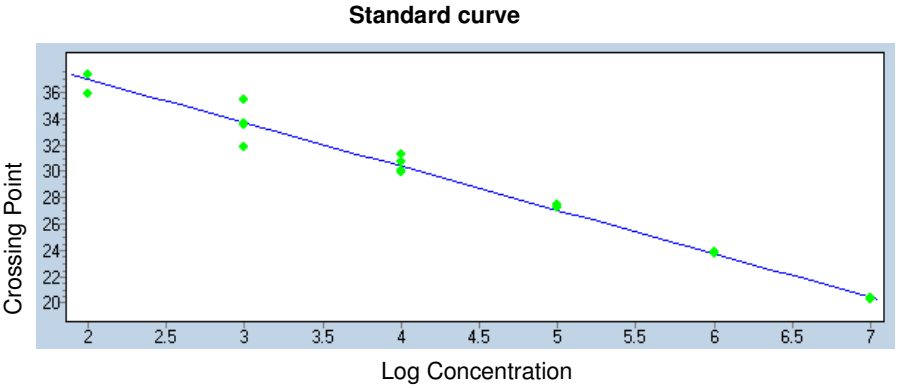


Figure 24: Example of standard curves generated from the amplification plots of serially diluted (6 fold) cDNA for A: interleukin 2 (*IL2*), B: interferon gamma (*IFNG*) and C: colony stimulating factor 2 (granulocyte-macrophage) (*CSF2*). Crossing point values were plotted against log cDNA concentrations.

5.2 Effect of immunosuppressive drugs on NFAT-regulated gene expression

Because the investigated drugs were dissolved in methanol, the potential drug effects were determined in relation to control samples with methanol only. Following exposure to CsA 1000 nmol/L (1203 µg/L) for 3 hours, the NFAT-regulated gene expression (combined response of *IL2*, *IFNG* and *CSF2*) was reduced by 90% (Figure 25). A similar response was observed for Tac, resulting in 80 – 90% and 97% inhibition of expression after exposure to Tac 50 nmol/L (41 µg/L) and Tac 100 nmol/L (82 µg/L), respectively (n = 2, Figure 26). Lower CNI concentrations (Tac 10 nmol/L and CsA 100 nmol/L) were associated with less inhibition (0 – 50%) and higher expression levels, suggesting an inverse relation between NFAT-regulated gene expression and CNI concentrations. Prednisolone 1000 nmol/L (360 mg/L) tended to slightly reduce the *IL2*, *IFNG* and *CSF2* expression levels, while mycophenolic acid 10 µmol/L (3 mg/L) did not have any clear impact on expression of NFAT-regulated genes (Figure 25). The responses after drug exposure differed between the individual cytokines, and CsA and Tac appeared to have the strongest impact on *IL2* and *CSF2* expression levels (Figure 25 and 27).

Comparison of expression levels in control samples with and without methanol demonstrated that methanol alone (final concentration of 2%, v/w) reduced NFAT-regulated gene expression levels by approximately 30% (data not shown). This implies that the results of the *ex vivo* drug exposure experiments are not directly transferable to drug response *in vivo*. Furthermore, the expression observed after 3 hours *ex vivo* incubation with drugs in a mixture of whole blood and activation medium may not necessarily reflects the response *in vivo* (*e.g.* differences in drug binding and exposure). Because of this, the following experiments involved samples from patients with *in vivo* exposure to CNIs.

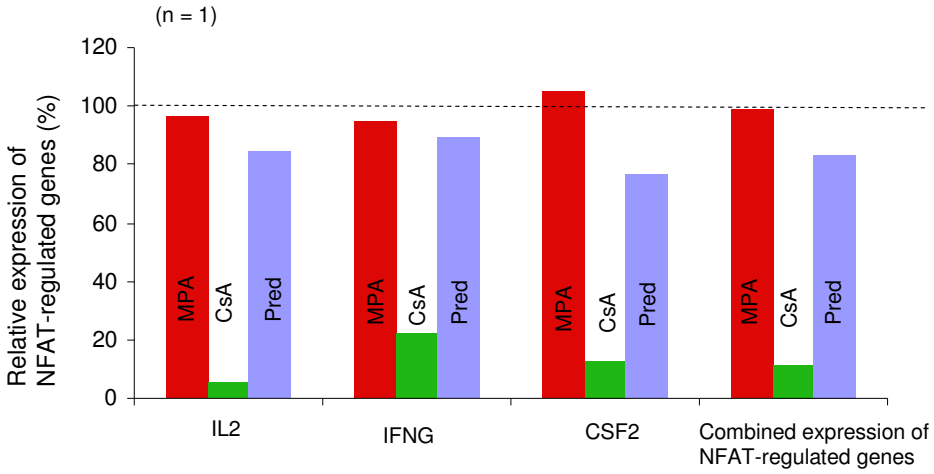


Figure 25: Relative expression nuclear factor of activated T cells (NFAT)-regulated genes. Ex vivo activation in 3 hours with addition of phorbol 12-myristate 13-acetate and ionomycin. Effects of mycophenolic acid (MPA) 10 µmol/L, cyclosporine (CsA) 1000 nmol/L and prednisolone (Pred) 1000 µmol/L. The results were normalized to control samples where the CNI/methanol solution was replaced by methanol only.

CSF2; colony stimulating factor 2 (granulocyte-macrophage), *IFNG*; interferon gamma, *IL2*; interleukin 2

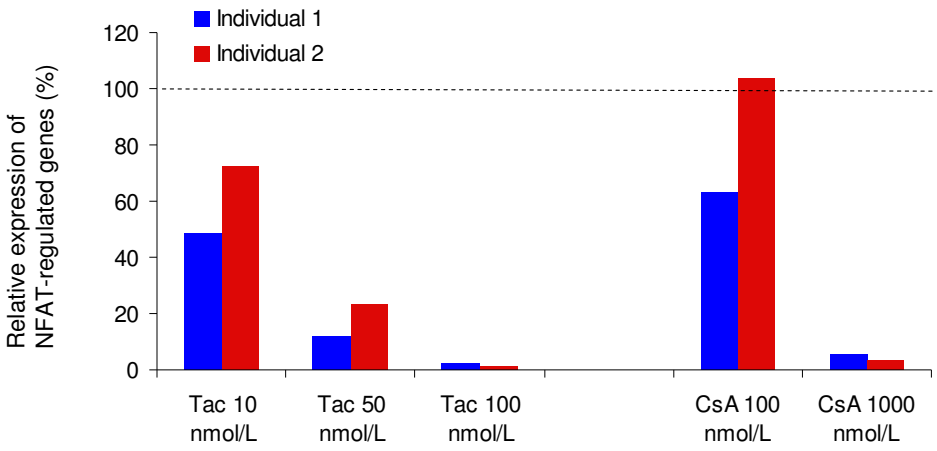


Figure 26: Relative expression nuclear factor of activated T cells (NFAT)-regulated genes. Quantitative analysis of the total NFAT-regulated gene expression after *ex vivo* exposure to different concentrations of tacrolimus (Tac) and cyclosporine (CsA). The results were normalized to control samples were the CNI/methanol solution was replaced by methanol only.

5.3 Expression of NFAT-regulated genes in Tac treated renal transplant recipients

Before starting the enrollment of patients in the pilot study, the utility of the assay was confirmed by measurement of NFAT-regulated gene expression in blood samples from a single renal transplant recipient treated with Tac. In this patient the residual expression of the combined NFAT-regulated genes (expression 1.5 hours post dose versus pre dose) was 14% as shown in Figure 27. The pre dose and 1.5 hours post dose concentrations of Tac were 5.1 µg/L and 15.9 µg/L, respectively.

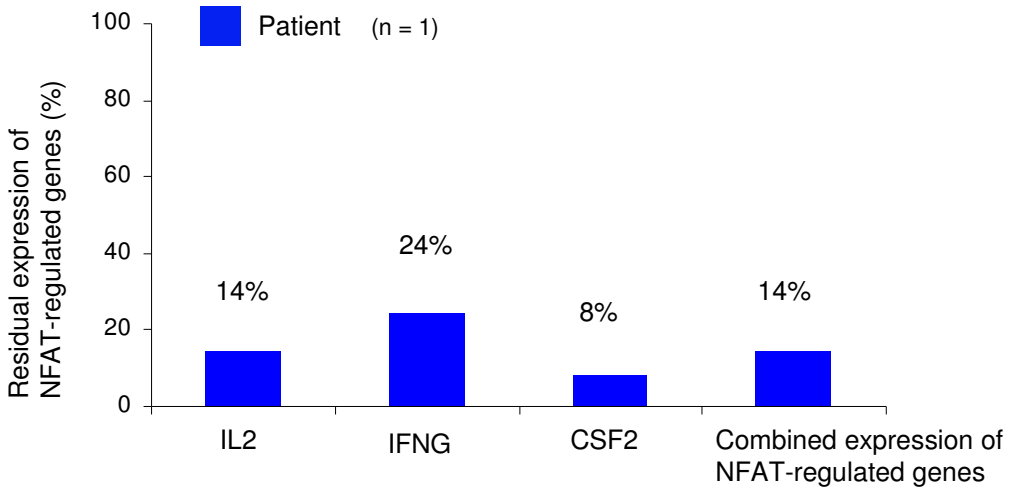


Figure 27: Residual expression of nuclear factor of activated T cells (NFAT)-regulated genes. The expression measured in a transplant patient before and 1.5 hours after administration of tacrolimus. See Section 4.6.1 for calculation.

