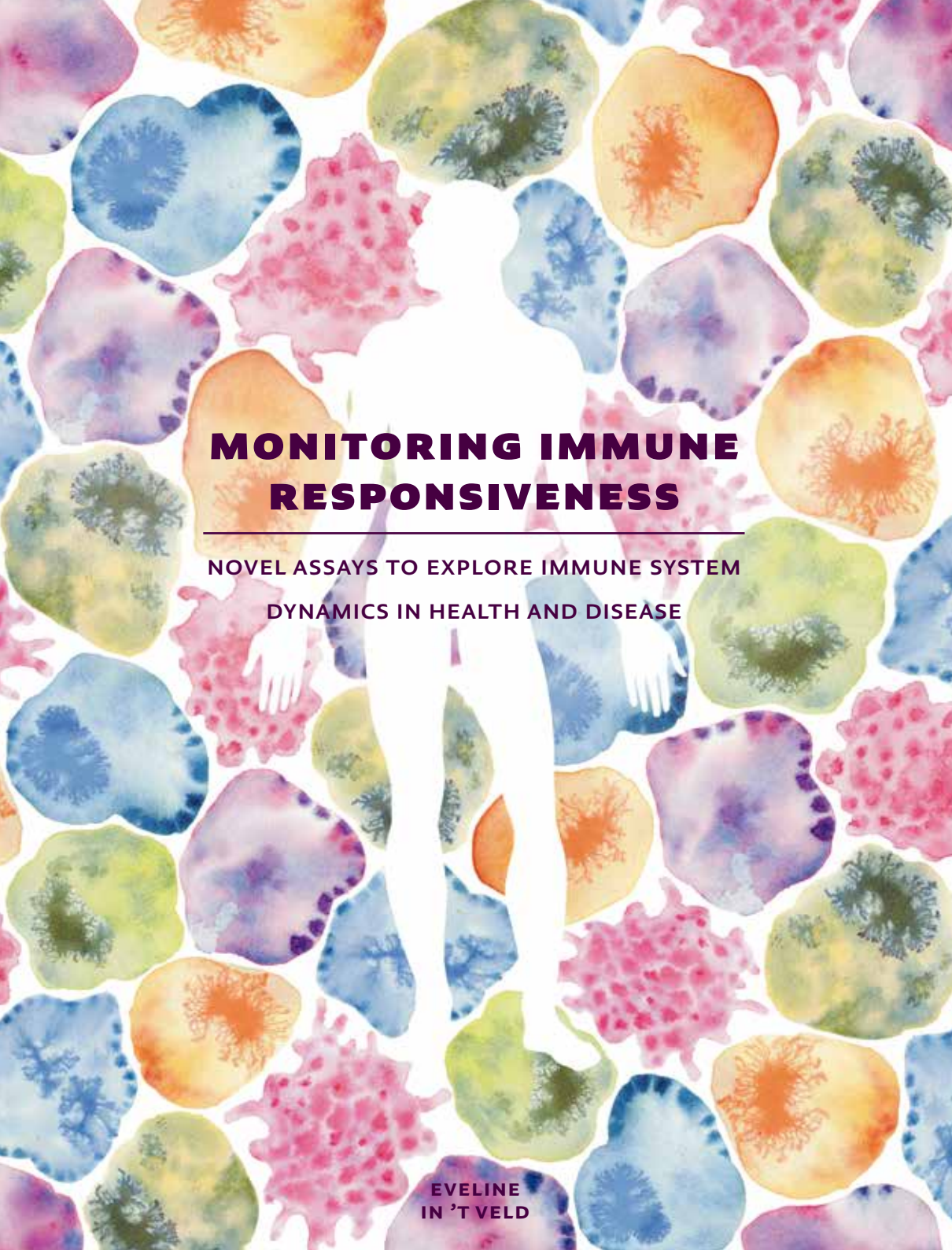

MONITORING IMMUNE RESPONSIVENESS

NOVEL ASSAYS TO EXPLORE IMMUNE SYSTEM DYNAMICS IN HEALTH AND DISEASE

EVELINE
IN 'T VELD

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DESIGN Caroline de Lint, Den Haag
COVER & CHAPTER IMAGES Roos in 't Veld

Publication of this thesis was financially supported by the Foundation Centre for Human Drug Research (CHDR), Leiden, the Netherlands

MONITORING IMMUNE RESPONSIVENESS

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Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op donderdag 14 november 2024
klokke 14:30 uur

door
Aliede Eveline in 't Veld
geboren te Alphen aan den Rijn
in 1993

PROMOTOR

Prof. Dr. J. Burggraaf

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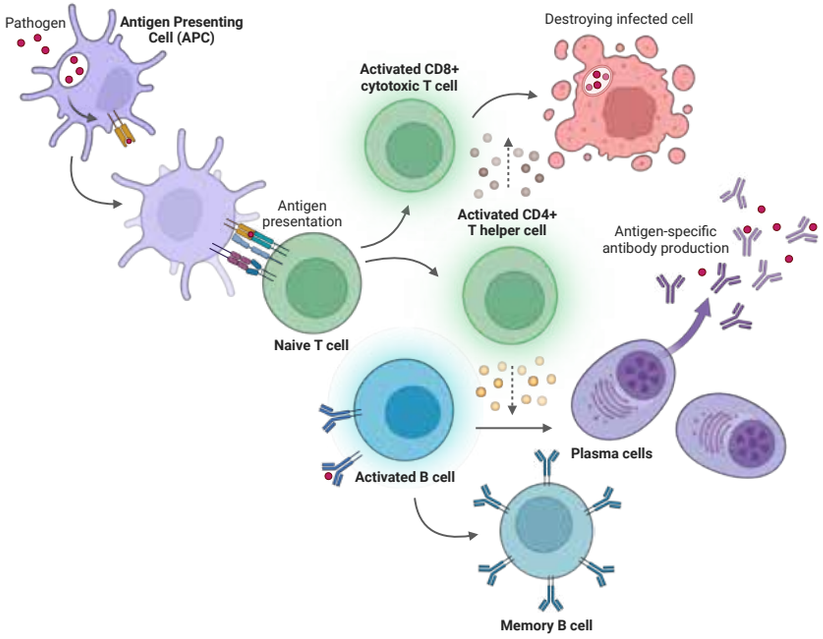
The human immune system is an intricate system that is based on the interaction between different types of immune cells and molecules. It can be broadly classified into two categories: the innate and adaptive immune system. The innate immune system consists of a variety of cell types (e.g. macrophages, dendritic cells, NK cells, neutrophils) which have the capability to recognize and respond to invading pathogens, attract additional immune cells and actively eliminate the pathogens. Among the innate immune cells, macrophages and dendritic cells belong to the group to antigen presenting cells (APCs). Their primary function is the uptake of pathogens, processing them to antigens and subsequently presenting it on the cell surface via MHC class II molecules. This presentation is crucial for activation of the adaptive immune system (T cells and B cells). Upon encountering the antigens presented by the APCs in the presence of co-stimulatory molecules and cytokines, antigen-specific T cells are activated and undergo proliferation. Activated T cells can exert different functions. Cytotoxic T cells (CD8+) identify and destroy infected cells, while T helper cells (CD4+) play a supporting role for other lymphocytes by producing cytokines and promoting the activation of cytotoxic T cells and B cells. B cells undergo activation and differentiation, leading to antibody production and the establishment of immune memory.[1] The role of the different types of immune cells is depicted in **Figure 1**.

Although the immune system is a well-regulated system, there are cases where the interaction between the immune cells is disturbed. Diseases (e.g. allergies, autoimmune disease, cancer), pathogens (e.g. Human Immunodeficiency Virus, HIV), or the use of medication (e.g. immunosuppressants) can impact the immune response, resulting in an over- or underactive immune system.[2-6] An excessive immune response results in the breakdown of healthy cells and tissue, while a suppressed immune response allows for infections or cancers to develop. To treat patients suffering from these immune disruptions, immunomodulatory drugs can be used. Like diseases that modify the immune response, immunomodulatory drugs can have the same consequences if misused.[7] Applying the right medication for the right diagnosis at the right dose is important, but as the immune system consists of so many different cells and molecules, this can also be a challenge. To understand how immunomodulatory drugs work and which pathways they influence, the immune system must therefore be closely monitored.

Figure 1 Overview of the interactions between the main innate and adaptive immune cells



In general, there are two different methods of immunomonitoring. First, the immune system can be monitored by taking blood or tissue samples and examining which immune cells are present and what genes or molecules they express. The pathophysiology, or the effect of the drug on the immune cells, can be studied by comparing patients with healthy controls, treated with non-treated patients, or pre- and post-treatment samples. The second method is functional immunomonitoring by challenging the immune system, and studying how the immune cells respond to the challenge. This challenge can be performed in the patient (referred to as *in vivo*), by a vaccine, allergen, or pathogen. But this challenge can also be performed on biological material derived from the drug-treated patient (referred to as *ex vivo*), by isolating immune cells from the patient and incubating them with a stimulus. Finally, the challenge can be performed on immune cells of patients or

healthy volunteers who are not treated with the investigational drug and incubating these cells with the stimulus and the drug simultaneously (referred to as *in vitro*).

This thesis will focus on immunomonitoring of immunomodulatory drugs that have been clinically approved and are widely used, using a combination of *in vitro* and *ex vivo* immune challenges. The original aim of this thesis was to focus on monitoring immunosuppressive treatment of transplant patients, especially kidney transplantation. However, the COVID-19 pandemic made it difficult to conduct clinical studies in this immune suppressed patient population, and the aim of the thesis was expanded to include immunomonitoring of treatments potentially relevant for COVID-19.

This thesis is therefore split into two sections. **Section I** describes how functional monitoring of the immune response may contribute to personalizing immunosuppressive treatment in transplantation patients. **Section II** presents a comparable approach of immunomonitoring for elucidating the mechanism of action and exposure-effect relationship of the immunosuppressant hydroxychloroquine, initially in the context of COVID-19, but potentially supporting other therapeutic applications of this drug.

Section I

KIDNEY TRANSPLANTATION AND THERAPY

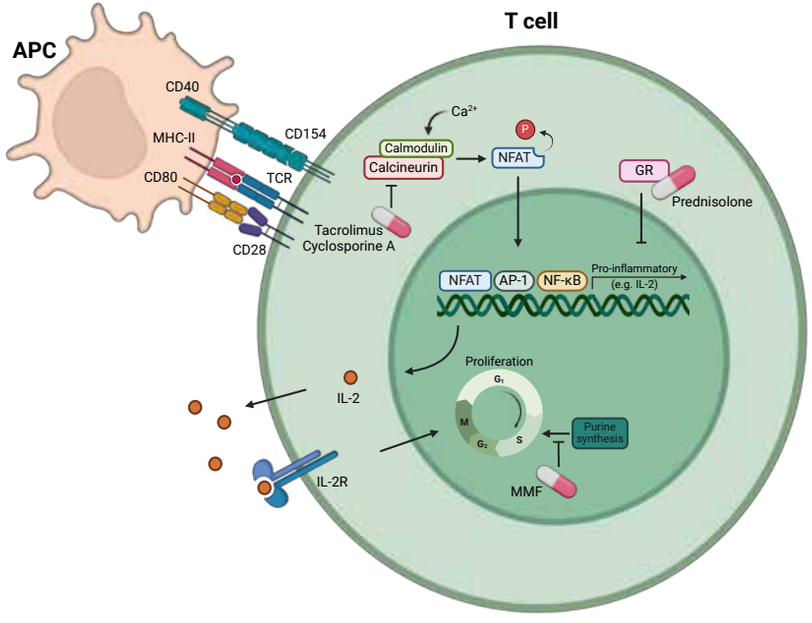
Kidney function can be impaired in different pathophysiological conditions, including diabetes, high blood pressure, and kidney inflammation (e.g. glomerulonephritis or interstitial nephritis). When damage to the kidney cannot be reversed it will ultimately lead to so called end-stage kidney disease for which the only treatment options are dialysis or transplantation. Kidney transplantation is often considered to be the best treatment option, because of the improved quality of life and life expectancy.[8] With a 10-years graft survival rate of 78%, the long-term graft survival has significantly improved over the last decades.[9] Mostly because the crossmatching of donor and recipient, surveillance of infections and cancer, and therapeutic treatment of rejection, infection, and cardiovascular diseases have been improved.[10] Upon organ transplantation, the recipient's immune cells will always induce an immune response against the graft. The antigen presenting cells of the recipient recognize graft tissue as foreign and will present donor antigens to the T cells of the recipient. The T cells proliferate, differentiate into subsets and will start an immune response to destroy the transplanted organ[11], referred to as the alloimmune response.

To prevent rejection, the alloimmune response must be suppressed. Immunosuppressive therapy after transplantation can be divided into two categories: induction therapy and maintenance therapy. Induction therapy is a powerful immunosuppressive treatment that is given at the time of transplantation to prevent acute damage to the allograft. It aims to completely inhibit the potential T cell response that can arise after surgery, at the moment of first antigen presentation. Since IL-2 is the cytokine mainly responsible for T cell proliferation, IL-2 receptor antagonists (e.g. basiliximab and daclizumab) are the recommended immunosuppressant for induction therapy. For patients with a higher risk for rejection, a lymphocyte depleting treatment may be used, such as anti-thymocyte globulin or anti-CD52 antibodies.[12] Maintenance immunosuppressive therapy is less potent than induction therapy. It is used to inhibit the alloimmune response on the long-term, by using a combination of immunosuppressive drugs to target different immune pathways. This treatment regimen usually consists of a combination of a calcineurin inhibitor (i.e. tacrolimus of cyclosporine A), antiproliferative agent (i.e. mycophenolate mofetil) and the corticosteroid prednisolone. The combination of these three drugs is also referred to as 'triple immunosuppressive treatment'. After the first three months of treatment, when the risk of allograft rejection decreases, the dose of maintenance immunosuppressive drugs is gradually lowered to avoid toxicity on the longer term.[12-13]

Calcineurin inhibitors (CNI) exert their function by inhibiting the enzyme calcineurin. When a T cell is activated by an antigen presenting cell, there is an increase in intracellular calcium which leads to calcineurin activation via the binding of calmodulin. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), allowing it to translocate to the nucleus where it induces pro-inflammatory gene expression, such as interleukin 2 (IL-2), and induces T cell proliferation.[14] By inhibiting calcineurin phosphatase activity, CNIs prevent NFAT-mediated gene transcription and subsequent pro-inflammatory immune response. Mycophenolate mofetil (MMF) has a different mechanism of action. Following oral administration, the pro-drug MMF is rapidly converted into mycophenolic acid (MPA). MPA is an inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH), which is important for the de novo synthesis of purine nucleotides.[15] Since

lymphocytes greatly depend on this method of DNA synthesis during proliferation, MPA is a selective inhibitor of lymphocyte proliferation. CNIs and MMF are usually combined with low-dose prednisolone as maintenance immunosuppressive therapy after transplantation. Prednisolone is a glucocorticoid that passages through cell membranes where binds to the intracellular glucocorticoid receptor (GR). Upon binding, the prednisolone-GR complex translocates to the nucleus where it induces anti-inflammatory and blocks pro-inflammatory gene expression. Prednisolone is known to inhibit multiple inflammatory pathways, including nuclear factor-Kappa B (NF-κB), Activator protein 1 (AP-1), and nuclear factor of activated T-cells (NFAT). The effect of all three types of maintenance immunosuppressive drugs on activated T cells is summarized in **Figure 2**.

Figure 2 Activation of the T cell via the T cell receptor (TCR) by an activated antigen presenting cell (APC). The three types of immunosuppressants that are mostly used as maintenance immunosuppressive therapy (i.e. calcineurin inhibitors tacrolimus and cyclosporine A, antiproliferative agent MMF and the corticosteroid prednisolone) are depicted inside the T cell to clarify their mechanism of action.



MONITORING OF IMMUNOSUPPRESSIVE THERAPY

To maintain the transplanted kidney, patients need lifelong immunosuppressive therapy. The current strategy of triple immunosuppressive therapy with a calcineurin inhibitor, MMF and prednisolone gives the best outcome and survival rates. Some of these drugs, however, show a large variability in drug concentrations between patients and within the patient, making it difficult to find the right dose for each individual patient. Pharmacological modulation of the immune system is challenging; too high drug exposure is associated with side effects and toxicity and too low drug exposure increases the risk of rejection of the transplanted organ.[16] It is therefore very important for each patient to receive the right dose of immunosuppressants to achieve optimal drug concentrations and subsequent immune suppression. To do so in clinical practice, the exposure to immunosuppressive drugs is carefully monitored by therapeutic drug monitoring (TDM). For calcineurin inhibitors (i.e. tacrolimus and cyclosporine A), regular TDM is performed based on trough levels (C_0), the drug concentration right before the next dose, or based on a measure of exposure (area under the curve, AUC). If a patient has a blood trough concentration outside the predefined ranges, the dosage is uptitrated to achieve the correct blood concentrations.[17] For the antiproliferative agent MMF, monitoring of drug concentrations is less common than for CNIs, but is now more frequently done in clinical practice. An exposure (area under the curve, AUC) of 30-60 mg x h/L mycophenolic acid (MPA), which is the active component of MMF, is considered safe.[18]

While monitoring drug concentrations has contributed to the prevention of rejection episodes and better survival rates over the years, long-term immunosuppressive treatment still comes with a broad range of side effects, including diabetes, cancer, cardiovascular disease, opportunistic infections, and rejection of the transplanted organ.[7] Patients with similar drug concentrations can have a very different clinical outcome, due to the differences in immune responsiveness between patients. The immune system is a complex network of interacting cells and molecules and its responsiveness can be affected by various factors, including age, gender, genetic variability, and drug interactions.[19-23] The current method of TDM, in which drug concentrations are regularly measured, does not necessarily reflect the immune responsiveness of the individual patient. To prevent side effects due to long-term immunosuppression, it is key to find the optimal balance between over- and under immune suppression for each individual

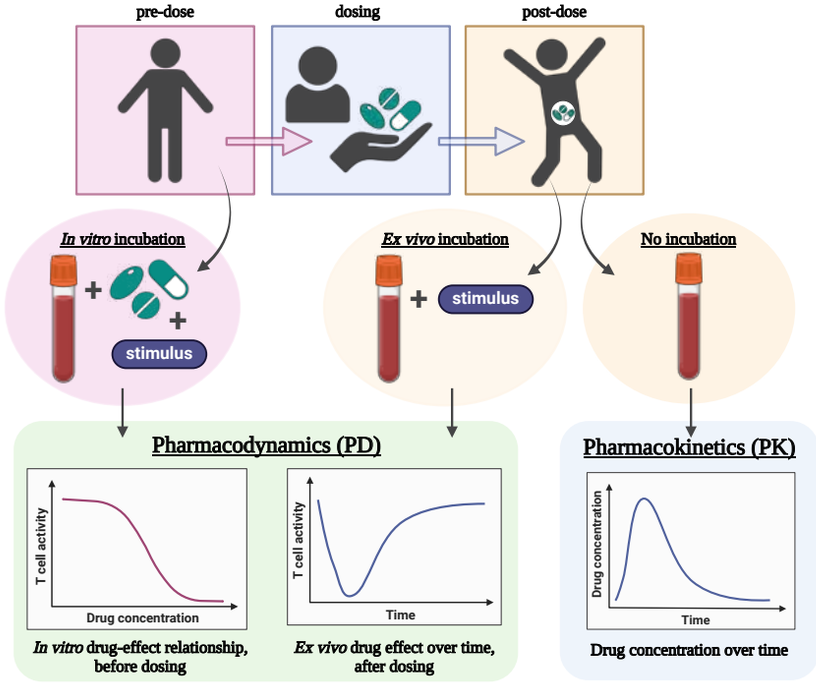
patient. To do so, it might be better to focus on monitoring immune responsiveness, rather than drug concentrations, to help personalizing immunosuppressive therapy.