CSF2; colony stimulating factor 2 (granulocyte-macrophage), *IFNG*; interferon gamma, *IL2*; interleukin 2

5.3.1 MarkIt pilot study

During the time of the present master project, five participants receiving their first graft were included in the pilot study. Demographic and clinical data are summarized in Table 9. The limited number of patients included in the study so far did not permit statically testing and the results are presented as individual data or median and range of values.

There was a large variability (approximately 5 fold) in NFAT-regulated gene expression before Tx between the five participants of the pilot study. Approximately one week post Tx, the gene expression (pre dose) was considerably reduced among three patients and relatively stable among the other two patients, compared to pre Tx levels (Figure 28).

Following Tac (Prograf) dosing, the expression of the NFAT-regulated genes was rapidly down-regulated and the response differed between patients. Four patients demonstrated residual gene expression levels below 13%, while one patient presented a residual expression of 27% at 1.5 hours after Tac dosing (Figure 29).

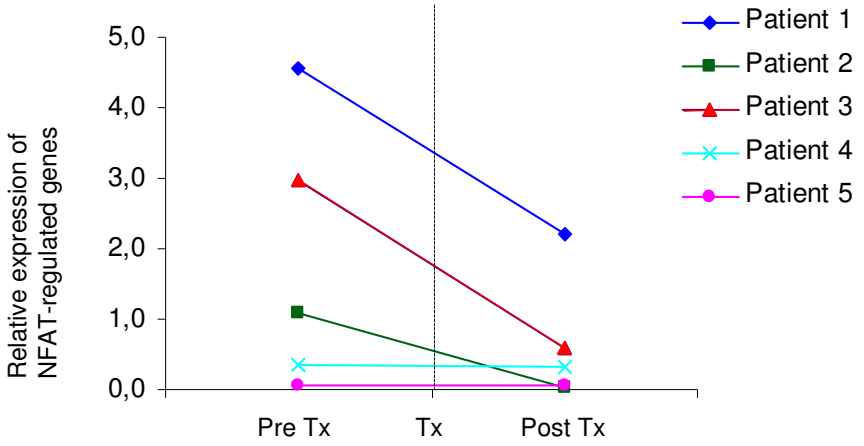


Figure 28: Relative expression of nuclear factor of activated T cells (NFAT)-regulated genes. The expression measured in 0 – 4 days pre transplantation (Tx) and pre dose at approximately one week post Tx following the initiation of standard tacrolimus therapy.

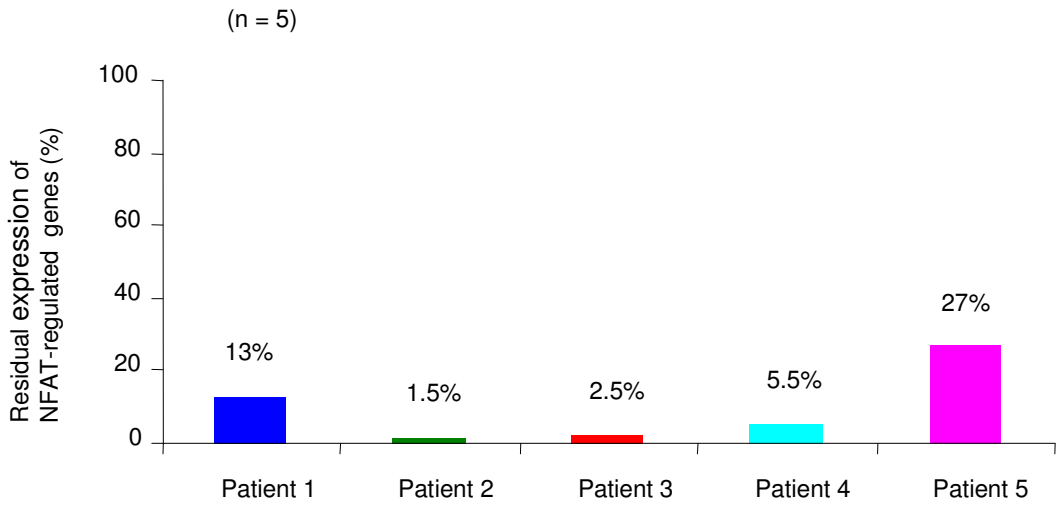


Figure 29: Residual expression of nuclear factor of activated T cells (NFAT)-regulated genes 1.5 hours after tacrolimus dosing. Five renal transplant recipients approximately one week after transplantation.

Tac treatment was initiated on the day of Tx, starting with fixed doses of approximately 0.04 mg/kg twice a day, followed by dose adjustments to obtain pre dose concentrations within the therapeutic range (< 6 months post Tx: 3 – 7 µg/L and 3 – 5 µg/L after 6 months) according to the Tx protocol at the time of the study [24]. The pre dose and 1.5 hours post dose Tac concentrations of the patients approximately one week post Tx are listed in Table 10.

Table 9: Demographic characteristics and clinical data in the MarkIt pilot study

	Patient (n = 5)				
	901	**902**	**903**	**904**	**905**
Sex (M/F)	M	M	F	M	F
Age (year)	57	37	67	20	54
Bodyweight (kg)	97	96.5	74	76	58.9
Dialysis pre transplant (yes/no)	no	no	no	yes	yes
CYP3A5	*3/*3	*3/*3	*1/*3	*3/*3	*3/*3
HLA - mismatch (DR)	4 - 2	3 - 1	1 - 1	1 - 1	0 - 0
Donor specific antibody	–	–	–	Anti-DQ7 Crossmatch negative	–
Immunosuppression post transplant	Standard regimen	Standard regimen	Standard regimen	DSA protocol [A]	HLA protocol [B]
Complications first weeks post transplant	–	4 days: Increase of creatinine [C] and rejection [D]	72 days: Polyomavirus (BK) positive	–	3 days: Ureteral leak Reoperation

– Standard regimen: Mycophenolat mofetil (MMF), Tacrolimus (Tac: Prograf, Advagraf), Prednisolon (Pred) and Basiliximab

[A]: DSA protocol: Standard regimen + Rituximab 375 mg 2 – 4 weeks pre Tx + MabThera (one dose) and Kiovig for 5 subsequent days post Tx

[B]: HLA Protocol: Standard regimen without MMF

[C]: Increase of serum creatinine from 189 – 250 µmol/L within one day.

[D]: Biopsy-proven acute rejection: both cellular and humoral. Reversed following Solu-Medrol (500 mg daily) for 4 subsequent days post Tx.

Abbreviations: F; female, HLA; human leukocyte antigen, M; male

---

Table 10: Pre and 1.5 hours post dose concentrations and doses of Tac approximately one week after Tx

	Tac C_0 (µg/L)	Tac $C_{1.5}$ (µg/L)	Dose (mg/kg)
Patient 1	6.4	15.0	0.03
Patient 2	5.3	22.9	0.05
Patient 3	5.2	16.4	0.05
Patient 4	10.6	>30.0	0.08
Patient 5	4.6	6.3	0.04

Abbreviation: Tac; tacrolimus

6 Discussion

The introduction of CNIs has contributed to reduce the incidence of acute rejection episodes to as low as 10% the first year after Tx and CNIs are now included in most immunosuppressive regimens after organ Tx. However, despite the efficacy of CNIs, their use is restricted by severe side effects such as nephrotoxicity, neurotoxicity, hypertension, diabetes and gastrointestinal disorders [109,110]. The long-term outcomes after Tx have not improved in parallel with short-term outcomes [79], and this may, at least partially, be explained by the negative impact of CNI toxicity on long-term outcomes.

The risks and benefits of low-dose CNI or elimination of CNI after Tx have been examined in several studies. More *et al.* showed that the use of mycophenolate mofetil (prodrug of mycophenolic acid) in CNI sparing regimens was associated with improved short-term renal function and possibly improved graft survival on long-term outcomes [111]. In the BENEFIT (Belatacept Evaluation of Nephroprotection and Efficacy as First-Line Immunosuppression Trial) study, 686 patients were randomized to receive a more intensive regimen of belatacept (Nulojix), a less intensive regimen of belatacept or CsA. Patients receiving either a more or less intense regimen of belatacept experienced better renal function and overall patient/graft survival but had higher incidence and grade of acute rejection compared to CsA treated patients [112].

Both CsA and Tac have a narrow therapeutic window and a close monitoring of these drugs is required to avoid the risk of over- or underimmunosuppression in the individual patient. Adequate immunosuppression is especially important during the first days post Tx. The results of monitoring based on C_0 are suboptimal and full $AUC_{0\text{-}12h}$ and abbreviated AUCs (0 – 6 hours or 0 – 4 hours) have been reported to better correlate with clinical outcomes. However, AUC monitoring in each patient is not feasible in clinical routine practice. Several studies have proposed that C_{max} levels of CsA and Tac, measured 2 and 1.5 hours post dose, respectively, better predict the clinical response of the drugs as compared to C_0 levels [29,74,113]. However, a systematic review showed that the benefit of C_2 with respect to C_0 levels could not be demonstrated and monitoring of C_0 levels is still regarded the standard of care in many Tx centers [114]. Recently, Zahn *et al.* confirmed that the maximum inhibition of PMA and ionomycin-induced expression of NFAT-regulated genes was obtained 1.5 hours after oral administration of Tac [77].