Section 1 of this thesis describes the search for biomarkers that inform on the immunosuppressive state of transplantation patients. Since T cells are the main mediators of rejection, and most immunosuppressive therapies aim to inhibit T cell activation, we focused on the development of biomarkers that reflect general T cell functionality. By culturing whole blood with a T cell stimulus, the responsiveness of T cells was studied using T cell proliferation, T cell activation marker expression (CD69, CD25, CD71, CD154) and cytokine production (IL-2 and IFN-γ) as readout measures. IL-2 is one of the first cytokines to be produced upon T cell activation, mediated by NFAT, and an important inducer of anti- and pro-inflammatory gene expression.[24] IFN-γ is not only produced by T cells, but essential in the immune response and strongly affects T cell function. CD69 and CD25 are two immediate-early activation markers, whereas CD71 and CD154 are two mid-early activation markers. CD69 is a type II C-lectin receptor, and CD25 is the alpha chain of the IL-2 receptor, both are rapidly expressed after T cell activation and are important for proliferation and activation.[25-26] CD40 ligand (CD154) is a co-stimulatory molecule that interacts with CD40 to activate other cell types, like macrophages and B cells.[27] Transferrin receptor 1 (CD71) is upregulated after T cell activation to increase the iron uptake, which is essential for proliferation.[28]

Pharmacological activity was studied at two different levels; *in vitro* and *ex vivo*. For the *in vitro* response monitoring, whole blood was incubated with a T cell stimulus in combination with a concentration range of the immunosuppressant, to study the concentration-effect relationship of the selected drug. For the *ex vivo* response monitoring, blood samples obtained from subjects that had been dosed with the immunosuppressant were incubated with a T cell stimulus. By measuring the *ex vivo* drug effect over time after intake of the drug, the *ex vivo* concentration-effect relationship can be established (**Figure 3**).

Chapter 2 describes a clinical study in which these functional T cell biomarkers were studied in healthy volunteers receiving a single dose of the calcineurin inhibitor tacrolimus. In **chapter 3** a similar healthy volunteer study is described. For this study, the functional readout measures from chapter 2 have been improved, and their effectiveness in demonstrating

Figure 3 Overview of the clinical study design, highlighting the difference between *in vitro* and *ex vivo* drug effect. *In vitro* drug effect was studied by incubating whole blood with a T cell stimulus in combination with a concentration range of the immunosuppressant, before dosing. The *ex vivo* drug effect was studied by incubating blood samples obtained from subjects that had been dosed with the immunosuppressant. These blood samples were taken at multiple time points after drug intake and stimulated with the exact same T cell stimulus as the *in vitro* blood samples. For both *in vitro* and *ex vivo* incubations, the effect of the immunosuppressant on the immune response was studied. This effect is referred to as pharmacodynamics (PD). At the same time points as the *ex vivo* pharmacodynamic measurements, we also measured the concentrations of the immunosuppressant in the blood of the study participants to assess its pharmacokinetics (PK).



drug effect was studied after a single dose of another calcineurin inhibitor, cyclosporin A. Since standard immunosuppressive therapy after transplantation consists of the combination of a calcineurin inhibitor (e.g. tacrolimus or cyclosporine A) with the antiproliferative agent MMF, in **chapter 4** the

effect of a single dose MMF on the selected readout measures is described. Finally, to test whether the biomarkers studied in healthy volunteers can also demonstrate the effect of these immunosuppressive drugs in patients, an observational study in kidney transplantation patient was performed. **Chapter 5** describes the variability of the immune responsiveness of stable kidney transplantation patients during the day, using the previously described functional T cell readout measures.

Section II

Chloroquine (CQ) was the first effective antimalarial drug to be mass-produced and has been widely used since the 1940s. Where CQ shows toxicity such as gastrointestinal issues, retinopathy and cardiotoxicity, this is less of an issue for hydroxychloroquine (HCQ), a chloroquine analogue. The difference between HCQ and CQ is a single hydroxyl-group, which greatly reduces the toxicity of the drug. During World War II antimalarials were used by millions of soldiers, resulting in the discovery that they also greatly improve skin conditions and inflammatory arthritis, starting research into antimalarials as treatment for autoimmune diseases.[29] Today, HCQ is approved and used for the treatment of autoimmune diseases such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), and systemic lupus erythematosus (SLE).

After oral administration, HCQ is absorbed and widely distributed throughout the body. HCQ is known to bind strongly to tissues such as liver, kidneys, lungs and melanin-containing cells, leading to a large volume of distribution.[30] HCQ is an effective and relatively safe treatment in SLE and is also frequently used in RA patients in combination with other treatments.[31-32] RA is an autoimmune disorder characterized by painful and swollen joints caused by inflammation, while SLE is a systemic autoimmune disease affecting multiple organs. In both diseases, activated T and B cells and their pro-inflammatory cytokine production play a critical role in the inflammatory response and subsequent damage to the inflamed tissue.[33-34] The main reason for HCQ's treatment in these auto-immune diseases is its immunosuppressive effect.[35-36] The exact working mechanism of HCQ, however, is complex and not yet completely understood. Various immunosuppressive mechanisms of HCQ have been described in literature, including the inhibition of toll-like receptor signalling, antigen-presentation, and pro-inflammatory cytokine production37. Besides these immunosuppressive effects, HCQ and CQ have also been investigated as anti-viral therapy[38-40], have shown beneficial effects in lipid metabolism and cardiovascular disease[41-42], and have been studied as anti-cancer therapy.[43] Because of the wide range of diseases for which HCQ can potentially be used as therapy, it is increasingly important to better understand of its immunomodulatory effects and the concentration at which these occur. There are various studies describing HCQ's concentration-effect relationship on cell lines *in vitro*, but there is limited literature available on the exposure-effect relationship of HCQ in a clinical setting, which can be evaluated in primary human cells as *ex vivo* drug activity.

In **section II** of this thesis, we aim to gain a better insight into the immunosuppressive concentration-effect relationship of HCQ. This was especially relevant during the COVID-19 pandemic, when HCQ was studied as prophylactic antiviral therapy. Based on HCQ's inhibiting effect on SARS-COV-2 replication *in vitro*, investigators began testing its efficacy in the prevention of SARS-COV-2 infection in people with a high risk of infection.[44-45] However, the immunosuppressive effects of HCQ remained largely underexposed in these studies, while administering an immunosuppressive drug during a pandemic could potentially lead to an increased risk of infection. **Chapter 6** therefore gives an overview on HCQ's immunosuppressive effects and summarizes its potential risks in the use of HCQ as prophylactic antiviral treatment. In **chapter 7**, the results of a clinical trial are described in which the *in vitro* and *ex vivo* immunomodulatory effects of HCQ were studied to gain a better insight in the role of HCQ on the innate and adaptive immune response. Finally, all findings of section I and II are summarized and discussed in **chapter 8**.

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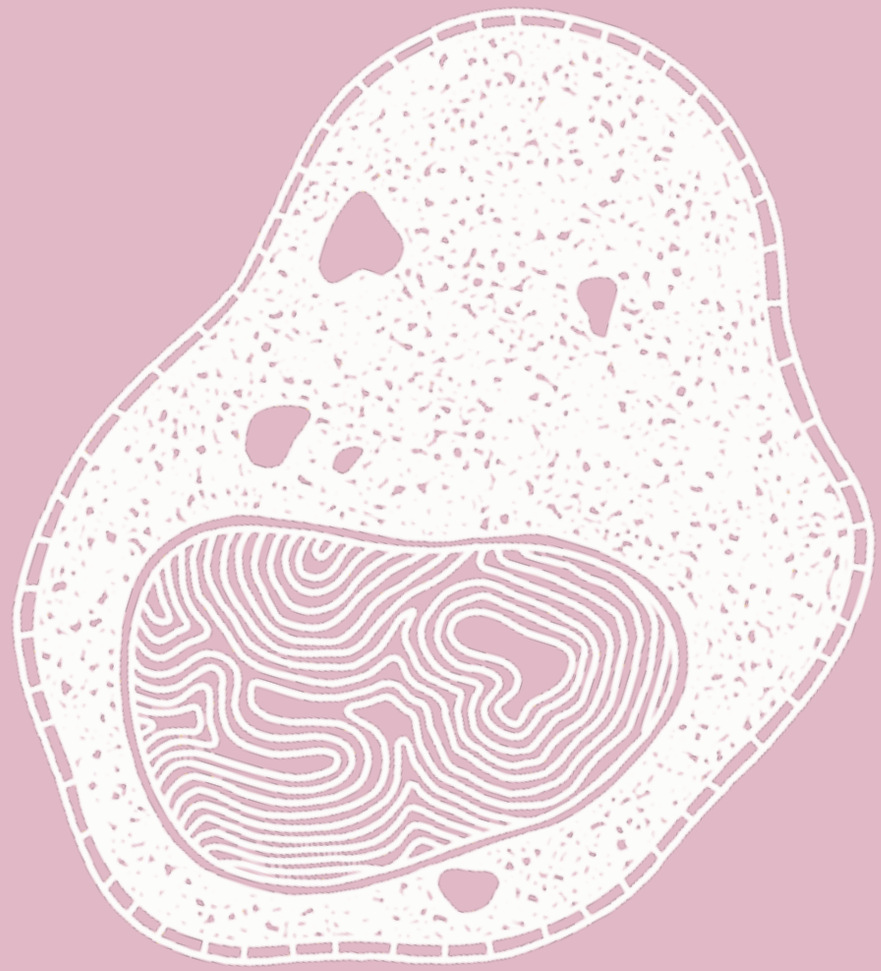
SECTION 1

CHAPTER 2 IMMUNOMONITORING OF TACROLIMUS IN HEALTHY VOLUNTEERS: THE FIRST STEP FROM PK- TO PD-BASED THERAPEUTIC DRUG MONITORING?

International Journal of Molecular Science. 2019 Sep 23, 20(19):4710. doi: 10.3390/ijms20194710.



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Abstract

Therapeutic drug monitoring is routinely performed to maintain optimal tacrolimus concentrations in kidney transplant recipients. Nonetheless, toxicity and rejection still occur within an acceptable concentration-range. To have a better understanding of the relationship between tacrolimus dose, tacrolimus concentration, and its effect on the target cell, we developed functional immune tests for the quantification of the tacrolimus effect. Twelve healthy volunteers received a single dose of tacrolimus, after which intracellular and whole blood tacrolimus concentrations were measured and were related to T cell functionality. A significant correlation was found between tacrolimus concentrations in T cells and whole blood concentrations ($r = 0.71$, $p = 0.009$), while no correlation was found between tacrolimus concentrations in peripheral blood mononuclear cells (PBMCs) and whole blood ($r = 0.35$, $p = 0.27$). Phytohaemagglutinin (PHA) induced the production of IL-2 and IFN-γ, as well as the inhibition of CD71 and CD154 expression on T cells at 1.5 h post-dose, when maximum tacrolimus levels were observed. Moreover, the *in vitro* tacrolimus effect of the mentioned markers corresponded with the *ex vivo* effect after dosing. In conclusion, our results showed that intracellular tacrolimus concentrations mimic whole blood concentrations, and that PHA-induced cytokine production (IL-2 and IFN-γ) and activation marker expression (CD71 and CD154) are suitable readout measures to measure the immunosuppressive effect of tacrolimus on the T cell.

Introduction

A combination of tacrolimus, mycophenolate mofetil (MMF) and glucocorticoids is the standard treatment of choice for kidney transplant recipients. However, despite the excellent survival rate (>90%) in the first year after transplantation in treated recipients, long-term clinical outcomes remain poor.[1-2] Calcineurin inhibitors (CNIs), like tacrolimus, suffer from large intra- and interpatient variability in pharmacodynamic (PD) activity, complicating optimization of an individual dosing strategy.[3] Underexposure can lead to acute organ rejection and formation of donor-specific antibodies, while overexposure is associated with an increased risk of infection, toxicity and malignancies.

The calcineurin inhibitor tacrolimus (FK506) is generally used after allogeneic organ transplantation, but also in other T cell-mediated diseases such as eczema and psoriasis. In order to maintain optimal levels of tacrolimus and to minimize the risk of overexposure, therapeutic drug monitoring (TDM) of pre-dose trough levels (C_0) in whole blood is routinely performed in kidney transplant recipients. Nonetheless, toxicity and rejection still occur in patients within the $C_{0\text{-range}}$, which indicates that the relationship between C_0 measurements and the occurrence of rejection or tacrolimus-related toxicity is debatable.[4-5] Alternatively, the monitoring of intracellular drug concentrations in peripheral blood mononuclear cells (PBMCs) can be informative, although its correlation with clinical outcomes is suboptimal.[6]

The mechanism of action of tacrolimus involves complex formation with the intracellular FK506 binding protein (FKBP12). This complex binds and inhibits calcineurin phosphatase activity, which causes a reduction in the expression of nuclear factor of activated T cells (NFAT)-mediated pro-inflammatory genes, such as interleukin 2 (IL-2) and interferon gamma (IFN-γ).[7] The quantitative relationship between tacrolimus concentration and the effect on T cell functionality has been extensively studied, also in primary human cells.[8-12] However, the effect of tacrolimus in fresh human whole blood samples has been left unattended, while the availability of a whole blood PD assay could be the missing link in TDM of tacrolimus. Unravelling the relationship between T cell functionality and tacrolimus dose, tacrolimus concentration in whole blood and in the target cell would enable a PD- rather than pharmacokinetic (PK)-based approach for future TDM of tacrolimus.

In the current study we aimed to investigate the relationship between tacrolimus dose, tacrolimus concentration, and its effect on the target cell. We developed functional immune tests for the quantification of tacrolimus effects in stimulated human whole blood samples. In an open label study, healthy volunteers received a single dose of tacrolimus, where after tacrolimus concentrations were measured in whole blood and isolated cells (PBMCs and T cells) and were related to T cell functionality (*ex vivo* cytokine production and flow cytometry-based cell activation). This study may be a first step towards the identification of functional PD readout measures for future immunomonitoring of transplantation patients, allowing adjustment of treatment regimens according to the needs of individual patients.

Materials and Methods

STUDY DESIGN

In this open label study, 12 healthy volunteers received a single oral dose of 0.05 mg/kg Prograf®, rounded up to the available dosage forms (0.5 mg, 1 mg, and 5 mg Prograf®). The dosage was based on the recommended dose for renal transplant patients receiving both tacrolimus and mycophenolate mofetil treatment. The healthy volunteers were both male and female, between 18 and 55 years of age and non-smoking. All subjects gave written informed consent and did not have any disease associated with immune system impairment or evidence of any other active or chronic disease. Volunteers were excluded when taken any other drugs within 21 days prior to study start. This study was approved by the independent medical ethics committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands), and is registered with the Dutch Trial Registry (Nederlands Trial Register) under study number NTR7420.

WHOLE BLOOD AND INTRACELLULAR PK

Blood samples were drawn pre-dose and 1.5, 48, 96 and 192 hours after drug administration. The samples for whole blood PK measurement were collected in K2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, US) and stored at -80°°C. PBMCs were collected using sodium heparin CPT tubes (Becton Dickinson) and T cells were isolated from heparinized whole blood by immunomagnetic cell sorting. The RoboSep human T cell isolation kit was used in combination with RoboSep (StemCell Technologies) to label unwanted cells with antibody complexes and magnetic particles, after which T cells were be isolated by automated magnetic sorting. After PBMC and T cell isolation, the cells were washed, and the remaining red blood cells were removed using RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, US). PBMCs and T cells were counted with a MacsQuant 10 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored in PBS at 20 x 10^6 cells/mL at -80°C. The purity of the isolated T cell population was assessed with an anti-CD45-FITC and anti-CD3-VioGreen staining (Miltenyi Biotec).

Whole blood and intracellular tacrolimus concentrations were measured using a Waters Acquity UPLC–MS/MS system by the Department of Hospital Pharmacy, Erasmus Medical Center, as described previously[13].