Measurement of whole blood concentrations of CNIs does not necessarily reflect the biological response in immune cells, and measurement of PD response biomarkers may better predict the clinical response of the drugs. Although differing in chemical structure (Figure 3), both CNIs prevent activation of the transcription factor NFAT and thereby the transcription of several cytokines essential for lymphocyte activation and proliferation.

The majority of studies have investigated NFAT-regulated gene expression responses during CsA therapy in stable renal Tx patients. Compared to CsA, Tac has been associated with a lower risk of acute rejection in the early phase after Tx [29-36]. Currently, the use of Tac is increasing although only few data exist addressing the outcome in Tac treated patients in early phase post transplant. Tac has replaced CsA in many immunosuppressive protocols, including the standard immunosuppressive protocol for renal Tx at Oslo University Hospital. In parallel to CsA, NFAT-regulated gene expression has been suggested as a promising tool for PD monitoring of Tac therapy in different transplant patient populations [30,76,77,80,92,105]. The patients included are both *de novo* pediatric, adult and senior transplant patients (*e.g.* renal, heart, liver or lung) and stable transplant patients [56,90-92]. The variability in gene expression responses between patients suggests individual differences in immunosuppressive responsiveness to CNI. However the gene expression response appears to differ between CsA and Tac [30], and further studies are needed to characterize potential associations between NFAT-regulated gene expression and clinical outcomes in Tac treated patients. The initial part of the present project focused on the development of a useful assay for determination of NFAT-regulated gene expression in whole blood. Subsequently, the assay was used to investigate the NFAT-regulated gene expression as potential PD biomarkers among Tac treated patients in the early phase after renal Tx.

6.1 Assay for determination of NFAT-regulated gene expression in whole blood

Traditionally, gene expression measurements involved Northern blotting techniques or semi-quantitative RT-PCR. The more recently introduced RT-qPCR provides rapid and reproducible quantification of mRNA over a wide range of concentrations. The purpose of

this project was to develop and validate a real-time PCR based assay for the quantification of *IL2, IFNG* and *CSF2* gene expression to monitor Tac response in renal transplant patients.

6.1.1 Sample preparations

In the initial studies, peripheral blood mononuclear cells were isolated before *ex vivo* activation [105]. The isolation procedure may affect cellular responses and eliminate plasma components and thereby influence the measured NFAT-regulated gene expression. Compared to activation of isolated cells, activation of lymphocytes in whole blood minimizes sample-handling steps, is less time-consuming and may better reflect the *in vivo* physiological environment since all cellular blood components, including drugs and the metabolites are present [115,116]. CNIs are extensively bound to erythrocytes and since approximately 90% of the drugs are bound in the cellular component in blood it was decided to activate the lymphocytes and thereby measure the CNI concentration in whole blood [117,118].

Cytokines play a crucial role in the immune response towards a transplanted allograft. Their expression levels affect many signal cascades, including the differentiation of naive T cells into effector T cells. Exposure to clinically relevant concentrations of CsA and Tac has been reported to down-regulate the expression of *IL2*, *TNF-α*, *INFG* and *CSF2* [92]. The expression of the individual cytokines may be influenced by different factors, and measurement of a combined response of multiple cytokines probably provides more robust assessment of the CNI response compared with measurement of single cytokines [119]. Because the expression of *TNF-α* in whole blood is low and variable, NFAT-regulated gene expression responses were calculated based on *IL2*, *INFG* and *CSF2* gene expression levels only [92]. Giese *et al.* confirmed that the measurement of these three cytokines in whole blood is reproducible and that these biomarkers can be effectively used in gene expression [105].

In the previously published gene expression assays, lymphocytes were activated *ex vivo* with PMA and ionomycin [30,76,77,80,92,105,120]. The present project is part of a bigger study involving measurement of several potential biomarkers. Different principles of lymphocyte activation were tested to identify a common activation strategy suitable for the different assays. Corresponding to previous findings, the highest expression levels of NFAT-regulated

genes were obtained after 3 hours incubation with PMA and ionomycin. Incubation for 48 and 72 hours was associated with pronounced hemolysis and degradation of RNA. The relatively short incubation period of 3 hours is favorable considering potential use of the assay in clinical routine. PMA and ionomycin was also a suitable lymphocyte activation principle for the assessment of other potential PD biomarkers in parallel studies. Using the same lymphocyte activation principle in studies of different biomarker responses, allows more direct comparison of the results.

Compared to the previously published assays, the present method involves a simplified one-step lysis procedure. This provides a simpler, faster and more robust sample preparation procedure, as well as increased yields of mRNA. The time to RNA stabilization is reduced and potential regulation of gene expression during the red blood cell lysis procedure is avoided. Altogether, the sample preparation steps were easy to perform and could be carried out within one day with high intra-and interassay reproducibility.

6.1.2 Gene expression assay

The MagNA Pure LC™ instrument allowed automated RNA isolation from up to 32 different samples in one batch. Furthermore, the pipetting function of the instrument was utilized to transfer cDNA and PCR reagents into LightCycler 96-well plates. The use of a closed and automated system for the RNA isolation and PCR preparation steps minimizes manual pipetting errors, reduces the risk of contamination, reduces hands-on time and improves the assay precision.

The two-step RT-PCR procedure allowed individual optimization of the RT and PCR reactions. This provides greater flexibility in the selection of polymerases and priming strategies and allows multiple PCR reactions to be performed from a single cDNA sample.

In preliminary NFAT gene expression assays, RT was performed by avian myeloblastosis virus reverse transcriptase and oligo(dT) primers [77,105]. The present assay utilized the Transcriptor First Strand cDNA Synthesis Kit to ensure efficient RT of both target and reference genes. The thermostability of the Transcriptor enzyme allowed RT reactions to be performed at temperatures up to 65°C, reducing potential RNA secondary structures.

Furthermore, in contrast to the avian myeloblastosis virus enzyme, the RNase H positive Transcriptor enzyme degrades RNA in RNA-DNA hybrids, preserving the quantity of the original material. A combination of random hexamer and anchored-oligo$(dT)_{18}$ primers generally improved RT efficiency, demonstrated by lower Cp values, compared to using a single priming strategy. Oligo$(dT)_{18}$ primers initiate cDNA synthesis at the poly(A) tail of mRNA, generating full-length cDNAs. However, the efficacy of oligo$(dT)_{18}$ priming may vary depending on the distance from the poly(A) tail. Random hexamer primers bind to many sites throughout the length of RNA. The present assay involves analysis of several sequences with different locations in relation to poly(A) tails. Thus, combining random hexamer and anchored-oligo$(dT)_{18}$ primers are thought to provide a more uniform representation of the RNA sequences of interest.

Our previously established reference gene assays are based on product detection using hybridization probes. The same format was also selected for the detection of target gene amplicons, facilitating the use of a single PCR program and automated data analysis. Hybridization probes offer an additional level of selectivity as the FRET process and signal emission only occurs if both probes are bound to their complementary sequences simultaneously. However, compared to the SYBR Green I format used in the reported assays [30,77,92,105,120], hybridization probes are more expensive.

Following identification of suitable primers and probes, the assay design and optimization procedure included titration of the primer concentrations. Too high primer concentrations may result in the formation of primer dimers and non-specific products, while too low concentrations may reduce the PCR-efficiency. The optimal primer concentrations were selected based on SYBR Green I analysis and Cp values of amplification plots, indicating primer selectivity and PCR-efficiency, respectively.

Normalization of the expression of target genes to reference genes corrects for non-biological variation due to *e.g.* differences in template input quality or quantity and the yields of RNA isolation and RT. An optimal reference gene should be stably expressed under all experimental conditions and the expression levels should be in the same range as the expression of the target gene of interest. However, as there is no single reference gene with constant expression under all conditions, use of a combination of at least three different validated reference genes has been recommended [101,121]. The selection of stable reference genes is critical for a reliable and robust normalization of the target gene expression. In the

preliminary published NFAT-regulated gene expression assays, target gene expression was normalized to the mean expression of beta-actin and cyclophilin B [76,77,105]. Several studies have shown that the expression of reference genes like beta-actin is altered under different experimental conditions [100,122,123]. To minimize the impact of potential gene regulation and strengthen the accuracy of the present assay, gene expression was normalized to the geometric mean expression of three different reference genes. The reference genes *ALAS1*, *B2M* and *RPL13A* have been reported to be constitutively expressed in most cell types [99] and belong to different functional classes, thereby minimizing the risk of coregulation. Moreover, the expression of the selected reference genes in blood cells has been validated in previous experiments in our laboratory [106,107]. See Section 1.6.4.1 for more details.

To further minimize potential non-biological variability, the target/reference ratio of the sample was normalized to the target/reference ratio of the calibrator sample included in each run. This corrects for differences in detection sensitivity between target and reference genes caused by differences in probe annealing, FRET efficiency, or dye extension coefficients. Additionally, such normalization provides a constant calibration point between runs and makes the processing assay relatively robust. Because of the instability of RNA, a cDNA calibrator was used for normalization in the present assay.