IN VITRO AND *EX VIVO* WHOLE BLOOD CULTURE

On the same time points as the PK samples, blood was drawn for PD assessments, including whole blood challenges. All incubations were started within one hour after blood withdrawal. For the measurement of cytokine production and surface marker expression, heparinized whole blood (Becton Dickinson) was stimulated with 10 µg/mL phytohaemagglutinin (PHA) (Sigma Aldrich, St. Louis, MO, US). To generate an *in vitro* tacrolimus concentration-effect curve for every individual subject, pre-dose samples were incubated with PHA and a concentrations of 100, 33, 11, 3.7, and 1.2 µg/L tacrolimus (Prograf® for injection). To study the immunosuppressive effect of tacrolimus *ex vivo*, whole blood samples were incubated with PHA only.

Samples for the measurement of calcineurin activity were collected in EDTA tubes (Becton Dickinson). Pre-dose samples were first incubated 1 h at 37°C, 5% CO_2 with a concentration range of tacrolimus. For post-dose samples the analysis was started directly (within one hour) after the blood sample was taken.

CYTOKINE PRODUCTION

Whole blood samples were cultured for 24 h, after which supernatant was collected and stored at -80°C until analysis. IFN-γ and IL-2 concentrations were measured by Meso Scale Discovery Vplex-2 method by Ardena Bioanalytical Laboratory in Assen, the Netherlands.

SURFACE MARKER EXPRESSION

Following 48 h whole blood incubation, RBC lysis buffer (Thermo Fisher Scientific) was used to lyse the red blood cells. After washing with PBS, cells were stained with anti-CD3-Viogreen, anti-CD69-APCVio770, anti-CD95-PEVio770, anti-CD25-PE, anti-CD71-FITC, and anti-CD154-VioBlue (Miltenyi Biotec). The samples were measured after a final washing step, using a MaqsQuant10 analyzer. Before measurement, propidium iodide (PI) (Miltenyi Biotec) was added to assess viability. Analysis of the cell populations was performed with Flowlogic software (Inivai Technologies, Mentone VIC, Australia). For each time point the unstimulated samples was used to set the correct gating. The gating strategy is shown in **Figure S1** in the supplemental material.

CALCINEURIN ACTIVITY

The method described by Sellar et al. was used for the measurement of calcineurin activity.[14] Red blood cells were lysed using ACK lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), after which two million cells were lysed in freshly prepared lysis buffer (50 mM Tris-HCL, 1.0 mM 1,4-Dithiothreitol, 5.0 mM L-ascorbic acid, 0.02% Igepal CA-630, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin). After lysis, the samples underwent three freeze-thaw cycles and were stored at -80°C until use.

A calcineurin phosphatase activity kit (Enzo Life Sciences, Brussels, Belgium) was used to measure calcineurin activity according to the protocol described by Sellar et al. Phosphatase activity of calcineurin (pmol min-1 x 10^6 cells) was calculated by subtracting the phosphate activity that was measured in the presence of Ca^{2+} and calmodulin from the phosphatase activity measured in the presence of EGTA.

DATA ANALYSIS

Data are presented as mean value ± standard deviation (SD). Correlations between tacrolimus concentrations, surface marker expression and cytokine production were analyzed by Spearman's rank-order correlation with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). IC50 of *in vitro* cytokine production and surface marker expression was calculated using GraphPad Prism 6.05 (GraphPad software Inc., San Diego, CA, US).

Results

SUBJECT CHARACTERISTICS

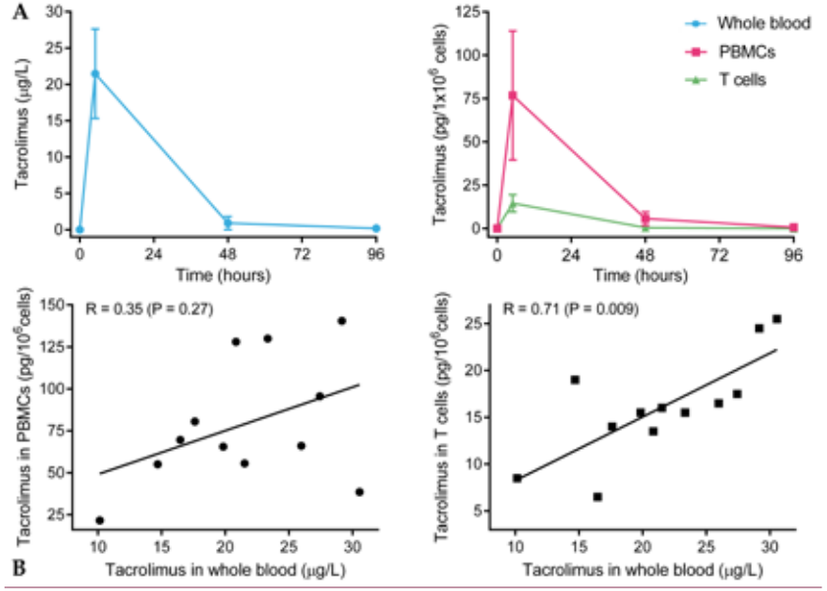
Twelve healthy volunteers, 6 men and 6 women, with a mean age of 31.5 years (range 18-54), participated in the study. Tacrolimus was well tolerated and there were no treatment-related adverse events.

WHOLE BLOOD AND INTRACELLULAR PHARMACOKINETICS

PK profiles of tacrolimus in whole blood, PBMCs and T cells are shown in **Figure 1**. In all matrices, the highest tacrolimus levels were observed at 1.5 h post-dose, and there was a considerable variation between subjects (whole blood: 21.5 ± 6.2 µg/L, PBMCs: 76.8 ± 37.3 pg/10^6 cells, T cells: 14.5 ± 4.9 pg/10^6 cells). At 48 h after administration, tacrolimus concentrations almost returned to baseline levels. PK profiles were not significantly different in whole blood, PBMCs and T cells. Intracellular tacrolimus concentrations, however, differed largely between T cells and PBMCs. The tacrolimus concentration in T cells was on average 5.3x lower compared to the concentration in PBMCs at 1.5 h post-dose.

Whole blood concentrations are generally used for therapeutic drug monitoring in renal transplantation patients. To determine whether whole blood levels may serve as proxy for drug concentrations that enter the target cell, whole blood concentrations were correlated to the intracellular concentrations. Tacrolimus levels in PBMCs showed no correlation with tacrolimus levels in whole blood, whereas tacrolimus levels in T cells were significantly correlated with tacrolimus levels in whole blood (r = 0.71, p = 0.009).

Figure 1 Tacrolimus Pharmacokinetics. (A) Mean tacrolimus concentration over time in whole blood, PBMCs and T cells. Samples were taken at 0 h, 1.5 h, 48 h and 96 h post-dose. (B) Correlation between tacrolimus concentrations at 1.5 h post-dose in whole blood and PBMCs, and between whole blood and T cells.



CYTOKINE PRODUCTION

To study the immunosuppressive effect of tacrolimus, cytokine production was measured after 24 h of phytohaemagglutinin (PHA) stimulation in whole blood. PHA is a lectin known for its membrane glycoproteins binding, including the T cell receptor (TCR), which leads to the activation of T cells[15]. PHA stimulation was used to induce a general T cell response. **Figure 2** shows the *in vitro* tacrolimus concentration-response curve that was generated pre-dose for each individual subject, and the *ex vivo* tacrolimus effect on cytokine production after dosing. A clear *in vitro* concentration-response relationship between tacrolimus and IL-2 and IFN-γ production was found (IC50 of 5.6 µg/L and 18.6 µg/L, respectively), with a 95% inhibition of both cytokines at a tacrolimus concentration of 100 µg/L. *Ex vivo*, tacrolimus strongly inhibited cytokine production at 1.5 h post-dose (10.0% IL-2 and 36.3% IFN-γ production remaining). The observed *ex vivo* cytokine inhibition corresponded well to the *in vitro* cytokine inhibition (**Figure 2**, left panels versus right panels, for tacrolimus concentration of 21.5 µg/L and 1.5 h time point).

Figure 2 *In vitro* and *ex vivo* tacrolimus effect on cytokine production. (A) IL-2 production and (B) IFN-γ production in PHA-stimulated whole blood. *In vitro* tacrolimus effect: pre-dose cytokine production after incubation with a dose range of tacrolimus (100-33-11-3.7-1.2 µg/L). *Ex vivo* tacrolimus effect: cytokine production at 0 h, 1.5 h, 48 h and 96 h after dosing. The cytokine production is calculated as percentage of baseline, and is displayed as mean ±SD.

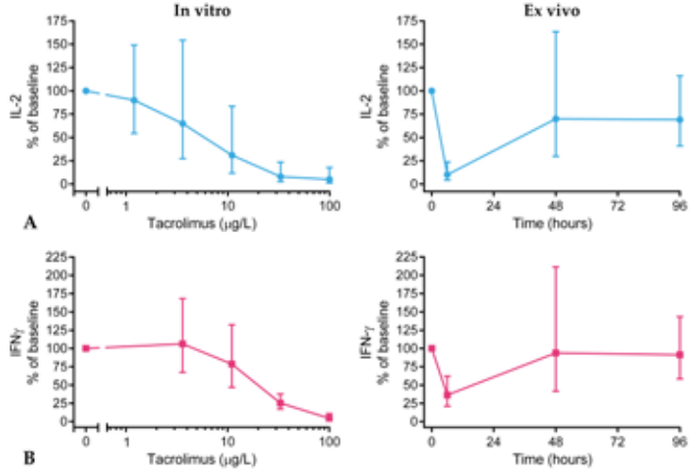
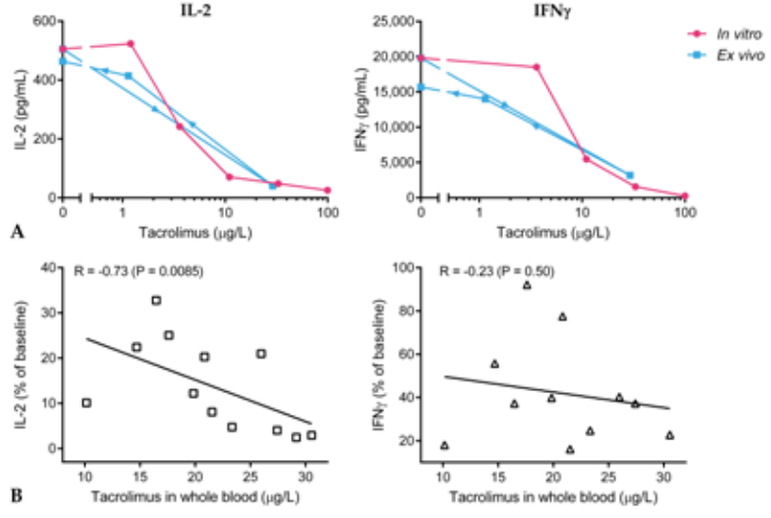


Figure 3 shows the *in vitro* and *ex vivo* effect of tacrolimus in a single subject. At this individual subject level, the *in vitro* concentration-effect curve appears to be a good predictor of the *ex vivo* tacrolimus effect. Cytokine production and tacrolimus concentrations at 1.5 h were correlated, and relative IL-2 production was found to correlate significantly with whole blood tacrolimus concentration (**Figure 3B**, r = -0.73, p = 0.0085). T cell tacrolimus concentration also correlated with relative IL-2 production (r = -0.51, p = 0.09), while this was not confirmed for tacrolimus concentration in PBMCs (r = -0.03, p = 0.92). A stronger inhibition of IL-2 production was observed in subjects with increased tacrolimus levels at 1.5 h post-dose, as compared to subjects with decreased levels of tacrolimus. This correlation could not be confirmed for IFN-γ production, which indicates that the inter-subject variability in IFN-γ production at 1.5 h cannot be explained by the difference in tacrolimus concentration.

Figure 3 Relationship between *in vitro* and *ex vivo* tacrolimus effect. (A) Overlay of *in vitro* and *ex vivo* tacrolimus effect on cytokine production after 24 h whole blood stimulation with for one subject. *In vitro* tacrolimus effect: pre-dose cytokine production after incubation with a dose range of tacrolimus (100-33-11-3.7-1.2 µg/L). *Ex vivo* tacrolimus effect: cytokine production at 0 h, 1.5 h, 48 h and 96 h after dosing. Arrows indicate time lapse. (B) Correlation between tacrolimus concentrations at 1.5 h post-dose in whole blood and relative IL-2 and IFN-γ production.



SURFACE MARKER EXPRESSION

Tacrolimus concentration-effect curves were generated, showing the surface marker expression in pre-dose blood samples (*in vitro* drug effect), and in PHA-stimulated whole blood collected over time (*ex vivo* drug effect). **Figure 4** shows that tacrolimus substantially and concentration-dependently suppressed the expression of CD154 and CD71 *in vitro*. In contrast to PHA-induced cytokine release of IL-2 and IFN-γ, tacrolimus did not fully inhibit PHA-driven surface marker expression of CD154 and CD71. Even at a concentration of 100 µg/L tacrolimus, a concentration that is never achieved in patients, a remaining surface marker expression of approximately 50% was found. Compared to the average expression found in unstimulated samples (a relative expression of 15% CD71, 17% CD154, and 24% CD25, compared to the stimulated sample), there was still surface marker expression remaining that could not be suppressed. The relative expression found in unstimulated samples was the background expression after 6 h incubation of the whole blood samples.

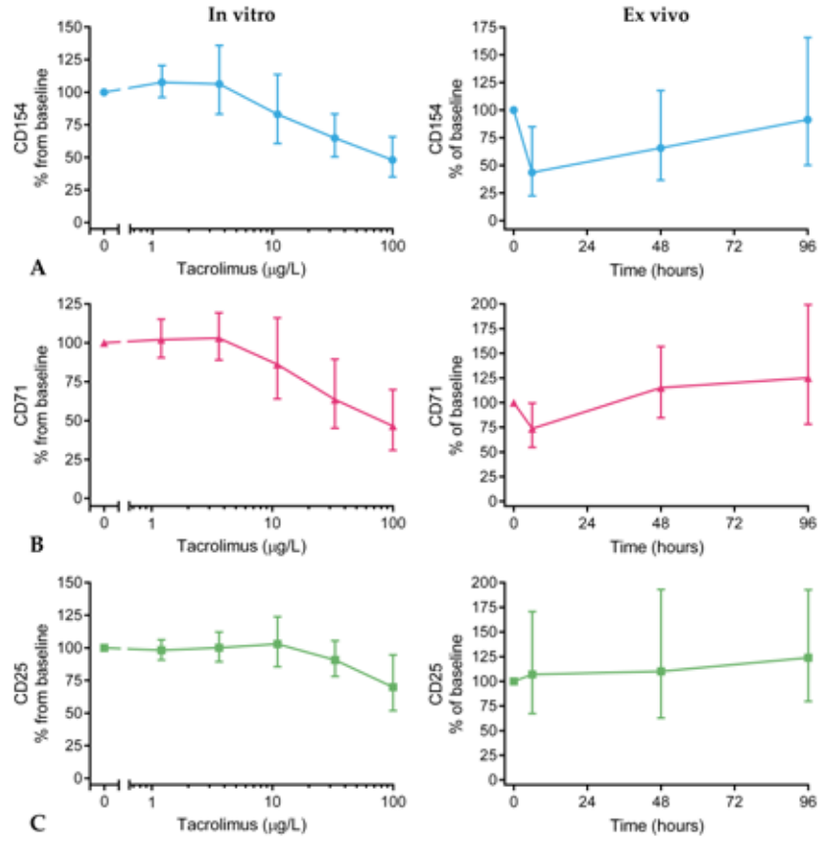
Of all measured activation markers, the expression of CD154 and CD71 on T cells was most strongly inhibited after dosing (44% and 73% remaining, respectively). The maximal *ex vivo* drug effect was observed at a whole blood concentration of 21.5 µg/L tacromlimus at 1.5 h post-dose, which corresponded with the observed *in vitro* effect size. CD25 expression, on the other hand, was not affected by tacrolimus dosing, which corresponded with the observed minor *in vitro* drug effect. A small *in vitro* tacrolimus effect on CD95 and CD69 expression was found, but CD95 and CD69 expressions were not significantly altered by *ex vivo* tacrolimus concentrations (data on file).

No correlation was found between tacrolimus levels in whole blood and CD154 and CD71 expression at 1.5h (data on file).

CALCINEURIN ACTIVITY

Calcineurin phosphatase activity assessed as described by Sellar et al.[14], allows for a PD readout measure more proximal to the drug target. However, this method proved to be unfeasible in the current study, mostly because sample handling was laborious and time-consuming. Data showed a high degree of variability and no dose-response relationship. The spectrophotometric readout of calcineurin activity was properly performed (CV of duplicates < 20%).

Figure 4 *In vitro* and *ex vivo* tacrolimus activity on T cell activation marker expression. Tacrolimus effect on expression of (A) CD154, (B) CD71, and (C) CD25 on CD3+ T cells after 48 h whole blood stimulation with PHA. *In vitro* tacrolimus effect: pre-dose surface marker expression after co-incubation with a tacrolimus concentration range (100-33-11-3.7-1.2 µg/L). *Ex vivo* tacrolimus effect: surface marker expression over time, after PHA stimulation of whole blood samples collected from tacrolimus-exposed subjects. The expression was calculated as percentage of baseline, and is displayed as mean ±SD.



Discussion

Despite routinely performed therapeutic drug monitoring (TDM) in kidney transplant recipients, transplant rejection, infection and (nephro)toxicity are still prevalent among patients with tacrolimus concentrations within the target range. Tacrolimus trough concentrations in whole blood are roughly targeted between 5 and 10 µg/L, based on a few randomized clinical trials showing correlations between target concentration and clinical outcome.[16] To improve the understanding of the relationship between tacrolimus concentration and the effect on clinical outcomes, new readout measures are of profound importance. We conducted a clinical study in healthy volunteers receiving a single dose of tacrolimus. Tacrolimus concentrations were quantified in whole blood, PBMCs, and T cells, and correlated with proximal drug effects (i.e. effects on the target cell).