The calculation of relative gene expression levels is based on Cp values and PCR efficiencies of target and reference genes. Generally, most amplification reactions do not reach 100% efficiency. The PCR efficiency may vary due to the presence of PCR inhibitors or inappropriate primer and/or probe design, *e.g.* targeting GC-rich sequences or resulting in coamplification of nonspecific PCR products. Compounds known to inhibit PCR include SYBR Green I, heparin, hemoglobin, lipids, polysaccharides and various sample preparation reagents (phenol, ethanol, proteinase K and paramagnetic silica beads) as mentioned in Section 1.6.4. The estimated PCR efficiencies were above 97.5% for all target and reference gene reactions, and this is well within the acceptable range of 90 to 110% efficiency according to the Roche manual. To correct for differences in PCR efficiency, the actual gene specific efficiencies were determined by dilution series experiments and included in the relative gene expression calculations (Equation 3 and 4 Section 4.6.1).

6.2 Assay validation

The quality of every PCR assay depends on the use of selective primers. Although the selectivity of primers and probes was evaluated *in silico* by different software tools, selective amplification and detection of products should also be confirmed experimentally. The selectivity of the primers and probes of the present assay was confirmed by three different techniques, SYBR Green I analysis, gel electrophoresis and direct sequencing, providing complementary information of the present amplicons. Furthermore, the suitability of the primer design was confirmed by the efficient amplification of target and reference gene sequences (discussed in Section 6.1.2).

Transplanted patients demonstrated a wide range in their leukocyte counts. Furthermore, the relative expression levels of *IL2, IFNG* and *CSF2* may vary considerable due to the immunosuppressive therapy and immunological conditions. This requires reliable quantification of gene expression over a wide range of mRNA concentrations. Analysis of repeated generation of standard curves demonstrated linearity over a range of 10^7 – 10^2 templates per reaction, indicating constant amplification efficiencies over the concentration range studied. Single-template detection is theoretically possible with real-time PCR. However, the distribution statistics at low template concentrations influence the actual lower limit of detection. The accuracy and the precision levels have been calculated on the basis of Cp values. The log–linear inverse relationship between concentrations and Cp values indicates that CVs based on relative concentrations will be nominally higher than CVs based on Cp values. The CVs of the present assay were calculated in terms of relative concentrations. These CVs are directly applicable and relevant for the interpretation of results presented in terms of relative concentrations. The within and between assay CVs were below 19.8% (10^7 – 10^4 templates per reaction) and 14.8%, respectively, which is regarded highly acceptable for this type of assay. Analysis of cytokine expression in patient samples resulted in Cp values < 35 (corresponding to 10^4 templates per reaction). This confirms precise quantification in the range of clinically relevant template concentrations.

6.3 Effect of *ex vivo* exposure to immunosuppressive drugs

NFAT-regulated gene expression was quantified in blood samples after incubation with different immunosuppressive drugs and concentrations *ex vivo*. A previous report by *Giese et al.* showed a CNIs exposure-dependent regulation of NFAT-regulated genes [92]. A similar concentration dependent NFAT-regulated gene expression response was demonstrated, whereas up to 100% inhibition was observed at the highest concentration of each CNI (Figure 26). As previously reported, *IL2* and *CSF2* gene expression appears to demonstrate a higher sensitivity to CNIs compared to *IFNG* expression [91]. Only smaller alterations of NFAT-regulated genes were observed after incubation with Pred, while the gene expression was relatively stable following exposure to mycophenolic acid (Figure 25). NFAT-regulated genes appear to be most strongly expressed to the effects of CNI *in vivo* compared to prednisolone and mycophenolic acid. This is in agreement with previous studies [87,124].

The observed effect of methanol alone on the NFAT-regulated gene expression represents a major limitation of the *ex vivo* drug exposure experiments. Although the gene expression response after *ex vivo* drug exposure was related to the control sample with methanol, the observed responses to different immunosuppressive drugs and concentrations may not reflect the actual immune response *in vivo*. Furthermore, the intracellular concentration of CNIs within lymphocytes is likely to differ after *ex vivo* versus *in vivo* drug exposure due to differences in matrix effects and drug distribution.

6.4 NFAT-regulated gene expression in renal transplant recipients treated with Tac

To further confirm the utility of the assay, the NFAT-regulated gene expression was determined in samples from a renal transplant recipient receiving Tac therapy. Several studies have reported an association between residual gene expression, calculated by normalizing the expression at maximum CNI exposure to pre dose expression, and clinical outcomes post Tx [30,76,77,80,92,105]. The expected time to reach the C_{max} after Tac dosing is about

1.5 hours. Samples for NFAT-regulated gene expression analysis were therefore drawn before and 1.5 hours after the administration of Tac. The patient demonstrated residual gene expression of 14% 1.5 hours after Tac dosing. The Tac concentration was 15.9 µg/L 1.5 hours post dose ($C_{1.5}$) and 5.1 µg/L pre dose (C_0). The C_0 level was well within the target range of Tac C_0 between 3 to 7 µg/L. The NFAT gene expression response during *in vivo* Tac therapy was further investigated in the MarkIt pilot study.

6.4.1 MarkIt pilot study

Despite comparable Tac blood concentrations, the clinical response varies considerably among patients. The level of residual NFAT-regulated gene expression has been associated with clinical events. Previous studies have proposed that an optimal range of residual NFAT-regulated gene expression during CsA therapy should be in the range of 20 – 30% [27,77,80]. Stronger inhibition has been associated with an increased risk of infection and malignancies, while residual gene expression above 30% may increase the risk of rejection episodes. However, the optimal range is primarily based on studies of CsA treated long-term stable renal transplant patients and may not directly be relevant for the response in Tac treated patients in the early phase after Tx. Mortensen *et al.* compared the inhibition of calcineurin activity in Tac treated patients early after Tx and 5 years later. Exposure to therapeutic Tac concentrations resulted in stronger inhibition of calcineurin activity in the early phase post transplant compared to 5 years after Tx [125].

Several studies have demonstrated that the residual gene expression levels are better correlated to clinical events compared to CNI blood concentrations [30,76,77,80,92,105]. A recent study by Sommerer *et al.,* investigating Tac treated stable renal allograft recipients, demonstrated residual NFAT-regulated gene expression levels below 20% to be associated with an increased risk of infections (*e.g.* CMV), while residual expression above 20% was associated with an increased risk of acute rejection [126]. However, another study reported patients with residual expression above 80% and still not experiencing rejection [30]. A PD monitoring study by Billing *et al.* demonstrated a higher inhibition of NFAT-regulated genes in pediatric renal transplant recipients compared to the inhibition in adults. The authors

emphasize the need to define the specific target ranges of residual NFAT-regulated gene expression in different patient populations [91].

All five renal transplant recipients included in the MarkIt pilot study demonstrated more than 70% inhibition of NFAT-regulated gene expression around one week post Tx. Previous studies have demonstrated an inverse correlation between the residual NFAT-regulated gene expression and serum creatinine levels [127]. One of the participants experienced an acute rejection episode four days after Tx. One week post Tx, the residual gene expression level in this patient was 1.5%. The almost complete inhibition of NFAT-regulated gene expression may be related to the rejection treatment, including four days of high dose i.v. glucocorticoid therapy and an increase of Tac doses.

Sommerer *et al.* demonstrated a correlation between a high degree of NFAT-regulated genes inhibition and an increased risk of adverse effects such as infections and malignancies [127]. Among the patients in the MarkIt pilot, four patients had residual NFAT-regulated gene expressions of 13% or less, suggesting a high degree of gene expression inhibition in the early period after Tx. Three of the patients demonstrated Tac pre dose concentrations within the therapeutic range, while one patient presented Tac C_0 above target. According to Sommerer and colleagues, these patients might benefit from Tac dose reductions [126]. One of the patients in the present study demonstrated residual expression of 2.5% and developed polyomavirus (BK) infection approximately 2 months post Tx. However, further data are needed to characterize potential associations between residual gene expression levels and the risk of infections.

There was a large interindividual variability in the relative gene expression levels (without normalization to pre dose values). Before Tx, up to 5 fold differences in NFAT-regulated gene expression was observed between individuals (Figure 28). This may be related to individual differences in immune status, which in turn may be influenced by previous dialysis, uremia, infections, previous blood transfusion, comedication or genetics. Comparing the relative gene expression before Tx versus pre dose after Tx, three patients demonstrated a reduction in expression levels. This may be explained by the presence of Tac in the pre dose samples after Tx. Among the two patients with the lowest pre Tx expression levels, Tx and initiation of immunosuppression did not appear to have any profound effect on the relative gene expression pre dose (Figure 28). One of these patients had donor specific antibodies (DSA) and received immunosuppressive therapy before Tx (Table 9). The considerable

variability in NFAT-regulated gene expression levels between patients may indicate different immune activation potentials and thereby different requirements for immunosuppression.

Finally, there has been extensive evidence that CNI PKs may be influenced by pharmacogenetic factors within the CYP system. The effects of CYP3A5-genotype on CNI PKs have been reviewed in detail by Staatz *et al.* and more recently by Picard *et al.* [128,129]. A recent study demonstrate that recipients with CYP3A5*1 (CYP3A5 expressers) required a higher dose of Tac than CYP3A5*3 homozygote (CYP3A5 nonexpressers) to reach C_0 concentrations [130]. Genotype was determined once for each participant and a genetic polymorphism in CYP3A5 was observed, which contributes to variation in distribution of Tac. Genotyping in the MarkIt pilot revealed the CYP3A5*3/*3 genotype in four patients, while one patient was found to be heterozygous CYP3A5*1/*3. Pharmacogenetic information should be taken into consideration as genotyping may identify which patients may need higher initial doses for effective immunosuppression during the critical first days after Tx.