At 1.5 h after drug administration, the highest tacrolimus concentrations were detected in whole blood, PBMCs, and T cells. At the next time point, 48 h after administration, tacrolimus concentrations had almost returned to baseline levels in all three matrices. This is in line with the reported PK profile of tacrolimus in healthy volunteers.[17] Our data indicate that tacrolimus did not stay significantly longer in target cells than freely circulating in blood. The intracellular tacrolimus concentrations were significantly different in PBMCs and T cells, even though it is known that the majority of PBMCs consist of T cells (60%).[18] Presumably, the difference between tacrolimus concentrations in PBMCs and T cells were not caused by the isolation procedure, but might be caused by another PBMC subpopulation with significant tacrolimus uptake. Washing steps during PBMC isolation are known to diminish the effect of tacrolimus[19], but the number of washing steps were kept similar for both isolations. Since intracellular tacrolimus concentrations have never been measured in cell populations other than PBMCs, the identity of the other cell subpopulation remains unknown. There was no correlation between tacrolimus concentrations in whole blood and PBMCs at 1.5 h post-dose. Evidence supporting this finding is scarce and conflicting; some papers do report a correlation in transplantation patients[13, 20], whereas other papers do not.[21-22] Tacrolimus levels in T cells, on the other hand, did correlate significantly with whole blood concentrations. This finding supports the chosen whole blood-based TDM strategy, and indicates that tacrolimus concentration in whole blood is a good representation of the concentration in the target cell.

Clinical practice nonetheless showed that whole blood tacrolimus concentration is far from ideal as a primary measure of TDM. We hypothesized that drug activity rather than drug concentration may be more appropriate for future TDM. We took a first step towards this future perspective by selecting, optimizing and qualifying functional assays for the quantification of the tacrolimus effect on the T cell. The results of the current study showed that the production of PHA-induced IL-2 and IFN-γ, and the expression of CD71 and CD154 on T cells were the most promising pharmacodynamic readout measures for the quantification of the *in vitro* and *ex vivo* tacrolimus effect. PHA-induced cytokine release was almost completely inhibited at an *in vitro* concentration of 100 µg/L tacrolimus. At an *in vitro* concentration of 20-25 µg/L, which is the peak tacrolimus whole blood concentration in healthy volunteers, the estimated inhibition of cytokine production was 80% for IL-2 production and 50% for IFN-γ production (25% and 50% cytokine release remaining, respectively). At this peak concentration, 1.5 h after administration, tacrolimus inhibited IL-2 production by 90% and IFN-γ production by 64% *ex vivo* (10% and 36% cytokine release remaining, respectively). Though PK and PD data were not formally modelled and integrated (due to limited sample size), this indicates that the *in vitro* tacrolimus effect on cytokine production corresponds decently with the *ex vivo* tacrolimus effect.

Tacrolimus significantly reduced the PHA-induced expression of CD71 and CD154 on T cells. For surface marker CD25, the tacrolimus effect was less obvious. Transferrin receptor (CD71), co-stimulatory molecule CD40 ligand (CD154), and IL-2 receptor (CD25) are all upregulated upon T cell activation[23], and their expression on lymphocytes has been associated to clinical outcomes in transplant recipients.[24-26] Our data showed that all surface markers were expressed by non-stimulated T cells (expression levels of 15%, 17%, and 24%, for CD71, CD154 and CD25, respectively, compared to a PHA-stimulated condition, set as 100%). At a concentration of 100 µg/L, tacrolimus reduced CD71 and CD154 expression to approximately 50% *in vitro*, which means that at this very high tacrolimus concentration, T cell activation was still not fully inhibited. The *ex vivo* tacrolimus effect on the expression of CD71 and CD154 in drug-exposed volunteers was 27% and 56% for CD71 and CD154 expression, respectively (73% and 44% remaining). The *in vitro* and *ex vivo* tacrolimus effect on activation marker expression corresponded well, after having compared the drug effect at 1.5 h (*ex vivo*; tacrolimus peak) with the drug effect at a tacrolimus concentration of 20-25 µg/L (*in vitro*).

Due to logistical reasons, the number of data points with significant tacrolimus concentrations and substantial T cell inhibition were limited, which hampered an analysis involving quantitative correlations or systematic PK/PD integration. However, the *ex vivo* tacrolimus effects in drug-exposed volunteers corresponded well with the *in vitro* tacrolimus effects quantified in baseline samples from the same volunteers. Since the *in vitro* concentration-effect curve seemed to predict the *ex vivo* tacrolimus effect in the current study, it might be an option to base future TDM in transplant recipients on the *in vitro* concentration-effect curve. A critical next step will be to investigate the correlation between the functional T cell measures and clinical outcomes, such as allograft survival/rejection and side effects, in a patient-based study.

Despite the explorative character of this study, it is tempting to speculate about the theoretical T cell activity profile over time in tacrolimus-treated transplantation patients, based on the current study outcomes. It is known that the PK profile of tacrolimus is highly variable between transplant recipients. Trough concentrations are measured 12 h after dosing and can vary between 0.6 and 50 µg/L, with an average of 5-10 µg/L.[27-30] Based on the *in vitro* tacrolimus effect we observed in the current study (which corresponded well with the *ex vivo* drug effect after treatment of healthy volunteers), such concentrations would translate into a minor inhibition of T cell activity: at a tacrolimus whole blood concentration of 5 µg/L, even none of the PD T cell measures were inhibited, except for IL-2 production (approximately 40% inhibition). These findings suggest that with the conventional tacrolimus dosing regimen, some patients may experience time intervals in which their T cell activity is not inhibited (at least not by tacrolimus). On the other hand, in this study the effect of a single dose of tacrolimus was studied, while in patients the responsiveness of T cells might be different because of long-term repeated tacrolimus dosing. Moreover, tacrolimus is usually combined with MMF and corticosteroid treatment, drugs that also suppress T cell activity, but that were not included in the current study. For these drugs, and combinations of these drugs, a dedicated PK/PD study in healthy volunteers should be performed.

In conclusion, this study showed that intracellular tacrolimus concentrations mimic the time course of whole blood concentrations, and that PHA-induced cytokine production (IL-2 and IFN-γ) and activation marker expression (CD71 and CD154) are suitable PD readout measures for the quantification of the immunosuppressive effect of tacrolimus on the T cell. Although the effect of tacrolimus on T cell activity has been studied before[8-11], this is the first study in which whole blood and intracellular tacrolimus concentrations are related to *ex vivo* drug effects, and in which *in vitro* and *ex vivo* tacrolimus effects are compared. As such, the current study may serve as the first step from PK- towards PD-based therapeutic drug monitoring in transplant recipients using tacrolimus.

SUPPLEMENTAL MATERIAL

All mentioned supplementary figures in this chapter can be found on the publisher's website by scanning the QR code.

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CHAPTER 3 MONITORING OF *EX VIVO* CYCLOSPORIN A ACTIVITY IN HEALTHY VOLUNTEERS USING T CELL FUNCTION ASSAYS IN RELATION TO WHOLE BLOOD AND CELLULAR PHARMACOKINETICS

Pharmaceutics. 2022 Sep 16, 14(9):1958. doi: 10.3390/pharmaceutics14091958

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Abstract

Therapeutic drug monitoring (TDM) of calcineurin inhibitors (i.e., tacrolimus and cyclosporin A) is standard of care after solid organ transplantation. Although the incidence of acute rejection has strongly decreased, there are still many patients who experience severe side effects or rejection after long-term treatment. In this healthy volunteer study we therefore aimed to identify biomarkers to move from a pharmacokinetic-based towards a pharmacodynamic-based monitoring approach for calcineurin inhibitor treatment. Healthy volunteers received a single dose of cyclosporine A (CsA) or placebo, after which whole blood samples were stimulated to measure *ex vivo* T cell functionality, including proliferation, cytokine production, and activation marker expression. The highest whole blood concentration of CsA was found at 2 h post-dose, which resulted in a strong inhibition of interferon gamma (IFN-γ) and interleukin-2 (IL-2) production and expression of CD154 and CD71 on T cells. Moreover, the *in vitro* effect of CsA was studied by incubation of pre-dose whole blood samples with a concentration range of CsA. The average *in vitro* and *ex vivo* CsA activity overlapped, making the *in vitro* dose–effect relationship an interesting method for prediction of post-dose drug effect. The clinical relevance of the results is to be explored in transplantation patients on calcineurin inhibitor treatment.

Introduction

Therapeutic drug monitoring (TDM) of immunosuppressive treatment is standard of care after kidney transplantation. TDM is mostly used for individualized dosing of calcineurin inhibitors (i.e., tacrolimus and cyclosporin A) since these are known for their large pharmacokinetic intra- and interpatient variability and small therapeutic window. While overexposure to calcineurin inhibitors (CNIs) can lead to adverse events such as nephrotoxicity, neurotoxicity, malignancies, and opportunistic infections, underexposure can result in allograft rejection and loss of the transplanted organ.[1] To prevent toxicity and rejection, patients are being strictly monitored based on whole blood CNI concentrations, especially in the first year after transplantation. Although the incidence of acute rejection has strongly decreased after implementation of CNI therapy with TDM, there are still many patients that experience severe side effects or rejection after several years of treatment, indicating that the current monitoring strategy needs to be improved.[2-3]

TDM is based on monitoring pharmacokinetic (PK) variability either by measurement of pre-dose concentrations (C_0) or 'limited-sampling' area under the curve (AUC). More sophisticated PK-based biomarkers have been studied, such as CNI concentrations inside the target cell, but the correlation with clinical outcome is debatable.[4-6] None of these parameters, however, do explain the large inter- and intra-patient pharmacodynamic (PD) variability.[7] Hence, new PD-based biomarkers that reflect the immunological status of the patient should be developed to allow monitoring of the individual to the immunosuppressive treatment response and improve personalized dosing. As the first step in this effort, we choose to develop PD-markers for CNIs such as tacrolimus (Tac) and cyclosporin A (CsA), because over the last decade, several promising biomarkers have been identified to monitor drug activity of CNIs. Since both Tac and CsA exert their function by inhibiting the enzyme calcineurin, the most drug-specific biomarker for CNI therapy is measurement of calcineurin enzymatic activity. Several methods to measure calcineurin activity in patients have been studied.[8-11] However, these methods require cell preparation that results in washing out of the drugs, are laborious, or use radioactive labeling, making them rather cumbersome in clinical practice. Besides enzymatic monitoring of the target enzyme, also general immune markers have been studied as a potential pharmacodynamic monitoring strategy. These include cytokine production[12-14], surface marker expression[15], and nuclear factor of activated T cells (NFAT)-mediated gene expression[16], providing insight into the immunosuppressive effect of CNIs at different levels. Although these markers have shown to be informative, none of them has been implemented in clinical practice yet. Overall, the ideal biomarker for TDM correlates well with (prediction of) the occurrence of toxicity and organ rejection and is analytically straightforward. Since transplantation patients receive a combination of immunosuppressive drugs, ideally, these biomarkers reflect the general immune status of the patient rather than drug-specific activity. Because T cells are the main mediators of rejection, and most immunosuppressive therapies aim to inhibit T cell activation, the focus will be on monitoring the functional T cell status. This can be evaluated by cell culture-based assays using whole blood or peripheral blood mononuclear cells (PBMCs), triggered with a T cell agonist. This approach allows quantification of the *ex vivo* activity of T cell suppressive drugs.

We aimed to develop and select PD biomarkers for future evaluation of the general T cell function of transplantation patients on immunosuppressive therapy, which could eventually be used to support TDM. We focused

on whole blood-based biomarkers for evaluation of *ex vivo* drug activity. As a proof-of-concept, we performed a clinical study on healthy volunteers receiving a single dose of CsA. The aim of this study was to evaluate the PK/PD relationship of CsA, a well-known and widely used T cell-suppressive drug. Various T cell function assays were evaluated in parallel, with the goal of selecting assays for future use in transplantation patients. In addition, CsA concentrations were compared between whole blood, isolated PBMCs and T cells to explore potential differences in CsA PK between matrices.

Materials and Methods

STUDY DESIGN

In total, sixteen male and female healthy volunteers were enrolled in this randomized, double-blind, placebo-controlled study. All subjects received a single oral dose of 5 mg/kg CsA (Neoral® capsules, Novartis Pharma, Basel, Switserland), rounded up to the available dosage forms (100 mg and 25 mg Neoral®) or placebo. The dosage was based on the recommended daily dose for renal transplant patients receiving cyclosporine as maintenance immunosuppressive therapy (2–6 mg/kg per day in two equal doses). The inclusion criteria were healthy male or female subjects, 18–55 years of age, which gave written informed consent prior to any study-related procedure. The main exclusion criteria were any disease associated with immune system impairment, evidence of any other active or chronic disease, and intake of any nutrients known to modulate CYP enzyme activity. Of the sixteen subjects enrolled in this study, four subjects received placebo, and twelve subjects received active treatment. The subjects were divided into four groups of four subjects and had a total of 3 visits. Both PK and PD samples were taken pre-dose (0 h), 2 h, 3 h, 6 h, 24 h, and 7 days post-dose. This study was approved by the independent medical ethics committee "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) on 4 March 2019, and is registered in the International Clinical Trials Registry Platform (ICTRP) under study number NL7601. The study was performed in compliance with the Dutch laws on drug research in humans.

WHOLE BLOOD AND INTRACELLULAR PK

For measurement of CsA concentrations in whole blood, samples were collected in K2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and directly frozen at -80 °C until use. For quantification of intracellular CsA concentrations, PBMCs were isolated using sodium heparin CPT tubes (Becton Dickinson). After isolation, red blood cells were lysed with RBC lysis buffer (Biolegend®, San Diego, CA, USA) and PBMCs were resuspended in phosphate-buffered Saline (PBS) (Gibco™, Waltham, MA, USA) and frozen at -80 °C until use. T cells were isolated from sodium heparinized blood (Becton Dickinson) by automated magnetic sorting using RoboSep human T cell isolation kit in combination with RoboSep (Stemcell Technologies Inc., Vancouver, Canada). After a RBC lysis step (Biolegend®) the T cells were frozen in PBS (Gibco™) at -80 °C until use.

The quantification of CsA concentrations in whole blood, PBMC and T cell samples was performed by the department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, the Netherlands. CsA concentration in whole blood was quantified using a previously validated LC-MS/MS assay.[17-18] For the quantification of intracellular CsA concentration in PBMCs and T cells, a new method was developed and validated according to EMA bioanalytical method validation guideline. In short, the calibration standards and quality controls were prepared using different stock solutions and cyclosporin-free PBMCs. Stock solutions of cyclosporin A (Supelco®, Sigma Aldrich, St. Louis, MO, US) and cyclosporin A-D12 (Alsachim, Illkirch-Graffenstaden, France) (1 mg/mL) were prepared in acetonitrile (Merck, Darmstadt Germany) and stored at -20 °C. Substock solutions of 10 mg/L were prepared by diluting the stock solution in acetonitrile. Calibration standards were 0.1, 0.2, 0.5, 1, 5, 10, 20, 50, and 100 ug/L. The lower limit of quantification was set at 0.1 ug/L. QC's low, medium, and high of 0.5, 5, and 50 ug/L, respectively, were used in every analytical run. All were prepared by diluting the working solution with cyclosporin-free PBMC's. Samples that were expected to exceed the calibration curve were diluted 1:1. 100 µL of sample was mixed with 20 µL of internal standard solution (200 µg/L), 40 µL of 0.1 M zinc sulfate, and 100 µL acetonitrile and vortexed for 5 min at 2000 rpm. Subsequently, the samples were centrifuged for 5 min at 1300 rpm, and a 200 µL aliquot of the supernatant was transferred to an autosampler insert vial. A volume of 40 µL was injected into the LC system. All samples were analyzed on a Quantiva UPLC-MS/MS system, consisting of a Dionex Ultimate 3000 series UHPLC system, coupled to a TSQ Quantiva triple stage quadrupole mass spectrometer, all from ThermoFisher Scientific (Waltham, MA, USA). Data were acquired and processed using ThermoFisher Scientific Chromeleon software version 7.2. Chromatographic separation was achieved using an Acquity UPLC BEH C18

1.7 µm 2.1 × 50 mm column, coupled to an Acquity UPLC BEH C18 1.7 µm 2.1 × 5 mm precolumn, both from Waters. The column heater was set to 65 °C. Gradient elution was performed with a mobile phase consisting of a mixture of 0.1% formic acid and 2 mM ammonium in water (eluent A) or methanol (eluent B). Ultrapure water was produced onsite using a PURELAB® Flex purification system from ELGA LabWater (Lane End, UK). The elution gradient (eluent A%/eluent B%) was 80/20 from initiation to 1.5 min, 98/2 from 1.5 min to 4.2 min, and 80/20 from 4.2 to completion of the run at 5.5 min, with a constant flow and pressure of 0.4 mL/min and 350 bar, respectively. The MS instrument was operated in the ESI+ mode, electrospray voltage was 4600 V, capillary temperature was 300 °C, and vaporizer temperature was 350 °C. Sheath, auxiliary, and sweep gas flow rates were set at 18.5, 9.3, and 3.3 arbitrary units, respectively.

The following mass transitions were used for multiple reaction monitoring acquisition (m/z): cyclosporine A 1202.8 → 1184.9, [$^2H_{12}$]-cyclosporine A 1214.8 → 1196.9. All analytical validation parameters were in accordance with the EMA bioanalytical method validation guideline.

WHOLE BLOOD CULTURE AND PD ASSESSMENTS

For all PD assessments, sodium heparinized whole blood (Becton Dickinson) was incubated at 37 °C, 5% CO_2, and stimulated with 10 µg/mL phytohemagglutinin (PHA) (Merck). At the pre-dose time point, the *in vitro* CsA concentration–effect relationship for each individual subject was studied by incubating whole blood samples with a concentration of 10, 3.3, 1.1, 0.36, and 0.12 µg/L CsA (Merck). To study the immunosuppressive effect of CsA *ex vivo*, all whole blood samples post-dose were incubated with PHA only.