6.5 Future perspectives

Despite the many PD monitoring proposals to individualize Tac therapy none of these has been implemented in routine clinical practice, an optimal approach still needs to be established. Monitoring of NFAT-regulated gene expression has been suggested as a promising tool to optimize CNI therapy [29]. However, so far, there are only a few data on *de novo* renal Tx recipients and on patients receiving Tac.

The optimal range of NFAT-regulated gene expression needs to be established in different patient populations and in different phases after Tx. To better characterize the NFAT-regulated gene expression in Tac treated patients early after Tx, the next step will be to perform the planned enrollment of 30 participants. The relative gene expression and the residual gene expression 1.5 hours post Tac dose will be investigated in relation to clinical parameters like infections, malignancies, rejection episodes. Furthermore, the gene expression response in the early phase (first 8 weeks) will be compared to the response at approximately 1 year post Tx.

Although NFAT-regulated gene expression is reported to be relatively CNI specific, knowledge of potential factors interacting with the gene expression response is still limited. Further studies are needed to study the potential impact of immunosuppressive co-medication, infections, the general immune status and genetic factors.

Several studies suggest measurement of NFAT-regulated gene expression to be a relevant tool for preventing over-immunosuppression and CNI induced adverse events [30,76,77,80,92,105,120,126,131]. However, data on the relation between residual NFAT-regulated gene expression and acute rejection episodes are less consistent. With the present immunosuppressive regimens, the number of acute rejection episodes is limited. This implies that relatively large studies are required to characterize the relation between NFAT-regulated gene expression and acute rejection episodes.

Before implementation in clinical practice, the value of monitoring NFAT-regulated gene expression needs to be confirmed in prospective studies. Monitoring of NFAT-regulated gene expression has been suggested as a complementary tool to PK monitoring.

CYP3A5 play an important role in metabolizing and distribution of Tac and is more recently suggested as useful marker to characterize Tac therapy. The correlation between Tac blood concentration and the presence of CYP3A5 should be further clarified.

7 Conclusion

To further improve long-term outcomes after solid organ Tx, there is a need for optimization of the immunosuppressive therapy. Currently, CNIs are included in most immunosuppressive regimens. Monitoring NFAT-regulated gene expression has been suggested as a promising approach for individualizing CNI therapy.

We have established and validated an assay for quantification of *IL2*, *IFNG* and *CSF2* gene expression after *ex vivo* immune activation. The assay was based on a previously reported procedure [30,76,77,80,92,105,120]. The simplified sample preparation involved 3 hours incubation of whole blood with PMA and ionomycin, followed by direct lysis of blood cells and extraction of total RNA. After RT and real-time PCR, the expression of the target genes was normalized to the expression of the reference genes *ALAS1, B2M* and *RPL13A*. Validation of the RT-qPCR assay confirmed specific and reproducible quantification of the target genes.

The present sample presentation steps improved workflow efficiency compared with previously published assay. Additionally, the experimental results could be obtained within one day with high intra-and interassay reproducibility.

Ex vivo exposure of whole blood samples from healthy individuals to clinically relevant CNIs concentrations demonstrated an inverse correlation between CNI exposure and NFAT-regulated gene expression. In contrast, prednisolone and mycophenolic acid did not appear to have any pronounced effect on the NFAT-regulated cytokines. Furthermore, the utility of the assay was confirmed by analyzing samples from renal transplant patients receiving Tac therapy. The large variability in NFAT-regulated gene expression before Tx may suggest interindividual differences in immune status and potentially different requirements for immunosuppression. Residual gene expression at 1.5 hours post dose, normalized to pre dose levels, has been reported to be associated with clinical outcomes like rejection episodes, infections and malignancies [30,77]. About one week post Tx, 4 of 5 patients demonstrated residual gene expression below the suggested optimal range. However, only one of these patients presented Tac C_0 levels above the therapeutic target range.

The preliminary findings support the use of NFAT-regulated gene expression as PD biomarker of Tac response. Combined PD and PK monitoring may allow better prediction of

the clinical response during Tac therapy. However, further studies are needed to determine the optimal PD monitoring strategy in different patient populations. Finally, the benefit of PD monitoring needs to be confirmed in larger prospective intervention trials.

References

1. Albrechtsen, D., Pfeffer, P.F. *En Gave for livet historien om organtransplantasjon i Norge*, Unipub, Oslo, 2011.

2. Sommerer, C., Morath, C., Andrassy, J., Zeier, M. The long-term consequences of living-related or unrelated kidney donation. Nephrol Dial Transplant 2004; 19 Suppl 4: iv45-iv47.

3. McFarlane, P.A. Should patients remain on intensive hemodialysis rather than choosing to receive a kidney transplant? Semin Dial 2010; 23: 516-519.

4. Meier-Kriesche, H.U., Schold, J.D. The impact of pretransplant dialysis on outcomes in renal transplantation. Semin Dial 2005; 18: 499-504.

5. Heldal, K., Hartmann, A., Leivestad, T., Lien, B., Foss, A.E., Midtvedt, K. Renal transplantation is also an option for patients over 70. Tidsskr Nor Laegeforen 2011; 131: 2004-2007.

6. Wolfe, R.A., Ashby, V.B., Milford, E.L., Ojo, A.O., Ettenger, R.E., Agodoa, L.Y., Held, P.J., Port, F.K. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med 1999; 341: 1725-1730.

7. Schnuelle, P., Lorenz, D., Trede, M., Van Der Woude, F.J. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence for reduced mortality risk compared with hemodialysis during long-term follow-up. J Am Soc Nephrol 1998; 9: 2135-2141.

8. Welberry Smith, M.P., Baker, R.J. Assessment and management of a patient with a renal transplant. Br J Hosp Med (Lond) 2007; 68: 656-662.

9. Cooke, G.S., Hill, A.V. Genetics of susceptibility to human infectious disease. Nat Rev Genet 2001; 2: 967-977.

10. Wang, P., Sidney, J., Kim, Y., Sette, A., Lund, O., Nielsen, M., Peters, B. Peptide binding predictions for HLA DR, DP and DQ molecules. BMC Bioinformatics 2010; 11: 568.

11. Douek, D.C., Altmann, D.M. T-cell apoptosis and differential human leucocyte antigen class II expression in human thymus. Immunology 2000; 99: 249-256.

12. Kumanovics, A., Takada, T., Lindahl, K.F. Genomic organization of the mammalian MHC. Annu Rev Immunol 2003; 21: 629-657.

13. Traherne, J.A. Human MHC architecture and evolution: implications for disease association studies. Int J Immunogenet 2008; 35: 179-192.

14. Parham, P., Janeway, C.A. *The immune system*, 3rd ed.edn. Garland Science, London, 2009.

15. van Besouw, N.M., Zuijderwijk, J.M., Vaessen, L.M., Balk, A.H., Maat, A.P., van der Meide, P.H., Weimar, W. The direct and indirect allogeneic presentation pathway during acute rejection after human cardiac transplantation. Clin Exp Immunol 2005; 141: 534-540.

16. Afzali, B., Lombardi, G., Lechler, R.I. Pathways of major histocompatibility complex allorecognition. Curr Opin Organ Transplant 2008; 13: 438-444.

17. Herrera, O.B., Golshayan, D., Tibbott, R., Salcido, O.F., James, M.J., Marelli-Berg, F.M., Lechler, R.I. A novel pathway of alloantigen presentation by dendritic cells. J Immunol 2004; 173: 4828-4837.

18. Afzali, B., Lechler, R.I., Hernandez-Fuentes, M.P. Allorecognition and the alloresponse: clinical implications. Tissue Antigens 2007; 69: 545-556.

19. Hashmi, S., Poommipanit, N., Kahwaji, J., Bunnapradist, S. Overview of renal transplantation. Minerva Med 2007; 98: 713-729.

20. Anonymous. KDIGO clinical practice guideline for the care of kidney transplant recipients. Am J Transplant 2009; 9 Suppl 3: S1-155.

21. Maluf, D.G., Mas, V.R., Archer, K.J., Yanek, K., Gibney, E.M., King, A.L., Cotterell, A., Fisher, R.A., Posner, M.P. Molecular pathways involved in loss of kidney graft function with tubular atrophy and interstitial fibrosis. Mol Med 2008; 14: 276-285.

22. Fishman, J.A., Rubin, R.H. Infection in organ-transplant recipients. N Engl J Med 1998; 338: 1741-1751.

23. Tanphaichitr, N.T., Brennan, D.C. Infectious complications in renal transplant recipients. Adv Ren Replace Ther 2000; 7: 131-146.

24. Anon. [Transplantasjonsprotokoll]. (11-1-2012).

25. Vethe, N.T., Midtvedt, K., Asberg, A., Amundsen, R., Bergan, S. [Drug interactions and immunosuppression in organ transplant recipients]. Tidsskr Nor Laegeforen 2011; 131: 2000-2003.