For analysis of T cell activation marker expression, the whole blood samples were incubated for 6 h. Red blood cells were lysed using RBC lysis buffer (Biolegend®), and the samples were stained for flow cytometry analysis with anti-CD3-Viogreen, anti-CD69-APCVIO770, anti-CD95-PEVIO770, anti-CD25-PE, anti-CD71-FITC, and anti-CD154-VioBlue (Miltenyi Biotec, Köln, Germany). Propidium iodide (Miltenyi Biotec) was added, and samples were measured using a MACSQuant 10 analyser (Miltenyi Biotec). Cytokine production was analysed after 24 h incubation, and supernatant was collected and stored at −80 °C until analysis. IFN-γ and IL-2 concentrations were measured by the Meso Scale Discovery Vplex-2 method by Ardena Bioanalytical Laboratory in Assen, the Netherlands.

To analyse T cell proliferation, the whole blood samples were incubated for 48 h with PHA and 20 µM of the labelled nucleoside analogue EdU (5-ethynyl-2'-deoxyuridine) (Thermo Fisher Scientific). After red blood cell lysis, the EdU assay was continued according to the manufacturer's instructions. The cells were stained with anti-CD3-VioGreen (Miltenyi Biotec) and viability dye eFluor780 (Thermo Fischer Scientific) for flow cytometry analysis and were analysed using MACSQuant 10 analyser.

DATA ANALYSIS

Analysis of flow cytometry data was performed with Flowlogic software (Inivai Technologies, Mentone VIC, Australia). The gating strategy is shown in **Figure S1**. Data of all plots are presented as mean value ± standard deviation (SD). No formal power analysis was performed for this explorative study with new cell-based biomarkers. For that reason, no formal statistics were applied to discriminate between active and placebo treatment. IC50s of *in vitro* CsA activity was calculated using Graphpad Prism 9.4 (GraphPad software Inc., San Diego, CA, USA).

Results

SUBJECT CHARACTERISTICS AND SAFETY

A total of 12 subjects received a single dose of Neoral (CsA), and 4 subjects received placebo. The baseline characteristics of the 16 healthy volunteers are summarized in Table 1. A total of 35 Treatment Emergent Adverse Events (TEAE) occurred during the study, of which 32 in the Neoral (CsA) group and 3 in the placebo group. All TEAE were mild in severity, transient, and resolved spontaneously (summary in **Table 1**). No clinically relevant changes in blood chemistry, hematology, urinalysis, vital signs, or ECG were identified during the study.

PHARMACOKINETICS

Concentrations of CsA were measured in three different matrices: whole blood, PBMCs, and T cells. All PK profiles are shown in **Figure 1**. The CsA levels in whole blood were highest (1615.3 ± 374 µg/L) at 2 h post-dose and almost returned to baseline levels at 24 h post-dose. The intracellular PK profiles followed a similar profile as CsA levels in whole blood, with peak concentrations of 6.2 ng/10^6 cells (± 2.0 ng/10^6 cells) in PBMCs and 4.4 ng/10^6 cells (± 1.4 ng/10^6 cells) in T cells at 2 h post-dose. Moreover, the CsA concentration in T cells was, on average, 70% of the concentration in PBMCs.

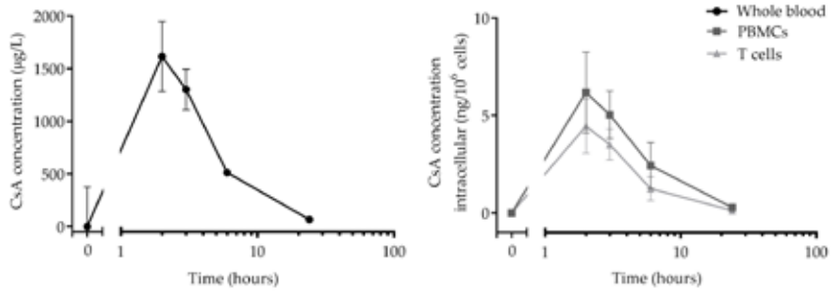
Table 1 Baseline subject characteristics and treatment emergent adverse events by treatment. All TEAEs were coded using the Medical Dictionary for Regulatory Activities (MEDDRA) version 24.1. The grey rows depict the system organ classes. Multiple TEAEs could be reported by the same subject.

Subject characteristics	5 mg/kg Neoral (N = 12)		Placebo (N = 4)	
Age (range)	28.9 (21–52)		25.5 (22–28)	
Gender (female/male)	4/8		2/2	
BMI (kg/m^2), mean (range)	23.3 (19–26.4)		24.0 (21.5–27.5)	
System Organ Class/Preferred term	**Events**	**Subjects (%)**	**Events**	**Subjects (%)**
Any events	32	11 (91.7)	3	3 (75.0)
Gastrointestinal disorders	5	5 (41.7)	-	-
Abdominal pain	1	1 (8.3)	-	-
Faeces pale	1	1 (8.3)	-	-
Nausea	2	3 (25.0)	-	-
General disorders and administration site conditions	18	7 (58.3)	-	-
Burning sensation	1	1 (8.3)	-	-
Fatigue	5	3 (25.0)	-	-
Feeling cold	1	1 (8.3)	-	-
Feeling hot	6	3 (25.0)	-	-
Hyperhidrosis	1	1 (8.3)	-	-
Peripheral coldness	4	3 (25.0)	-	-
Infections and infestations	1	1 (8.3%)	-	-
Candida infection	1	1 (8.3%)	-	-
Nervous system disorders	6	6 (50.0)	3	3 (75.0)
Dizziness	-	-	1	1 (25.0)
Headache	5	5 (41.7)	2	2 (50.0)
Somnolence	1	1 (8.3)	-	-
Renal and urinary disorders	1	1 (8.3)	-	-
Chromaturia	1	1 (8.3)	-	-
Respiratory, thoracic, and mediastinal disorders	1	1 (8.3)	-	-
Nasopharyngitis	1	1 (8.3)	-	-

CSA STRONGLY INHIBITS PD MARKERS POST-DOSE

To study the immunosuppressive effect of CsA administration on the selected PD markers, whole blood samples taken at 0, 2, 3, 6, and 24 h post-dose were stimulated with PHA. After incubation, *ex vivo* cytokine production (IL-2 and IFN-γ), T cell activation marker expression (CD71, CD154, CD69, and CD25), and T cell proliferation were measured. All markers, except for CD69 and CD25 (**Figure S2**), clearly decreased at 2- and 3-h post-dose and returned to baseline at 24 h in the CsA-treated group. The largest CsA effect (compared to baseline and placebo) was found for cytokine production and T cell activation markers (**Figure 2A,B**). Although the difference is small, also for T cell proliferation, it was possible to discriminate between the CsA and placebo group (**Figure 2C**). Interestingly, the level of inhibition of all PD markers was similar at the 2- and 3-h time points, while the CsA concentrations seemed to differ at these time points (**Figure 1**). This could indicate that the CsA concentrations 2- and 3-h post-dose both result in maximum inhibition of the PD markers or that the duration of the PD effect of CsA is longer than the presence of CsA in the cells.

Figure 1 Mean concentration of CsA in whole blood, PBMCs, and T cells. Samples were taken at 0 h, 2 h, 3 h, 6 h, and 24 h post-dose.



IN VITRO CONCENTRATION–EFFECT RELATIONSHIP OF CSA

Besides monitoring the *ex vivo* drug activity, also the *in vitro* concentration–effect relationship of CsA was studied. At timepoint 0 h, whole blood samples of each subject were stimulated with PHA in the presence of a concentration range of CsA, after which *in vitro* cytokine production, T cell activation marker expression, and T cell proliferation were measured. In **Figure 3** *in vitro* concentration-response relationship of CsA for all PD markers is shown. IL-2 and IFN-γ production, together with CD154 expression were most strongly affected by CsA (IC50 of 345, 309, and 385 µg/L, respectively, with 95% CI of 158–752, 120–792, and 256–581), reaching complete inhibition at 3300 µg/L CsA. For CD71 expression, the IC50 was slightly higher than for the other markers (487 µg/L), and its expression could not be completely inhibited, not even at the highest concentration of CsA. T cell proliferation, on the other hand,

showed the strongest dose–effect relationship with an IC50 of 294 µg/L but was more variable between subjects (IC50 95% CI of 62–1401). Absolute *in vitro* data, without the logistic regression model, are shown in **Figure S3**.

Figure 2 Ex vivo CsA activity. (A) *Ex vivo* cytokine production, (B) T cell activation marker expression, and (C) T cell proliferation after a single dose of 5 mg/kg Neoral (colored lines) or placebo (grey lines). Samples were taken at 0 h, 2 h, 3 h, 6 h, and 24 h post-dose.

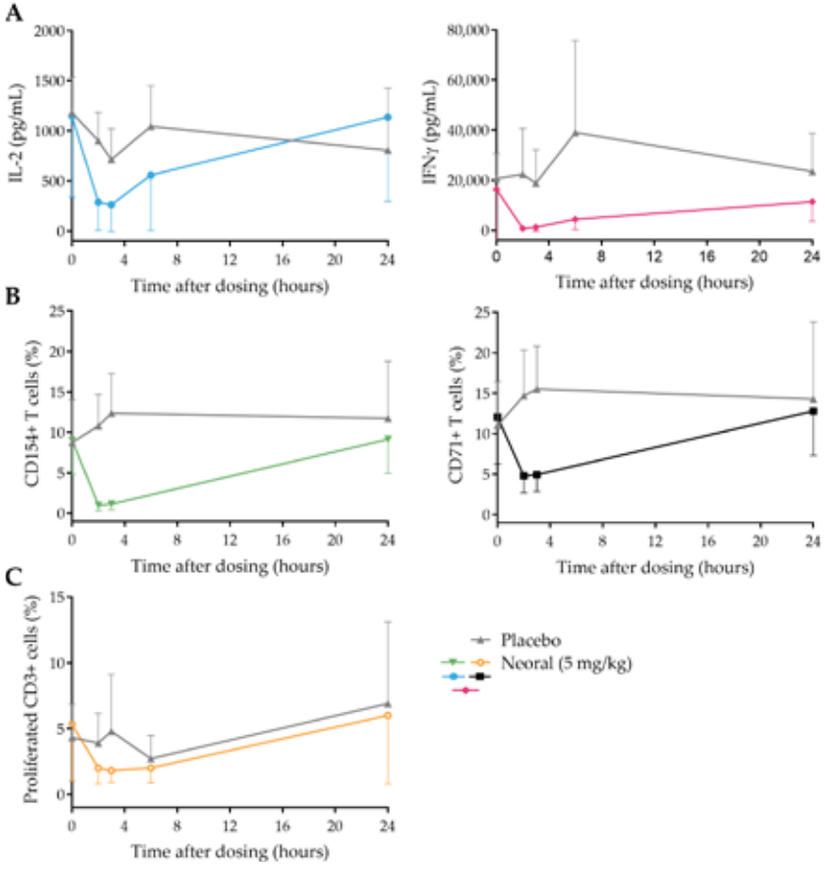
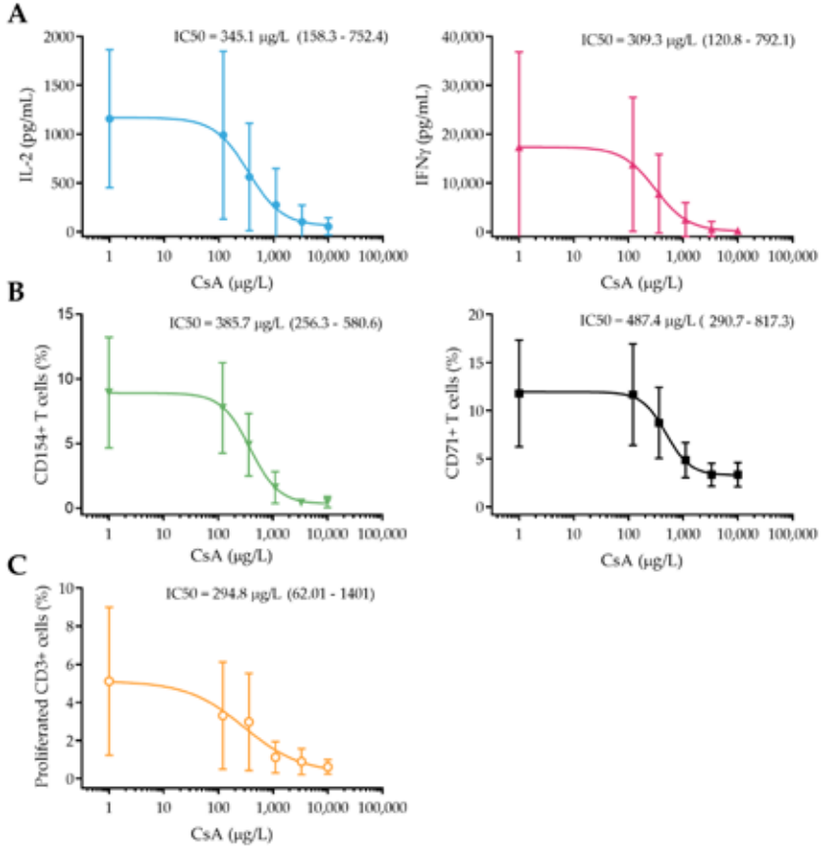


Figure 3 *In vitro* CsA activity. *In vitro* CsA effect on (A) cytokine production, (B) T cell activation marker expression, and (C) T cell proliferation. All whole blood samples were taken pre-dose, stimulated with PHA, and incubated with a concentration range of CsA (10,000, 3300, 1100, 360, 120 µg/L). Absolute data points (± SD) and logistic regression model are plotted.



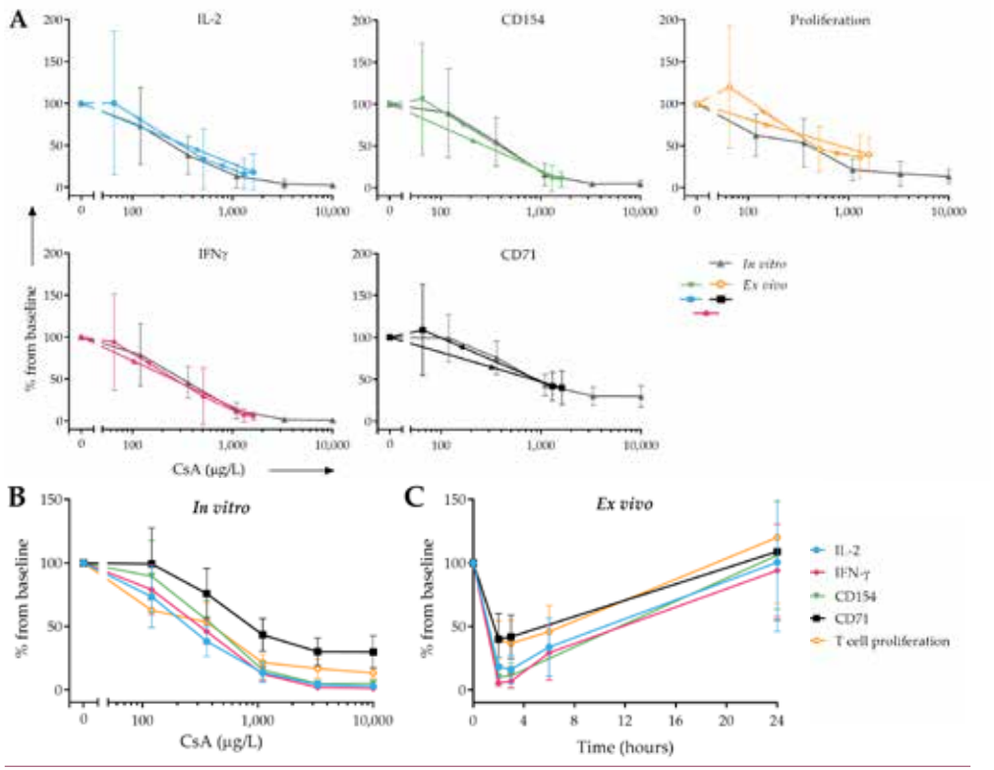
CORRELATION OF *IN VITRO* AND *EX VIVO* DRUG EFFECT

To study the association between the *in vitro* concentration–effect relationship of CsA (as shown in **Figure 3**) and the *ex vivo* CsA effect post-dose (as shown in **Figure 2**), an overlap of mean *in vitro* and *ex vivo* drug effect is plotted in **Figure 4A**. There is a clear overlap between both plots, indi-

cating that the *in vitro* dose–effect relationship seemed a good predictor of the *ex vivo* drug effect for all PD markers.

In **Figure 4B** all PD markers are expressed as percentage from baseline. With a maximum inhibition of >95% *in vitro* and >80% *ex vivo*, the strongest CsA-dependent inhibition was found for IFN-γ production, IL-2 production, and CD154 expression. CD71 expression and T cell proliferation showed a smaller CsA-dependent decrease (inhibition of 70% and 86.7% *in vitro* and 60.1% and 63.3% *ex vivo*, respectively), but still were clearly suppressed by CsA.

Figure 4 Overlay of *in vitro* and *ex vivo* CsA effect. (A) CsA effect on cytokine production, T cell activation marker expression, and T cell proliferation. In grey, the *in vitro* concentration–effect relationship of CsA for each individual PD marker is shown. In color (green, yellow, blue, black, and pink), the *ex vivo* effect of CsA on each PD marker is shown. Arrows indicate the time course of the samples (0 h, 2 h, 3 h, 6 h, and 24 h). (B) *In vitro* and (C) *ex vivo* effect of CsA on selected PD markers (IL-2, IFN-γ, CD154, CD71, and T cell proliferation), expressed as percentage from baseline.