26. Bergan, S., Albrechtsen, D., Bentdal, O. [Immunosuppressive agents in organ transplantation]. Tidsskr Nor Laegeforen 1999; 119: 3615-3620.

27. Giese, T., Sommerer, C., Zeier, M., Meuer, S. Approaches towards individualized immune intervention. Dig Dis 2010; 28: 45-50.

28. Opelz, G., Dohler, B. Influence of immunosuppressive regimens on graft survival and secondary outcomes after kidney transplantation. Transplantation 2009; 87: 795-802.

29. Sommerer, C., Meuer, S., Zeier, M., Giese, T. Calcineurin inhibitors and NFAT-regulated gene expression. Clin Chim Acta 2012; 413: 1379-1386.

30. Sommerer, C., Zeier, M., Meuer, S., Giese, T. Individualized monitoring of nuclear factor of activated T cells-regulated gene expression in FK506-treated kidney transplant recipients. Transplantation 2010; 89: 1417-1423.

31. Sommerer, C., Giese, T., Meuer, S., Zeier, M. New concepts to individualize calcineurin inhibitor therapy in renal allograft recipients. Saudi J Kidney Dis Transpl 2010; 21: 1030-1037.

32. Busuttil, R.W., Lake, J.R. Role of tacrolimus in the evolution of liver transplantation. Transplantation 2004; 77: S44-S51.

33. Margreiter, R. Efficacy and safety of tacrolimus compared with ciclosporin microemulsion in renal transplantation: a randomised multicentre study. Lancet 2002; 359: 741-746.

34. Radermacher, J., Meiners, M., Bramlage, C., Kliem, V., Behrend, M., Schlitt, H.J., Pichlmayr, R., Koch, K.M., Brunkhorst, R. Pronounced renal vasoconstriction and systemic hypertension in renal transplant patients treated with cyclosporin A versus FK 506. Transpl Int 1998; 11: 3-10.

35. Pirsch, J.D., Miller, J., Deierhoi, M.H., Vincenti, F., Filo, R.S. A comparison of tacrolimus (FK506) and cyclosporine for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Group. Transplantation 1997; 63: 977-983.

36. Mayer, A.D., Dmitrewski, J., Squifflet, J.P., Besse, T., Grabensee, B., Klein, B., Eigler, F.W., Heemann, U., Pichlmayr, R., Behrend, M., Vanrenterghem, Y., Donck, J., van, H.J., Christiaans, M., Morales, J.M., Andres, A., Johnson, R.W., Short, C., Buchholz, B., Rehmert, N., Land, W., Schleibner, S., Forsythe, J.L., Talbot, D., Pohanka, E., . Multicenter randomized trial comparing tacrolimus (FK506) and cyclosporine in the prevention of renal allograft rejection: a report of the European Tacrolimus Multicenter Renal Study Group. Transplantation 1997; 64: 436-443.

37. Hariharan, S., McBride, M.A., Cherikh, W.S., Tolleris, C.B., Bresnahan, B.A., Johnson, C.P. Post-transplant renal function in the first year predicts long-term kidney transplant survival. Kidney Int 2002; 62: 311-318.

38. Friman, S., Backman, L. A new microemulsion formulation of cyclosporin: pharmacokinetic and clinical features. Clin Pharmacokinet 1996; 30: 181-193.

39. Marcen, R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. Drugs 2009; 69: 2227-2243.

40. Knoll, G. Trends in kidney transplantation over the past decade. Drugs 2008; 68 Suppl 1: 3-10.

41. Pollard, S., Nashan, B., Johnston, A., Hoyer, P., Belitsky, P., Keown, P., Helderman, H. A pharmacokinetic and clinical review of the potential clinical impact of using different formulations of cyclosporin A. Berlin, Germany, November 19, 2001. Clin Ther 2003; 25: 1654-1669.

42. Fahr, A. Cyclosporin clinical pharmacokinetics. Clin Pharmacokinet 1993; 24: 472-495.

43. Aukrust, P and Hartmann, A. Norsk legemiddelhåndboka [Terapi 18 Immunsykdommer og transplantasjoner]. (20-6-2010).

44. Mayer, A.D. Chronic rejection and graft half-life: five-year follow-up of the European Tacrolimus Multicenter Renal Study. Transplant Proc 2002; 34: 1491-1492.

45. Ligtenberg, G., Hene, R.J., Blankestijn, P.J., Koomans, H.A. Cardiovascular risk factors in renal transplant patients: cyclosporin A versus tacrolimus. J Am Soc Nephrol 2001; 12: 368-373.

46. Kaufman, D.B., Shapiro, R., Lucey, M.R., Cherikh, W.S., Bustami, T., Dyke, D.B. Immunosuppression: practice and trends. Am J Transplant 2004; 4 Suppl 9: 38-53.

47. Artz, M.A., Boots, J.M., Ligtenberg, G., Roodnat, J.I., Christiaans, M.H., Vos, P.F., Moons, P., Borm, G., Hilbrands, L.B. Conversion from cyclosporine to tacrolimus improves quality-of-life indices, renal graft function and cardiovascular risk profile. Am J Transplant 2004; 4: 937-945.

48. Artz, M.A., Boots, J.M., Ligtenberg, G., Roodnat, J.I., Christiaans, M.H., Vos, P.F., Blom, H.J., Sweep, F.C., Demacker, P.N., Hilbrands, L.B. Improved cardiovascular risk profile and renal function in renal transplant patients after randomized conversion from cyclosporine to tacrolimus. J Am Soc Nephrol 2003; 14: 1880-1888.

49. Winkler, M., Christians, U. A risk-benefit assessment of tacrolimus in transplantation. Drug Saf 1995; 12: 348-357.

50. Kihara, Y., Matsuno, N., Mijiti, A., Nagao, T., Takeuchi, H., Unezaki, S., Hirano, T. Comparative study of the cellular pharmacodynamics of calcineurin inhibitors between patients with chronic renal failure awaiting renal transplantation and cirrhosis patients awaiting liver transplantation. Cell Transplant 2009; 18: 639-646.

51. Niioka, T., Satoh, S., Kagaya, H., Numakura, K., Inoue, T., Saito, M., Narita, S., Tsuchiya, N., Habuchi, T., Miura, M. Comparison of Pharmacokinetics and Pharmacogenetics of Once- and Twice-Daily Tacrolimus in the Early Stage After Renal Transplantation. Transplantation 2012.

52. Vicari-Christensen, M., Repper, S., Basile, S., Young, D. Tacrolimus: review of pharmacokinetics, pharmacodynamics, and pharmacogenetics to facilitate practitioners' understanding and offer strategies for educating patients and promoting adherence. Prog Transplant 2009; 19: 277-284.

53. Venkataramanan, R., Swaminathan, A., Prasad, T., Jain, A., Zuckerman, S., Warty, V., McMichael, J., Lever, J., Burckart, G., Starzl, T. Clinical pharmacokinetics of tacrolimus. Clin Pharmacokinet 1995; 29: 404-430.

54. de Jonge, H., de Loor, H., Verbeke, K., Vanrenterghem, Y., Kuypers, D.R. In Vivo CYP3A4 Activity, CYP3A5 Genotype, and Hematocrit Predict Tacrolimus Dose Requirements and Clearance in Renal Transplant Patients. Clin Pharmacol Ther 2012; 92: 366-375.

55. Wallemacq, P.E., Verbeeck, R.K. Comparative clinical pharmacokinetics of tacrolimus in paediatric and adult patients. Clin Pharmacokinet 2001; 40: 283-295.

56. Treede, H., Klepetko, W., Reichenspurner, H., Zuckermann, A., Meiser, B., Birsan, T., Wisser, W., Reichert, B. Tacrolimus versus cyclosporine after lung transplantation: a prospective, open, randomized two-center trial comparing two different immunosuppressive protocols. J Heart Lung Transplant 2001; 20: 511-517.

57. Lindenfeld, J., Miller, G.G., Shakar, S.F., Zolty, R., Lowes, B.D., Wolfel, E.E., Mestroni, L., Page, R.L., Kobashigawa, J. Drug therapy in the heart transplant recipient: part II: immunosuppressive drugs. Circulation 2004; 110: 3858-3865.

58. Taylor, A.L., Watson, C.J., Bradley, J.A. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005; 56: 23-46.

59. Andreoni, K.A., Brayman, K.L., Guidinger, M.K., Sommers, C.M., Sung, R.S. Kidney and pancreas transplantation in the United States, 1996-2005. Am J Transplant 2007; 7: 1359-1375.

60. Halloran, P.F. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004; 351: 2715-2729.

61. Anonymous. Mycophenolate mofetil for the treatment of a first acute renal allograft rejection: three-year follow-up. The Mycophenolate Mofetil Acute Renal Rejection Study Group. Transplantation 2001; 71: 1091-1097.

62. Behrend, M. Adverse gastrointestinal effects of mycophenolate mofetil: aetiology, incidence and management. Drug Saf 2001; 24: 645-663.

63. Waldmann, T.A., O'Shea, J. The use of antibodies against the IL-2 receptor in transplantation. Curr Opin Immunol 1998; 10: 507-512.

64. Padiyar, A., Augustine, J.J., Hricik, D.E. Induction antibody therapy in kidney transplantation. Am J Kidney Dis 2009; 54: 935-944.

65. McKeage, K., McCormack, P.L. Basiliximab: a review of its use as induction therapy in renal transplantation. BioDrugs 2010; 24: 55-76.

66. Nashan, B. Review of the proliferation inhibitor everolimus. Expert Opin Investig Drugs 2002; 11: 1845-1857.

67. Morath, C., Arns, W., Schwenger, V., Mehrabi, A., Fonouni, H., Schmidt, J., Zeier, M. Sirolimus in renal transplantation. Nephrol Dial Transplant 2007; 22 Suppl 8: viii61-viii65.

68. Thervet, E. Sirolimus therapy following early cyclosporine withdrawal in transplant patients: mechanisms of action and clinical results. Int J Nanomedicine 2006; 1: 269-281.

69. Conti, D.J., Petrov, R., Elbalhoul, O., Gallichio, M. Sirolimus-based, calcineurin-inhibitor sparing immunotherapy, long-term (6-year) results. Transpl Immunol 2008; 20: 12-13.

70. Rostaing, L., Kamar, N. mTOR inhibitor/proliferation signal inhibitors: entering or leaving the field? J Nephrol 2010; 23: 133-142.

71. Meier-Kriesche, H.U., Kaplan, B., Brannan, P., Kahan, B.D., Portman, R.J. A limited sampling strategy for the estimation of eight-hour neoral areas under the curve in renal transplantation. Ther Drug Monit 1998; 20: 401-407.

72. Schiff, J., Cole, E., Cantarovich, M. Therapeutic monitoring of calcineurin inhibitors for the nephrologist. Clin J Am Soc Nephrol 2007; 2: 374-384.

73. Mahalati, K., Belitsky, P., Sketris, I., West, K., Panek, R. Neoral monitoring by simplified sparse sampling area under the concentration-time curve: its relationship to acute rejection and cyclosporine nephrotoxicity early after kidney transplantation. Transplantation 1999; 68: 55-62.

74. Morris, R.G., Russ, G.R., Cervelli, M.J., Juneja, R., McDonald, S.P., Mathew, T.H. Comparison of trough, 2-hour, and limited AUC blood sampling for monitoring cyclosporin (Neoral) at day 7 post-renal transplantation and incidence of rejection in the first month. Ther Drug Monit 2002; 24: 479-486.

75. Kahan, B.D., Keown, P., Levy, G.A., Johnston, A. Therapeutic drug monitoring of immunosuppressant drugs in clinical practice. Clin Ther 2002; 24: 330-350.

76. Konstandin, M.H., Sommerer, C., Doesch, A., Zeier, M., Meuer, S.C., Katus, H.A., Dengler, T.J., Giese, T. Pharmacodynamic cyclosporine A-monitoring: relation of gene expression in lymphocytes to cyclosporine blood levels in cardiac allograft recipients. Transpl Int 2007; 20: 1036-1043.

77. Zahn, A., Schott, N., Hinz, U., Stremmel, W., Schmidt, J., Ganten, T., Gotthardt, D., Meuer, S., Zeier, M., Giese, T., Sommerer, C. Immunomonitoring of nuclear factor of activated T cells-regulated gene expression: the first clinical trial in liver allograft recipients. Liver Transpl 2011; 17: 466-473.

78. Filler, G., Feber, J. The transplanted child: New immunosuppressive agents and the need for pharmacokinetic monitoring. Paediatr Child Health 2002; 7: 525-532.

79. Meier-Kriesche, H.U., Schold, J.D., Srinivas, T.R., Kaplan, B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004; 4: 378-383.

80. Sommerer, C., Giese, T., Meuer, S., Zeier, M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009; 24: 21-27.

81. Bohl, D.L., Brennan, D.C. BK virus nephropathy and kidney transplantation. Clin J Am Soc Nephrol 2007; 2 Suppl 1: S36-S46.

82. Chapman, J.R., O'Connell, P.J., Nankivell, B.J. Chronic renal allograft dysfunction. J Am Soc Nephrol 2005; 16: 3015-3026.

83. Solez, K., Axelsen, R.A., Benediktsson, H., Burdick, J.F., Cohen, A.H., Colvin, R.B., Croker, B.P., Droz, D., Dunnill, M.S., Halloran, P.F., . International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. Kidney Int 1993; 44: 411-422.

84. Solez, K., Colvin, R.B., Racusen, L.C., Sis, B., Halloran, P.F., Birk, P.E., Campbell, P.M., Cascalho, M., Collins, A.B., Demetris, A.J., Drachenberg, C.B., Gibson, I.W., Grimm, P.C., Haas, M., Lerut, E., Liapis, H., Mannon, R.B., Marcus, P.B., Mengel, M., Mihatsch, M.J., Nankivell, B.J., Nickeleit, V., Papadimitriou, J.C., Platt, J.L., Randhawa, P., Roberts, I., Salinas-Madriga, L., Salomon, D.R., Seron, D., Sheaff, M., Weening, J.J. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 2007; 7: 518-526.

85. Wieland, E., Olbricht, C.J., Susal, C., Gurragchaa, P., Bohler, T., Israeli, M., Sommerer, C., Budde, K., Hartmann, B., Shipkova, M., Oellerich, M. Biomarkers as a tool for management of immunosuppression in transplant patients. Ther Drug Monit 2010; 32: 560-572.

86. Stein, C.M., Murray, J.J., Wood, A.J. Inhibition of stimulated interleukin-2 production in whole blood: a practical measure of cyclosporine effect. Clin Chem 1999; 45: 1477-1484.

87. Halloran, P.F., Helms, L.M., Kung, L., Noujaim, J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. Transplantation 1999; 68: 1356-1361.

88. Sottong, P.R., Rosebrock, J.A., Britz, J.A., Kramer, T.R. Measurement of T-lymphocyte responses in whole-blood cultures using newly synthesized DNA and ATP. Clin Diagn Lab Immunol 2000; 7: 307-311.

89. Augustine, N.H., Pasi, B.M., Hill, H.R. Comparison of ATP production in whole blood and lymphocyte proliferation in response to phytohemagglutinin. J Clin Lab Anal 2007; 21: 265-270.

90. Sommerer, C., Schnitzler, P., Meuer, S., Zeier, M., Giese, T. Pharmacodynamic monitoring of cyclosporin A reveals risk of opportunistic infections and malignancies in renal transplant recipients 65 years and older. Ther Drug Monit 2011; 33: 694-698.

91. Billing, H., Giese, T., Sommerer, C., Zeier, M., Feneberg, R., Meuer, S., Tonshoff, B. Pharmacodynamic monitoring of cyclosporine A by NFAT-regulated gene expression and the relationship with infectious complications in pediatric renal transplant recipients. Pediatr Transplant 2010; 14: 844-851.

92. Giese, T., Zeier, M., Schemmer, P., Uhl, W., Schoels, M., Dengler, T., Buechler, M., Meuer, S. Monitoring of NFAT-regulated gene expression in the peripheral blood of allograft recipients: a novel perspective toward individually optimized drug doses of cyclosporine A. Transplantation 2004; 77: 339-344.

93. Valentine, M.A., Tsoukas, C.D., Rhodes, G., Vaughan, J.H., Carson, D.A. Phytohemagglutinin binds to the 20-kDa molecule of the T3 complex. Eur J Immunol 1985; 15: 851-854.

94. Chilson, O.P., Kelly-Chilson, A.E. Mitogenic lectins bind to the antigen receptor on human lymphocytes. Eur J Immunol 1989; 19: 389-396.

95. Lagoo, A., Tseng, C.K., Sell, S. Molecular signals in B cell activation. I. Differential refractory effects of incomplete signaling by ionomycin or PMA relate to autocrine IL-2 production and IL-2R expression. Cell Immunol 1990; 127: 483-496.

96. Monis, P.T., Giglio, S., Saint, C.P. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 2005; 340: 24-34.

97. Arezi, B., Xing, W., Sorge, J.A., Hogrefe, H.H. Amplification efficiency of thermostable DNA polymerases. Anal Biochem 2003; 321: 226-235.

98. Lee, P.D., Sladek, R., Greenwood, C.M., Hudson, T.J. Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res 2002; 12: 292-297.

99. Ohl, F., Jung, M., Xu, C., Stephan, C., Rabien, A., Burkhardt, M., Nitsche, A., Kristiansen, G., Loening, S.A., Radonic, A., Jung, K. Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization? J Mol Med (Berl) 2005; 83: 1014-1024.

100. Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S.A., Orlando, C. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002; 309: 293-300.

101. Vandesompele, J., De, P.K., Pattyn, F., Poppe, B., Van, R.N., De, P.A., Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3: RESEARCH0034.

102. Beutler, E., Gelbart, T., Kuhl, W. Interference of heparin with the polymerase chain reaction. Biotechniques 1990; 9: 166.

103. Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci 1994; 39: 362-372.

104. Belec, L., Authier, J., Eliezer-Vanerot, M.C., Piedouillet, C., Mohamed, A.S., Gherardi, R.K. Myoglobin as a polymerase chain reaction (PCR) inhibitor: a limitation for PCR from skeletal muscle tissue avoided by the use of Thermus thermophilus polymerase. Muscle Nerve 1998; 21: 1064-1067.

105. Giese, T., Zeier, M., Meuer, S. Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. Nephrol Dial Transplant 2004; 19 Suppl 4: iv55-iv60.