Discussion

Calcineurin inhibitors (i.e., tacrolimus and cyclosporin A) have a large pharmacokinetic variability and small therapeutic window. To optimize dosing regimens, therapeutic drug monitoring (TDM) of calcineurin inhibitors is standard of care after solid organ transplantation. However, this PK-based monitoring strategy apparently provides limited information as transplantation patients still experience rejection of the transplanted organ or severe side effects after several years of treatment.[19] In this study, we therefore aimed to identify PD biomarkers that reflect T cell functionality and activity of immunosuppressive medication for future PD-focused TDM of calcineurin inhibitors in transplantation patients.

We performed a study on healthy volunteers receiving a single dose of cyclosporin A, after which drug concentrations were measured in whole blood, PBMCs, and T cells. To explore if drug concentrations in the target cell (PBMC and T cells) are more informative as a readout measure for TDM compared to the currently used whole blood concentrations, the PK between these three matrices were compared. The highest whole blood concentration of CsA was detected 2 h after drug administration, returning to baseline at 24 h post-dose, which is in line with previously reported PK profiles of CsA in healthy volunteers and patients.[20-21] The intracellular concentrations measured in PBMCs and T cells showed a comparable pharmacokinetic profile, peaking at 2 h post-dose and returning to baseline at approximately 24 h, similar to whole blood. Although for tacrolimus, there is an ongoing debate about the relevance of intracellular drug concentrations compared to whole blood concentrations[4], there is limited literature available for CsA.[22] Based on our results, we conclude that intracellular CsA concentrations do not carry additional value over whole blood concentrations, which is in line with what we previously found for tacrolimus.[23] The current whole blood-based TDM for CNIs seems to be a good representation of the concentrations found in the target cell.

Despite the good correlation between whole blood and intracellular concentrations, concentration-based TDM of calcineurin inhibitors is known to be suboptimal. We aimed to identify biomarkers that reflect the general immune status of the transplantation patient and that could be used for monitoring calcineurin inhibitor activity at a cellular level. Since T cells are the main mediators of rejection, most immunosuppressive therapies, including

CsA, aim to inhibit T cell activation. CsA inhibits the enzyme calcineurin, thereby preventing NFAT activation and subsequent anti- and pro-inflammatory gene expression, including cytokines, chemokines, growth factors, and metabolic regulators.[24] We evaluated various T cell function assays in parallel, with the goal of selecting assays for future use in transplantation patients. We stimulated whole blood with PHA to drive this T cell activation and evaluate *ex vivo* CsA activity at three different levels: cytokine production, T cell activation marker expression, and proliferation.

From a physiological point of view, IL-2 production is the most interesting PD biomarker. It is one of the first cytokines to be produced upon T cell activation, mediated by NFAT, and an important inducer of anti- and pro-inflammatory gene expression.[25] We found that whole-blood stimulated IL-2 production was strongly reduced (with 82% ± 22% compared to baseline, respectively) in CsA-treated subjects compared to placebo at 2 h post CsA administration. *In vitro,* a strong dose–effect relationship for CsA was also found, correlating with the inhibitory CsA effect that was measured *ex vivo*. For IFN-γ a similar reduction was found *ex vivo* (inhibition of 94% ± 5% compared to baseline at 2 h post-dose) and *in vitro* (maximum inhibition of 99% ± 1%). Although IFN-γ is not only produced by T cells, it is a pro-inflammatory cytokine that is essential in the innate and adaptive immune response and strongly affects T cell function.

While cytokine production is detectable a few hours after T cell activation, surface activation markers can be expressed within minutes after stimulation of the T cell receptor (TCR). In this study, we focused on four different surface markers as potential PD readout measures, two immediate-early (CD69 and CD25) and two mid-early T cell activation markers (CD71 and CD154). CD69 is a type II C-lectin receptor, and CD25 is the alpha chain of the IL-2 receptor, both are rapidly expressed after T cell activation and are important for proliferation and activation. Although CD69 and CD25 are strongly associated with T cell activation[26-27], with our experimental setup, no effect of CsA on these markers was found, neither *in vitro* nor *ex vivo*. The mid early activation marker CD154 (CD40 ligand) and CD71 (transferrin receptor 1) showed a strongly decreased expression after CsA administration (of 90 ± 9% and 60 ± 20%, respectively), which corresponded to the inhibitory effect of CsA that was found *in vitro*. CD40 ligand is a co-stimulatory molecule that interacts with CD40 and is primarily expressed by T helper cells. Inhibition of this interaction is currently studied as potential anti-rejection therapy for transplantation patients.[28] Transferrin receptor 1 is a marker that is upregulated after activation to increase the iron uptake of the activated T cell, which is essential for proliferation and known to be dependent on the presence of IL-2.[29]

The purpose of the increased cytokine production and expression of activation markers after TCR activation is to induce proliferation and differentiation of T cells and thereby start the adaptive immune response. To investigate whether a PD marker more distal to TCR stimulation could be a relevant readout measure for CsA activity, PHA-induced T cell proliferation was measured. Administration of CsA to healthy volunteers resulted in a strong inhibition of T cell proliferation, which is not surprising given the strong inhibition of IL-2, an important inducer of T cell proliferation. Overall, we conclude that IL-2 and IFN-γ production, CD154 and CD71 expression, and T cell proliferation are good biomarkers to monitor the immunosuppressive effect of CsA. T cell proliferation is the most laborious readout measure with the longest incubation times, while cytokine production and activation marker expression assays are simpler to execute and may be easier to standardize for clinical practice. Together with our findings that IFN-γ, IL-2, and CD154 showed the strongest dose-response relationship with the smallest variation, these readout measures appear to be most suitable for immunomonitoring of CNI in clinical practice.

When comparing the *in vitro* dose–effect relationship of CsA with our *ex vivo* results of the selected markers, there is a clear correlation. At the individual level, the overlap between *in vitro* and *ex vivo* plots can vary, but the mean *in vitro* dose-response curve seems to be a good predictor for the *ex vivo* inhibitory CsA effect observed after dosing. While whole blood and intracellular CsA concentrations started decreasing at 3 h post-dose (from 1615 µg/L at 2 h to 1300 µg/L at 3 h), this was not reflected at the level of cellular CsA activity. At three hours after administration, all PD endpoints still showed a maximal inhibitory effect of CsA. This suggests that at a concentration of 1300 ug/L the maximum possible inhibition of these markers was reached, which is in line with our *in vitro* data, where maximum inhibition of all markers is reached at 1100 µg/L CsA. As the CsA target ranges are trough level (C_0) of 100–200 µg/L, and a peak level (C_2) of 700–900 µg/L CsA in stable renal transplantation patients[30], it is likely that these patients have varying levels of immune suppression during the day and never reach maximum inhibition of T cell function. Moreover, the *in vitro*

concentration–effect curves of all cellular PD markers have a sigmoidal shape, indicating that the relationship between PK and PD is not a linear but a logistic regression. This suggests that measurement of PD markers, such as cytokine production and T cell activation marker expression, provides more insight into the immunosuppressive state of a patient than the measurement of whole blood drug concentrations. This relationship will be further studied using a PK/PD modelling approach.

In conclusion, we conducted a healthy volunteer study to characterize and select pharmacodynamic markers for monitoring CsA activity and assessment of functional T cell status. We showed that pharmacokinetic profiles for CsA were well comparable between whole blood, PBMCs and T cells, underlining the limited added value of monitoring of intracellular CsA concentrations. We identified several markers (IL-2, IFNγ, CD71, CD154, T cell proliferation) that convincingly showed the immunosuppressive effects of CsA. Moreover, the mean *in vitro* CsA concentration–effect relationship for these markers overlapped with the *ex vivo* drug effect. To evaluate the potential additional clinical value of these PD markers comparted to the current PK-based TDM strategy, a clinical study in renal transplantation patients is planned.

SUPPLEMENTAL MATERIAL

All mentioned supplementary figures in this chapter can be found on the publisher's website by scanning this QR code.

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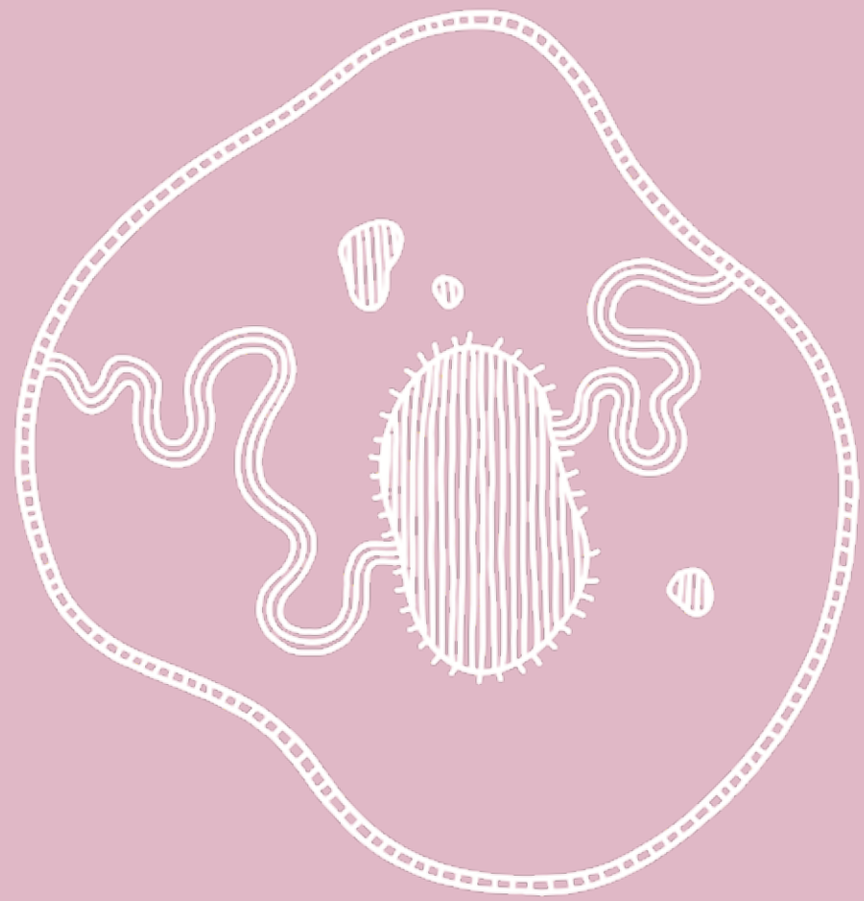
CHAPTER 4 IMMUNE MONITORING OF MYCOPHENOLATE MOFETIL ACTIVITY IN HEALTHY VOLUNTEERS USING *EX VIVO* T CELL FUNCTION ASSAYS

Pharmaceutics. 2023 May 31, 15(6):1635. Doi: 10.3390/pharmaceutics15061635



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Abstract

Mycophenolate mofetil (MMF) is part of the standard immunosuppressive treatment after transplantation and usually given as "one-dose-fits-all" together with a calcineurin inhibitor (CNI). Although drug concentrations are frequently monitored, there is still a group of patients who experience side effects related to excessive or insufficient immune suppression. We therefore aimed to identify biomarkers that reflect the overall immune status of the patient and might support individualized dosing. We previously studied immune biomarkers for CNIs and aimed to investigate whether these are also suitable to monitor MMF activity. Healthy volunteers received a single dose of MMF or placebo, after which IMPDH enzymatic activity, T cell proliferation, and cytokine production were measured and compared to MPA (MMF's active metabolite) concentration in three different matrices (plasma, peripheral blood mononuclear cells, and T cells). MPA concentrations in T cells exceeded those in PBMCs, but all intracellular concentrations correlated strongly with plasma concentrations. At clinically relevant MPA concentrations, IL-2 and IFN-γ production was mildly suppressed, while MPA T cell proliferation was strongly inhibited. Based on these data, it is expected that monitoring of T cell proliferation in MMF-treated transplantation patients may be a valid strategy to avoid excessive immune suppression.

Introduction

Mycophenolate mofetil (MMF) is an immunosuppressant that is usually combined with calcineurin inhibitors (CNIs) and corticosteroids to prevent rejection after kidney transplantation. Following oral administration, the prodrug MMF is rapidly taken up in the upper gastrointestinal tract and converted into mycophenolic acid (MPA). MPA is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), an essential enzyme for de novo guanosine synthesis. Since lymphocytes are greatly dependent on this guanosine synthesis during the S-phase of proliferation, MPA is a selective inhibitor of lymphocyte proliferation.[1]

Originally, the recommended dose for MMF after renal transplantation employed a "one-dose-fits-all" approach of 2 × 1 g per day. Over recent decades, several clinical centers have introduced monitoring of MPA exposure by measuring the area under the curve (AUC_{0-12h}) with a limited sampling strategy (e.g., 0 h, 0.5 h, and 2 h or 0 h, 2 h, and 4 h) to prevent acute rejection in the first year after transplantation.[2-4] The targeted exposure is between 30 and 60 mg*h/L MPA, which is based on the data from several clinical trials where individualized dosing using limited-sampling AUCs resulted in a reduction in acute rejection.[4-8] It was also shown that, similar to CNIs, MMF has large variability in exposure from patient to patient.[9-10] Most side effects found in renal transplantation patients are associated with general immune suppression, such as allograft rejection, infection, diabetes, and malignancies, and occur after several years of treatment. Although these effects cannot be directly correlated with MPA exposure,[11-13] MMF is a standard part of the immunosuppressive treatment regimen and, therefore, potentially contributes to excessive or insufficient immune suppression.

Ideally, the daily dose of immunosuppressive drugs should be adjusted to the individual needs of each transplant patient to prevent toxicity and rejection. In clinical practice, dosing is based on clinical symptoms of excessive or insufficient immune suppression (e.g., infection, toxicity) and monitoring of drug concentrations. Since the immunological response to these drugs can vary from patient to patient, monitoring of the individual patient should ideally be based on a biomarker reflecting the general immune status (level of immune suppression) rather than on drug concentrations. For CNIs, the search for these pharmacodynamics (PD)-based biomarkers has been ongoing for decades[14], but for MMF, only limited data are available. The only PD-based biomarker that has been studied in a clinical setting is the measurement of IMPDH activity, the enzyme that is directly inhibited by MPA.[15] This biomarker is specific for MPA activity and does not provide information on the effect of the other immunosuppressive drugs. Although IMPDH activity and the occurrence of rejection seem to correlate, the outcomes are highly variable.

We previously studied PD biomarkers for CNIs in healthy volunteers who received a single dose of tacrolimus or cyclosporine A. In these studies, we showed that production of IFN-γ and IL-2 in *ex vivo*-stimulated whole blood presented good biomarkers for the immunosuppressive effect of CNIs. Moreover, T cell proliferation and expression of CD154 and CD71 in T cells were also suitable to demonstrate CNI effects.[16-17] In our search for biomarkers that can reflect the overall immune status of the transplantation patient, we aimed to identify the effect of MMF treatment on these previously tested biomarkers.

Therefore, a clinical study was conducted with healthy volunteers evaluating the effect of a single dose of MMF on T cell proliferation and cytokine production. Moreover, MPA concentrations were measured in three different matrices (plasma, peripheral blood mononuclear cells, and T cells) to study the relationship with the PD biomarkers. IMPDH activity was measured to compare the selected biomarkers for MMF/MPA activity with those previously described in the literature. T cell activation was not evaluated in this study because, in our pre-clinical *in vitro* experiments this endpoint was not affected by MMF.

Materials and Methods

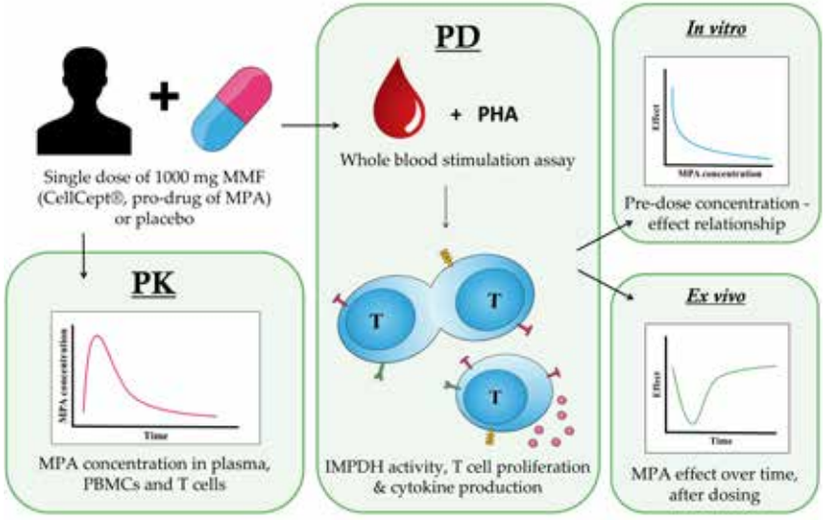
STUDY DESIGN

In this randomized, double-blind, placebo-controlled study, sixteen healthy volunteers were enrolled. Healthy male or female subjects 18–55 years of age who gave written informed consent and did not have any disease associated with immune system impairment were included. All subjects received a single oral dose of 1000 mg MMF (CellCept®, Roche Pharma AG, Grenzach Wyhlen, Germany), which is the recommended daily dose for renal transplant patients receiving MMF as maintenance immunosuppressive therapy (1000 mg CellCept® twice daily). A total of twelve subjects received active treatment and four subjects received the placebo. Both PK and PD samples were taken pre-dose (0 h) and at 0.5 h, 1 h, 2 h, 3 h, 4 h, 24 h, and 7 days post-dose (**Figure 1**). This study was approved by the "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, The Netherlands) on 30 April 2019 and is registered in the International Clinical Trials Registry Platform (ICTRP) under study number NL7804. The study was performed in compliance with the Dutch laws on drug research in humans.

PLASMA AND INTRACELLULAR PK

Concentrations of MPA, the active form of the pro-drug MMF, were measured in plasma, peripheral blood mononuclear cells (PBMCs), and T cells. Samples were processed as described previously[17], and the isolated cells were frozen in PBS until analysis. The quantification of MPA in plasma, PBMCs, and T cell samples was performed by the ISO15189-accredited Clinical Pharmaceutical Laboratory of the Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, the Netherlands.

Figure 1 Graphical representation of the clinical study design. Twelve (12) healthy volunteers received a single dose of 1000 mg MMF (CellCept®, pro-drug of MPA) and four (4) healthy volunteers received a single dose of placebo. PK and PD samples were taken pre-dose (0 h) and at 0.5 h, 1 h, 2 h, 3 h, 4 h, 24 h, and 7 days post-dose. PD samples were directly used for the measurement of IMPDH activity or first stimulated with PHA to measure cytokine production and T cell proliferation. The pre-dose PD samples were used to study the *in vitro* dose–effect relationship for MPA by adding a range of concentrations of 50, 10, 2, 0.4, and 0.08 µg/L MPA to the whole-blood stimulation. Post-dose PD samples were used to study *ex vivo* effects of MPA after a single dose of MMF.



MPA concentration in plasma was quantified using a previously validated LC-MS/MS assay.[18] A new method was developed for the quantification of intracellular concentrations in PBMCs and T cells, which was performed in a similar way as for the measurement of intracellular cyclosporine A concentrations.[17] In short, the calibration standards and quality controls used were mycophenolic acid (Alsachim, Illkirch-Graffenstaden, France) and mycophenolic acid-D3-C13 (Alsachim, France) prepared in acetonitrile (10 mg/L). Calibration standards of 0.1, 0.2, 0.5, 1, 5, 10, 20, 50, and 100 ug/L and QCs of 0.5, 5, and 50 ug/L were diluted in MPA-free PBMCs and used in every analytical run. Before measurement, 100 µL of sample was mixed with 20 µL of internal standard solution (20 µg/L) and vortexed for 15 min. Further sample processing and analysis were performed as previously described for

intracellular cyclosporine A measurement, with the following mass transitions for multiple reaction monitoring acquisition (m/z): mycophenolic acid 321.1→207.0, mycophenolic acid-D3-C13 325.1→211.0. All analytical validation parameters were in accordance with the EMA bioanalytical method validation guideline.

CYTOKINE PRODUCTION AND T CELL PROLIFERATION

For measurement of cytokine production, whole blood was stimulated for 24 h with 10 µg/mL phytohemagglutinin (PHA) (Sigma Aldrich, St. Louis, MO, US), as described previously.[17] At the pre-dose time point, the *in vitro* MPA concentration–effect relationship for each individual subject was studied by incubating whole-blood samples with concentrations of 50, 10, 2, 0.4, and 0.08 µg/L MPA (Sigma Aldrich). To study the immunosuppressive effect of MPA *ex vivo*, post-dose whole-blood samples were incubated with PHA only. IFN-γ and IL-2 concentrations were measured with the Meso Scale Discovery Vplex-2 method by Ardena Bioanalytical Laboratory in Assen, the Netherlands.

T cell proliferation was measured in the same way as in our previous study[17] using an EdU kit (Thermo Fisher Scientific, (Waltham, MA, USA). A MACSQUANT 16 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for flow cytometry analysis of EdU incorporation. T cell proliferation was expressed as the percentage of EdU-positive cells relative to the total number of T cells.

IMPDH ENZYME ACTIVITY

Preceding the start of the clinical study, the *in vitro* relationship between MPA concentration and IMPDH activity was evaluated in fresh whole blood from three healthy donors. Sodium-heparinized whole-blood samples were incubated for 1 h at 37 °C with 5% CO_2 with a range of MPA concentrations (50, 10, 2, 0.4, 0.08, and 0 µg/L). After incubation, PBMCs were isolated from lithium-heparinized whole blood using Lymphoprep and SepMate tubes (Stemcell Technologies Inc., Vancouver, Canada). After washing, the cells were frozen at −80 °C in distilled water. Furthermore, *ex vivo* IMPDH activity was monitored in the clinical study in freshly isolated and stored PBMCs that did not undergo incubation.

Final analysis of IMPDH enzymatic activity was performed by Ardena Bioanalytical Laboratory in Assen, the Netherlands, using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The PBMC lysate was incubated at +37 °C for 3 h in the presence of the IMPDH substrate inosine-5′-monophosphate (IMP) and NAD+. The amount of xanthosine-5′-monophosphase (XMP) was measured using LC-MS/MS. To express the IMPDH activity in µmol XMP/min/mg protein, the protein content of the PBMC sample lysates was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

DATA ANALYSIS

Flow cytometry data analysis was performed with Flowlogic software version 7.3 (Inivai Technologies, Mentone VIC, Australia), the gating strategy of which is shown in supplemental material, **Figure S1**. Data for all plots are presented as mean values ± standard deviation (SD). No formal power analysis was performed given the explorative character of the study. For that reason, no statistical analysis was applied to discriminate between active and placebo treatment. Repeated-measures correlation[19] (rmcorr) was used for determining the common within-individual association for repeated measures assessed at multiple time points for multiple individuals. Repeated-measures correlation was conducted using the rmcorr R package.[20-21]

Results

SUBJECT CHARACTERISTICS AND SAFETY

A total of 12 subjects received a single dose of CellCept (MMF) and 4 subjects received the placebo. The baseline characteristics of the 16 healthy volunteers are summarized in **Table 1**. A total of five adverse events (AEs) occurred during the study, which were fatigue and decreased platelet count. The AEs were all characterized as mild and only occurred in CellCept-treated subjects.

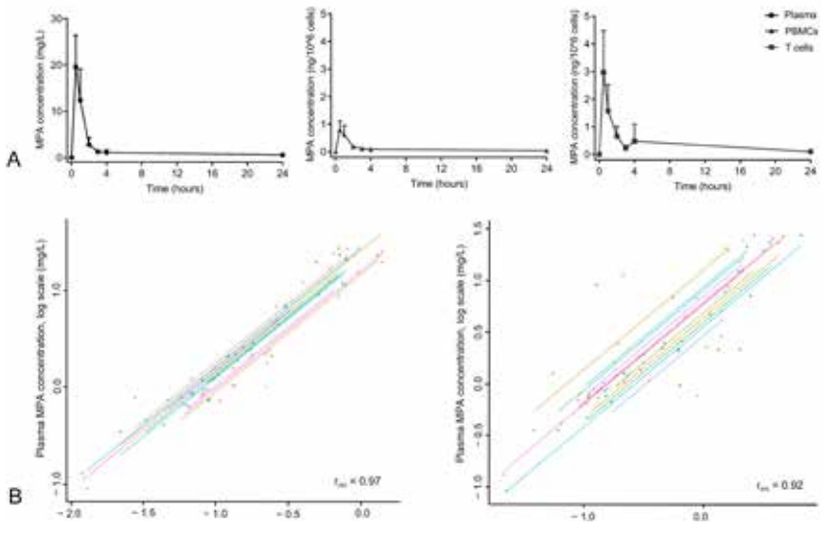
Table 1 Baseline subject characteristics

Subject Characteristics	1000 mg CellCept (N = 12)	Placebo (N = 4)
Age (range)	24.8 (18–39)	27.2 (18–46)
Gender (female/male)	5/7	3/1
BMI (kg/m^2), mean (range)	24.41 (18.7–28.9)	21.85 (20.1–24.3)

PLASMA AND INTRACELLULAR PHARMACOKINETICS

In **Figure 2A**, the MPA plasma concentration is shown. The highest plasma concentration was observed at 0.5 h post-dose (19.6 ± 6.8 mg/L), after which it strongly decreased to a concentration of 1.1 mg/L (± 0.97 mg/L) at 4 h post-dose. Intracellular MPA concentrations in PBMCs and T cells showed a comparable PK profile to the plasma concentration. The peak concentration in T cells was 3.75 times higher compared to the peak concentration in PBMCs, indicating a preferential uptake of MPA by T cells in the circulation, provided that different isolation procedures were not responsible for this difference.

Figure 2 MPA Pharmacokinetics. (A) Mean concentration of MPA in plasma, PBMCs, and T cells after a single dose of 1000 mg MMF. Samples were taken at 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, and 24 h. (B) Repeated-measures correlation for MPA in plasma vs. PBMCs and plasma vs. T cells. Each subject is represented by a different color, and pair concentration at each time point is indicated with a dot. Repeated-measures correlation (RRM) was calculated, and the correlation coefficient is shown in the plots.



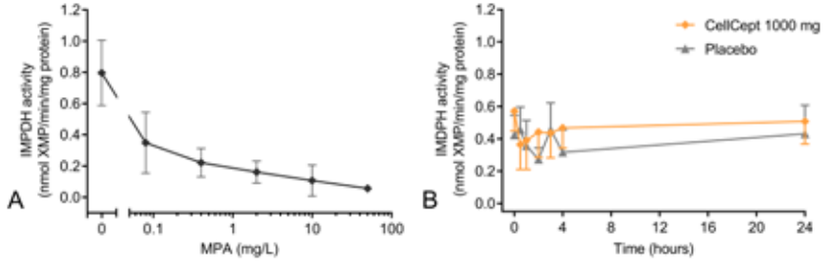
It has been stated that most of the administered MMF is metabolized into the inactive mycophenolic acid glucuronide (MPAG) and excreted in urine. Part of the MPAG is excreted in bile, after which it is back-converted into MPA and reabsorbed in the colon, leading to an increase in MPA plasma concentration.[22] In our data, this enterohepatic recirculation was clearly visible in the intracellular MPA concentrations in T cells, where a second peak at 4 h post-dose was found. Although not for all subjects, this second peak in MPA concentration was also visible in PBMCs (in PBMCs for three subjects and T cell concentrations for six subjects). For none of the subjects could a second peak in plasma concentration be detected.

Finally, the repeated-measures correlations between MPA plasma concentrations and intracellular concentrations are shown in **Figure 2B**. Intracellular MPA concentrations in both PBMCs and T cells showed strong repeated-measures correlations with plasma concentrations that were measured at the same time points (r_{rm} of 0.97 and 0.92, respectively), indicating that these intracellular PK parameters provide us with similar information as the plasma concentrations.

IMPDH ENZYMATIC ACTIVITY

Whole-blood incubations with a range of concentrations of MPA resulted in a strong inhibitory *in vitro* effect from MPA on inosine-5′-monophosphate dehydrogenase (IMPDH) activity (**Figure 3**). At a concentration of 0.08 mg/L MPA, IMPDH activity was inhibited by more than 50%, and a maximum inhibition of 93% was reached at a concentration of 50 mg/L. The *in vitro* dose–response relationship, which was studied in three donors, supported the inclusion of the IMPDH assay in the clinical study. The maximum *ex vivo* MPA effect was an inhibition of 28% at 30 min post-dose and could not be discriminated from the IMPDH activity in the placebo group at the same time point.

Figure 3 (A) In vitro and (B) ex vivo MPA effects on inosine-5′-monophosPHAte dehydrogenase (IMPDH) enzymatic activity. *In vitro* samples were incubated with a range of concentrations of MPA (50, 10, 2, 0.4, and 0.08 mg/L). *Ex vivo* samples were taken before and 0.5 h, 1 h, 2 h, 3 h, 4 h, and 24 h after subjects were dosed with MMF.



Moreover, the variability in the maximum *ex vivo* MPA effect (CV of 82%) indicates there was strong intersubject variability. At 30 min post-dose, a plasma concentration of 19.6 mg/L MPA was measured, which was expected to result in an inhibition of ~90% IMPDH activity based on the *in vitro* dose–response relationship, indicating that *in vitro* MPA effects were not predictive of the *ex vivo* MPA effect.

T CELL PROLIFERATION

In **Figure 4**, the *in vitro* and *ex vivo* effects of MPA on PHA-induced T cell proliferation are shown. With an IC50 of 0.113 mg/L, a strong dose–effect relationship was found *in vitro* (**Figure 4A**). Although the IC50 varied widely from subject to subject (95% CI of 0–0.78), nearly all subjects already reached the maximum inhibitory effect on T cell proliferation (inhibition of 95 ± 5%) at a concentration of 2 mg/L MPA.

Furthermore, a strong *ex vivo* MPA effect on T cell proliferation was observed compared to placebo (**Figure 4B**). At 30 min post-dose, the proliferation was completely inhibited (97% ± 11%), and even after 24 h, the proliferation was still reduced compared to baseline. However, there was variation in proliferation between subjects. To clarify the *in vitro* MPA concentration–effect relationship in relation to *ex vivo* MPA activity (measured post-dose), the overlay of the *in vitro* and *ex vivo* MPA effects on T cell proliferation is shown in **Figure 4C**. While an *in vitro* MPA concentration of 0.4 mg/L already resulted in an 89% inhibition of T cell proliferation, substantially higher MPA exposure was required to reach comparable T cell proliferation inhibition *ex vivo*. At the 3 h, 4 h, and 24 h time points, plasma concentrations of 1.3, 1.1, and 0.6 mg/L MPA were found, which resulted in inhibition of T cell proliferation of 74%, 75%, and 34%, respectively (**Figure S2**). Although the variability in T cell proliferation between subjects was high at these time points (CV of 130%, 191%, and 75%, respectively), the discrepancy between *ex vivo* and *in vivo* was visible for all individual subjects.

CYTOKINE PRODUCTION

The last PD biomarkers studied were represented by PHA-induced production of IFN-γ and IL-2. Incubation of pre-dose whole-blood samples with a range of MPA concentrations did not result in a strong *in vitro* effect on cytokine production (**Figure 5A**). For IL-2 production, inhibition at the higher MPA concentrations was found (inhibition of 53% and 70% at MPA concentrations of 10 and 50 mg/L), but the effect size was variable between subjects (inter-individual CV of 64% and 54%, respectively). For IFN-γ production, the *in vitro* MPA effect was less pronounced, with inhibition of 30% and 50% (inter-individual CV of 67% and 43%, respectively) at the highest MPA concentrations.

After MMF intake, a decrease in the PHA-induced production of IFN-γ and IL-2 was visible in the first 4 h post-dose (**Figure 5B**). In the placebo-treated subjects, however, a comparable decrease in cytokine production was found, making it impossible to discriminate between the placebo- and MMF-treated subjects. Change-from-baseline figures can be found in the Supplemental Material, **Figure S3**.

Figure 4 In vitro and ex vivo MPA effects on T cell proliferation. (A) *In vitro* samples were incubated with a range of MPA concentrations (50, 10, 2, 0.4, and 0.08 mg/L). The absolute data points (±SD, solid line) and logistic regression model (dotted line) are shown. (B) *Ex vivo* samples were taken before and 0.5 h, 1 h, 2 h, 3 h, 4 h, and 24 h after the subjects were dosed with MMF. (C) An overlay of the *in vitro* and *ex vivo* MPA effects on T cell proliferation expressed as percentage difference from baseline. Arrows indicate the time course of the samples.

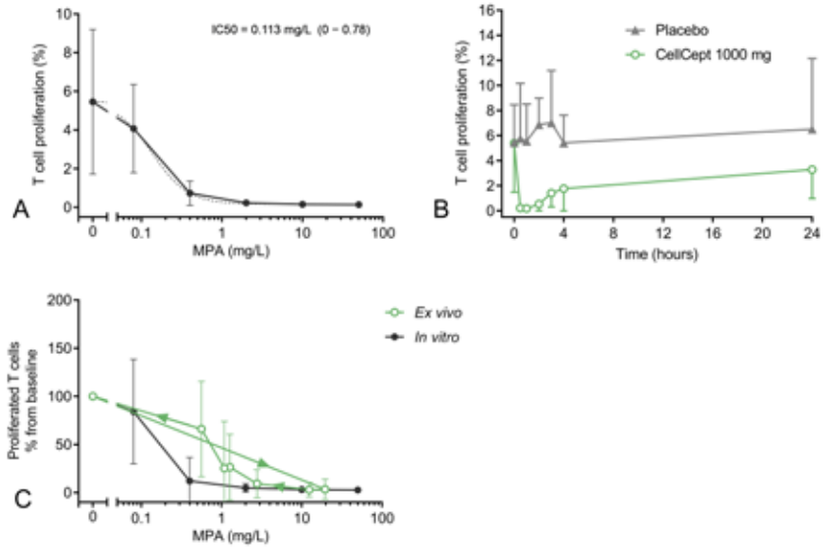
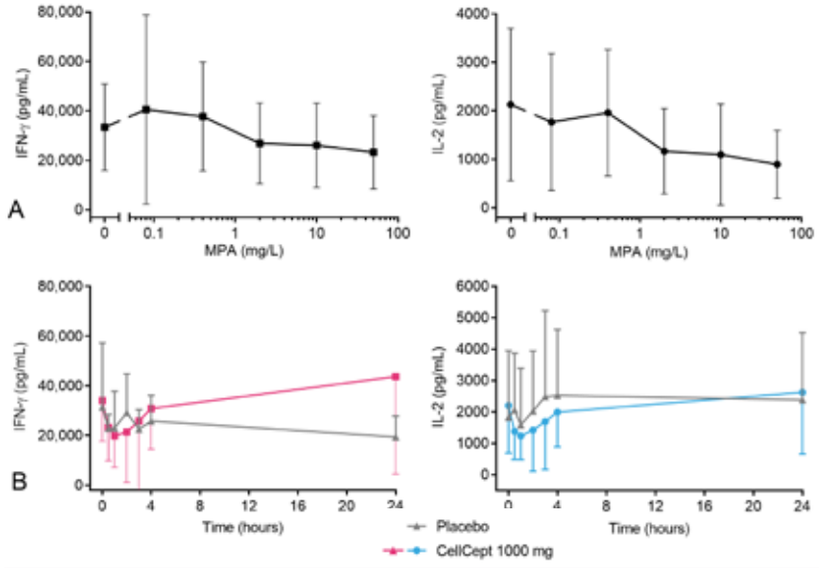


Figure 5 (A) In vitro and (B) ex vivo MPA effects on IFN-γ and IL-2 production. *In vitro* samples were incubated with a range of MPA concentrations (50, 10, 2, 0.4, and 0.08 mg/L). *Ex vivo* samples were taken before and 0.5 h, 1 h, 2 h, 3 h, 4 h, and 24 h after the subjects were dosed with MMF.