106. Bremer, S., Rootwelt, H., Bergan, S. Real-time PCR determination of IMPDH1 and IMPDH2 expression in blood cells. Clin Chem 2007; 53: 1023-1029.

107. Bremer, S., Ohlsson, A., Brodtkorb, E., Rootwelt, H., Rootwelt, T., Woldseth, B., Morkrid, L. A novel mucopolysaccharidosis type I associated splice site mutation and IDUA splice variants. Mol Genet Metab 2011; 104: 289-294.

108. Cheung, C.Y., Op den Buijsch, R.A., Wong, K.M., Chan, H.W., Chau, K.F., Li, C.S., Leung, K.T., Kwan, T.H., de Vrie, J.E., Wijnen, P.A., van Dieijen-Visser, M.P., Bekers, O. Influence of different allelic variants of the CYP3A and ABCB1 genes on the tacrolimus pharmacokinetic profile of Chinese renal transplant recipients. Pharmacogenomics 2006; 7: 563-574.

109. Hon, Y.Y., Chamberlain, C.E., Kleiner, D.E., Ring, M.S., Hale, D.A., Kirk, A.D., Mannon, R.B. Evaluation of tacrolimus abbreviated area-under-the-curve monitoring in renal transplant patients who are potentially at risk for adverse events. Clin Transplant 2010; 24: 557-563.

110. Scott, L.J., McKeage, K., Keam, S.J., Plosker, G.L. Tacrolimus: a further update of its use in the management of organ transplantation. Drugs 2003; 63: 1247-1297.

111. Moore, J., Middleton, L., Cockwell, P., Adu, D., Ball, S., Little, M.A., Ready, A., Wheatley, K., Borrows, R. Calcineurin inhibitor sparing with mycophenolate in kidney transplantation: a systematic review and meta-analysis. Transplantation 2009; 87: 591-605.

112. Vincenti, F., Charpentier, B., Vanrenterghem, Y., Rostaing, L., Bresnahan, B., Darji, P., Massari, P., Mondragon-Ramirez, G.A., Agarwal, M., Di, R.G., Lin, C.S., Garg, P., Larsen, C.P. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010; 10: 535-546.

113. Marin, J.G., Levine, M., Ensom, M.H. Is C2 monitoring or another limited sampling strategy superior to C0 monitoring in improving clinical outcomes in adult liver transplant recipients? Ther Drug Monit 2006; 28: 637-642.

114. Knight, S.R., Morris, P.J. The clinical benefits of cyclosporine C2-level monitoring: a systematic review. Transplantation 2007; 83: 1525-1535.

115. Caruso, R., Perico, N., Cattaneo, D., Piccinini, G., Bonazzola, S., Remuzzi, G., Gaspari, F. Whole-blood calcineurin activity is not predicted by cyclosporine blood concentration in renal transplant recipients. Clin Chem 2001; 47: 1679-1687.

116. Batiuk, T.D., Pazderka, F., Enns, J., De, C.L., Halloran, P.F. Cyclosporine inhibition of leukocyte calcineurin is much less in whole blood than in culture medium. Transplantation 1996; 61: 158-161.

117. Nagase, K., Iwasaki, K., Nozaki, K., Noda, K. Distribution and protein binding of FK506, a potent immunosuppressive macrolide lactone, in human blood and its uptake by erythrocytes. J Pharm Pharmacol 1994; 46: 113-117.

118. Kurokawa, N., Kadobayashi, M., Yamamoto, K., Arakawa, Y., Sawada, M., Takahara, S., Okuyama, A., Yanaihara, C. In-vivo distribution and erythrocyte binding characteristics of cyclosporin in renal transplant patients. J Pharm Pharmacol 1996; 48: 553-559.

119. Suormala, T., Wick, H., Bonjour, J.P., Baumgartner, E.R. Rapid differential diagnosis of carboxylase deficiencies and evaluation for biotin-responsiveness in a single blood sample. Clin Chim Acta 1985; 145: 151-162.

120. Sommerer, C., Giese, T., Schmidt, J., Meuer, S., Zeier, M. Ciclosporin A tapering monitored by NFAT-regulated gene expression: a new concept of individual immunosuppression. Transplantation 2008; 85: 15-21.

121. Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 2004; 26: 509-515.

122. Selvey, S., Thompson, E.W., Matthaei, K., Lea, R.A., Irving, M.G., Griffiths, L.R. Beta-actin--an unsuitable internal control for RT-PCR. Mol Cell Probes 2001; 15: 307-311.

123. Podevin, N., Krauss, A., Henry, I., Swennen, R., Remy, S. Selection and validation of reference genes for quantitative RT-PCR expression studies of the non-model crop Musa. Mol Breed 2012; 30: 1237-1252.

124. Millan, O., Brunet, M., Campistol, J.M., Faura, A., Rojo, I., Vidal, E., Jimenez, O., Vives, J., Oppenheimer, F., Martorell, J. Pharmacodynamic approach to immunosuppressive therapies using calcineurin inhibitors and mycophenolate mofetil. Clin Chem 2003; 49: 1891-1899.

125. Mortensen, D.M., Koefoed-Nielsen, P.B., Jorgensen, K.A. Calcineurin activity in tacrolimus-treated renal transplant patients early after and 5 years after transplantation. Transplant Proc 2006; 38: 2651-2653.

126. Sommerer, C., Zeier, M., Czock, D., Schnitzler, P., Meuer, S., Giese, T. Pharmacodynamic disparities in tacrolimus-treated patients developing cytomegalus virus viremia. Ther Drug Monit 2011; 33: 373-379.

127. Sommerer, C., Konstandin, M., Dengler, T., Schmidt, J., Meuer, S., Zeier, M., Giese, T. Pharmacodynamic monitoring of cyclosporine a in renal allograft recipients shows a quantitative relationship between immunosuppression and the occurrence of recurrent infections and malignancies. Transplantation 2006; 82: 1280-1285.

128. Staatz, C.E., Goodman, L.K., Tett, S.E. Effect of CYP3A and ABCB1 single nucleotide polymorphisms on the pharmacokinetics and pharmacodynamics of calcineurin inhibitors: Part I. Clin Pharmacokinet 2010; 49: 141-175.

129. Picard, N., Marquet, P. The influence of pharmacogenetics and cofactors on clinical outcomes in kidney transplantation. Expert Opin Drug Metab Toxicol 2011; 7: 731-743.

130. Shi, Y., Li, Y., Tang, J., Zhang, J., Zou, Y., Cai, B., Wang, L. Influence of CYP3A4, CYP3A5 and MDR-1 polymorphisms on tacrolimus pharmacokinetics and early renal dysfunction in liver transplant recipients. Gene 2012.

131. Sommerer, C., Hartschuh, W., Enk, A., Meuer, S., Zeier, M., Giese, T. Pharmacodynamic immune monitoring of NFAT-regulated genes predicts skin cancer in elderly long-term renal transplant recipients. Clin Transplant 2008; 22: 549-554.

Appendix

Appendix A: Reference gene sequences

Gene	Name	Sequence
ALAS1	ALAS1_F1	AATGAGTCGCCACCCACG
	ALAS1_R1	CAGCTCCCGCTCTAAGTCCA
	ALAS1_FL1	CCTGCCCCAGCACCATGTTGTTTC-Fluorescein
	ALAS1_LC1	LC Red640-GTGTCCATAACTGCCCCACACACC- Phosphate
B2M	B2M_F1	GTCTTTCTATCTCTTGTACTAC
	B2M_R1	GCATCTTCAAACCTCCATGA
	B2M_FL1	TGACTTTGTCACAGCCCAAGATAGT-Fluorescein
	B2M_LC1	LC Red640-AAGTGGGATCGAGACATGTAAGCA-Phosphate
RPL13A	RPL13A_F1	CGTGCGTCTGAAGCCTACA
	RPL13A_R1	GGAGTCCGTGGGTCTTGAG
	RPL13A_ FL1	CGGTAGTGGATCTTGGCTTTCTCTTTCC-Fluorescein
	RPL13A_ LC1	LC Red640-CTTCTCCTCCAGGGTGGCTGTCAC-Phosphate

Abbreviations: F1; forward primer, R1: reverse primer, FL1; forward probe, LC1; reverse probe

Appendix B: Optimized PCR reaction mixture for target genes

Master mix IL2		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	2.75 µL	
Primer: IL2_F1 (20 uM)	0.5 µL	0.5 µM
Primer: IL2_R1 (20 uM)	0.75 µL	0.75 µM
Probe: IL2_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: IL2_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	

Master mix IFNG		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	2.75 µL	
Primer: IFNG_F1 (20 uM)	0.5 µL	0.5 µM
Primer: IFNG_R1 (20 uM)	0.75 µL	0.75 µM
Probe: IFNG_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: IFNG_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	

Master mix CSF2		
Reagents	**Volume per reaction**	**Final concentration**
Water, PCR-quality	2.5 µL	
Primer: CSF2_F1 (20 uM)	0.75 µL	0.75 µM
Primer: CSF2_R1 (20 uM)	0.75 µL	0.75 µM
Probe: CSF2_FL1 (6 uM)	0.5 µL	0.15 µM
Probe: CSF2_LC1 (6 uM)	0.5 µL	0.15 µM
LightCycler® 480 Probes Master (2X)	10 µL	
Master mix total	15 µL	
Sample: cDNA	5 µL	
Total	20 µL	