Discussion

Ideally, the daily dose of immunosuppressive drugs prescribed to renal transplant patients (e.g., tacrolimus, MMF, and prednisolone) should be adjusted to the individual needs of the patient. As these patients will use immunosuppressive drugs for the rest of their lives, it is important to attain an optimal balance between over- and undersuppression of immunity. Individualized therapy could be advanced by the availability of biomarkers that reflect the immunosuppressive state of individual patients rather than dosing based on drug concentrations or the occurrence of side effects. In the search for such PD biomarkers, we previously selected functional T cell assays for monitoring of the immunosuppressive effects of cyclosporine A and tacrolimus.[16-17] In the current study, we investigated if these biomarkers are also suitable to monitor the immunosuppressive effect of MMF and examined their relationship with drug concentrations.

Twelve healthy volunteers received a single oral dose of 1000 mg MMF, after which MPA (the active form of MMF) concentrations were measured in plasma, PBMCs, and T cells. With a peak concentration of 19.6 mg/L MPA at 0.5 h and a concentration of 1.1 mg/L MPA at 4 h, the plasma PK profile was similar to what has been previously reported for healthy volunteers[23], with a slightly higher exposure than that found in stable renal transplantation patients (8–15 mg/L).[24-25] Little is known about the relationship between PBMC and plasma MPA concentrations. While in early renal transplantation patients, there was no correlation between plasma and PBMC MPA levels at C_0 (pre-dose), at later time points, a strong correlation at 1.5 and 3.5 h post-dose was found.[26] Although, in our study, the method of measuring intracellular drug concentrations was different, we found similar results. A strong repeated-measures correlation between plasma concentrations and the MPA concentrations inside the target cell (e.g., PBMCs and T cells) was found. Based on our data from healthy volunteers, there is no added value in measuring intracellular MPA concentrations rather than plasma concentrations.

Interestingly, the MPA concentrations in T cells were higher than those in PBMCs at the same time points, which contrasts with what we previously reported for intracellular tacrolimus and cyclosporine A concentrations.[16-17] T cells are the largest cell population present in PBMCs, suggesting that T cells take up more MPA into the cell compared to all other cell populations that are present in PBMCs (i.e., monocytes, B cells, NK cells, dendritic cells). This would be beneficial as lymphocytes are the targeted population for post-transplant immunosuppressants and are primarily affected by MPA. This is the first study in which MPA concentrations inside T cells have been measured. It should be noted that the methods of MPA measurement for PBMCs and T cells were identical, but the methods of cell isolation for these populations were different. Therefore, there is a possibility that the difference in PK between PBMCs and T cells was also affected by the technical differences.

The PD marker for MMF that is most frequently described in the literature is IMPDH enzymatic activity.[15] IMPDH catalyzes the oxidation of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), which is an essential step in DNA synthesis of proliferating lymphocytes. Since MPA is an IMPDH inhibitor, enzymatic IMPDH activity is the biomarker that is closest to the drug target. A strong *in vitro* concentration–response relationship with a maximum inhibition of 96% for IMPDH activity was found. Based on

these *in vitro* data, the plasma concentration of 19.6 mg/L MPA, observed at 0.5 h after MMF treatment, was expected to result in an inhibition of ~90% for IMPDH activity. However, no substantial *ex vivo* effect from MMF treatment on IMPDH activity was observed, indicating a large discrepancy between *in vitro* and *ex vivo* MPA effects for which we currently do not have an explanation. The method used to measure IMPDH activity was similar to what has been previously reported in the literature and was successful in demonstrating the inhibitory effect of MMF in renal transplantation patients, with a maximum inhibition of 75% at MPA plasma concentrations of ~7 mg/L.[27] We saw a comparable effect in the *in vitro* dose–response relationship but to a much lesser extent in the *ex vivo* data. Looking at the IMPDH activity in the placebo group of the study, there was large variability in IMPDH activity over time. This has also been described in the literature and is the reason that studies are currently more focused on measurement of IMPDH activity in erythrocytes than on using PBMCs.[15,28] Moreover, although IMPDH activity is an interesting readout measure to demonstrate direct MPA effects, it does not provide information about the activity of the overall immune response.

T cell proliferation is an immune biomarker that is directly affected by IMPDH activity, being more distal from the drug target. Of all the PD readout measures that were studied, proliferation had the strongest *in vitro* concentration–effect relationship, with an IC50 of 0.11 mg/L MPA. Interestingly, the concentration at which maximum inhibition of T cell proliferation was reached *in vitro* varied from subject to subject. While some subjects showed complete inhibition of T cell proliferation at 0.4 mg/L MPA, in other subjects, only 40% inhibition was found at this MPA concentration. These observations were consistent with the *ex vivo* MPA effect at 3–4 h post-dose, when lower plasma concentrations were measured. At these time points, intrasubject variability was higher than at the other time points (CVs of 78–100%), indicating that the concentration at which 100% inhibition was reached varied from subject to subject. In clinical practice, a target AUC_{0-12h} of 30–60 mg*h/L is recommended because this has been associated with lower occurrence of allograft rejection.[4] On average, these AUC reference values correspond roughly to a trough concentration (C_0) of ~2 mg/L.[29-30] At this concentration, both our *in vitro* and *ex vivo* data showed >90% suppression of T cell proliferation, indicating that, with the current AUC monitoring strategy, T cell proliferation in renal transplantation patients is most likely always completely suppressed. However, we did identify reasonable intersubject variability in the concentration at which maximal T cell inhibition was found. If this is also true in renal transplantation patients, it might be more relevant to find the lowest AUC at which an individual patient reaches maximum inhibition of T cell proliferation rather than maintaining the same AUC reference values for all patients.

Recognition of MHC/antigen complexes by T cell receptors and CD3 results in T cell activation and subsequent pro-inflammatory gene expression, including IL-2. By binding its receptor (CD25), IL-2 induces differentiation and proliferation of cytotoxic T cells and T helper cells, resulting in the production of more IL-2 and other pro-inflammatory cytokines, such as IFN-γ.[31] PHA-induced cytokine production, as measured in our study, therefore did not directly reflect IMPDH activity but was rather a more general measure of T cell responsiveness. While tacrolimus and cyclosporine A significantly impact IL-2 and IFN-γ production[16-17], no such effect was observed for the MPA exposures evaluated in this study *in vitro* or *ex vivo*. *In vitro*, MPA concentration-dependently inhibited IL-2 production with a maximum inhibition of 70%, while for IFN-γ production, the highest concentration of MPA resulted in an inhibition of 53%. This rather limited effect could be explained by the fact that tacrolimus and cyclosporine A directly act on T cell activation via suppression of nuclear factor of activated T cells (NFAT), while MPA only affects T cell proliferation but not activation. T cells present in the whole-blood culture can probably still produce cytokines, but the MPA-mediated reduction in proliferation resulted in a smaller number of T cells present in the culture. Despite the moderate inhibitory effect of MPA *in vitro*, no *ex vivo* MPA effect on cytokine production was seen. A dip in cytokine production was visible at 1 h post-dose in both the MMF and the placebo groups, potentially caused by the diurnal rhythmicity in circulating T cell numbers.[32]

T cell proliferation and PHA-induced IL-2 and IFN-γ production have previously been shown to be effective biomarkers in demonstrating the immunosuppressive effects of calcineurin inhibitors. Since renal transplantation patients often receive the combination of a calcineurin inhibitor with MMF, we aimed to investigate whether these biomarkers are also suitable for monitoring MMF treatment effects. We substantiated this biomarker panel with IMPDH activity, reflecting proximal MPA activity. PHA-driven production of IL-2 and IFN-γ was only mildly affected by MPA at clinically

relevant exposures, and we could not discriminate between placebo- and MMF-treated subjects in terms of IMPDH enzymatic activity. However, we showed that PHA-induced T cell proliferation is an excellent measure to monitor MPA activity. The only limitation of this study was the small subject population, which prevented formal statistical and PK/PD analyses from being performed and resulted in high intersubject variability for the readout measures.

In conclusion, based on the observed MPA plasma concentrations in a renal transplantation setting[33] and our *in vitro* and *ex vivo* MPA effects, it is expected that renal transplantation patients will always have completely inhibited T cell proliferation in conventional clinical MMF regimens, as well as at MPA trough levels. The MPA concentration at which maximum T cell inhibition was reached varied from subject to subject, implying that MMF dose reduction based on measurement of T cell proliferation in transplant patients may be a valid future strategy to avoid oversuppression of the immune system with MMF. Before this biomarker can be used in transplantation patients, however, we need to further validate these readout measures in a clinical setting. Since MMF is usually given in combination with CNIs, a follow-up study with renal transplantation patients to investigate the relationship between MMF and CNI dose, T cell proliferation, and clinical outcome would be valuable.

SUPPLEMENTAL MATERIAL

All mentioned supplementary figures in this chapter can be found on the publisher's website by scanning this QR code. The figures are listed on the bottom of the page as Appendix A.

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CHAPTER 5 IMMUNE RESPONSIVENESS IN STABLE KIDNEY TRANSPLANTATION PATIENTS: COMPLETE INHIBITION OF T CELL PROLIFERATION BUT RESIDUAL T CELL ACTIVITY DURING MAINTENANCE IMMUNOSUPPRESSIVE TREATMENT

Clinical and Translational Science. 2024 June 25, 17(6):e13860. doi: 10.1111/cts.13860

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Abstract

The recommended immunosuppressive treatment after kidney transplantation consists of tacrolimus, mycophenolate mofetil, and low-dose corticosteroid. Drug concentrations are monitored using therapeutic drug monitoring (TDM), which does not necessarily correlate with pharmacodynamic activity. To find the balance between optimal efficacy and minimal toxicity, it might be more informative to monitor patients' immunological status rather than drug concentrations. We selected a panel of T cell-based immune assays, which were used for immunomonitoring of fourteen stable kidney transplantation patients. Whole blood was incubated with a T cell stimulus, after which T cell proliferation, T cell activation marker expression and cytokine production were measured to study residual immune activity *in vitro* (before drug intake; drug added to the incubation) and *ex vivo* (after drug intake). T cell proliferation was completely suppressed in all patients over the full day, while IL-2, IFN-γ, CD71 and CD154 showed fluctuations over the day with a strong inhibition (75% - 25%) at 2 hours post-dose. The level of inhibition was variable between patients and could not be related to pharmacokinetic parameters or the presence of regulatory or senescence immune cells. Moreover, the level of inhibition didn't correlate with the *in vitro* tacrolimus drug effect as studied by incubating pre-dose blood samples with additional tacrolimus. Overall, IL-2, IFN-γ, CD71 and CD154 seem to be good markers to monitor residual immune activity of transplantation patients. To evaluate the correlation between these pharmacodynamic biomarkers and clinical outcome, prospective observational studies are needed.

Introduction

For patients with end stage renal failure, kidney transplantation is the best treatment option, greatly improving life quality and expectancy.[1-2] After kidney transplantation, long-term immunosuppressive treatment is required to prevent rejection. Currently, the recommended treatment regimen consists of a calcineurin inhibitor (CNI, tacrolimus or cyclosporine A) and an antiproliferative agent (mycophenolate mofetil, MMF) with or without low-dose corticosteroid (prednisolone).[3] Although these drugs are known to give the best outcome and survival rates, they are also known for their narrow therapeutic window and large inter-patient variability, making it difficult to identify the right dose for each individual patient. Current dosing strategies are therefore individualized to prevent rejection while minimizing exposure by using therapeutic drug monitoring (TDM).[4] This is particularly important for CNIs because of the many side effects that can arise with long-term use, such as nephrotoxicity, new onset diabetes, malignancies and hypertension.[5] Also for MMF (the pro-drug of mycophenolic acid, MPA), the therapeutic use has been changed in some clinical centers over the last decade, from standard to individualized dosing based on MPA exposure to prevent under- and over immunosuppression.[6-7]

TDM has been shown to be effective but does not necessarily correlate best with the biological activity of the CNIs and MMF. There are countless factors that can affect patients' sensitivity to the immunosuppressive treatment regimen, including age, gender, genetic variability, and drug-drug interactions.[8-12] Some of these factors (e.g., CYP-polymorphisms or drug-drug interactions), directly affect the drug concentration, and are usually accounted for by individual TDM. For other factors (e.g. age, lifestyle and immune variability), it is more difficult to understand and predict how these affect drug efficacy. To find the balance between optimal efficacy and minimal toxicity, it may be more informative to monitor biological drug activity with specific pharmacodynamic biomarkers rather than drug concentrations. We therefore focused on identifying T cell-based immune assays that can inform on the patient's immune status. This was supplemented by measuring torque teno virus (TTV) load, a marker that has shown to correlate with functional immunity in kidney transplant recipients.[13] For the T cell-based assays, whole blood was incubated with a T cell stimulus, after which T cell proliferation, T cell activation marker expression and cytokine production were measured to study the residual T cell activity. In previous studies, we showed that these immune assays could be used to measure drug effect of CNIs (calcineurin A and tacrolimus) and MMF in healthy volunteers.[14-16] To evaluate if these assays are also suitable for monitoring drug activity in a clinical setting, we conducted an observational trial in kidney transplant recipients.

Materials and Methods

STUDY DESIGN

Fourteen (14) kidney transplantation patients from the Transplant Center from Leiden University Medical Center (LUMC) were enrolled in this observational study. All patients had undergone a single kidney or kidney-pancreas transplantation > 2 years before study participation and were currently on maintenance immunosuppression therapy consisting of prednisolone, MMF and tacrolimus (twice daily) adjusted to target trough levels. The study consisted of a single visit, where blood samples were taken before (0 h) and 1, 2, 4, 6 and 8 hours after the patients' morning dose (**Figure 1**). This study was approved by the "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) on 9 April 2020, and is registered in the International Clinical Trials Registry Platform (ICTRP) under study number NL8639. The study was performed in compliance with the Dutch laws on drug research in humans.

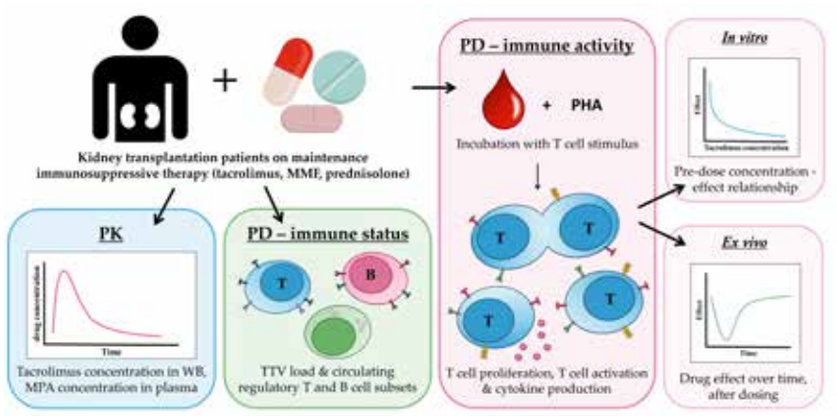
PHARMACOKINETIC ANALYSIS

At each time point, blood samples for pharmacokinetic monitoring of tacrolimus and MMF were taken. Concentrations of tacrolimus were measured in whole blood, whereas for MPA, the active form of the pro-drug MMF, plasma concentrations were measured. Samples were collected in K2EDTA tubes (Becton Dickinson), and stored at -80°C. Quantification of MPA and tacrolimus concentrations were performed by the ISO15189 accredited Clinical Pharmaceutical Laboratory of the department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, the Netherlands using previously validated LC-MS/MS assays.[17-18]

IN VITRO AND *EX VIVO* PHARMACODYNAMIC READOUT MEASURES FOR IMMUNE ACTIVITY

All pharmacodynamic (PD) readout measures were performed as described previously15. Sodium heparinized whole blood samples were incubated with 10 µg/mL phytohemagglutinin (PHA) (Sigma Aldrich) to study *ex vivo* drug activity. In addition, at the pre-dose time point (0 h), a concentration range of 100, 33, 11, 3 and 1 ng/mL tacrolimus (Prograf, Astellas Pharma) or a single concentration of 50 µg/mL MPA (Sigma Aldrich) was added to the whole blood cultures to study *in vitro* drug activity.

Figure 1 Graphical representation of the study methodology. Fourteen kidney transplantation patients were monitored for 8 hours. PK and PD samples were taken before and several times after intake of their immunosuppressive medication. PD samples were either used to study the immune status of the transplantation patients, by measuring circulating regulatory T and B cell subsets and TTV load, or to study residual immune activity of the transplantation patient, by incubating the samples with a T cell stimulus (PHA). *In vitro* immune activity was studied by incubating pre-dose (0 h) PD samples with PHA and a concentration range of tacrolimus or a single concentration of MPA. *Ex vivo* immune activity was studied by incubating post-dose PD samples with PHA only.



T cell activation marker expression was analysed after 6 hours incubation, after which the cells were stained for flow cytometry analysis with anti-CD3, anti-CD69, anti-CD25, anti-CD71, anti-CD154 and propidium iodide as listed in **Table S1**. The reason for choosing these T cell activation markers has been previously described 15. For measurement of cytokine production, the whole blood samples were stimulated for 24 hours. The supernatant was collected and stored at -80 °C until analysis. IFN-γ and IL-2 concentrations were measured by the Meso Scale Discovery Vplex-2 method by Ardena Bioanalytical Laboratory (Assen, the Netherlands).

The samples for measurement of T cell proliferation were incubated for 48 hours with PHA and 20 µM EdU (Thermo Fisher Scientific). After incubation, red blood cells were lysed and the remaining cells were stained with anti-CD3 and viability dye eFluor780 as listed in **Table S1.** Flow cytometry analysis of activation marker expression and T cell proliferation was performed on a MACSQuant 16 analyzer (Miltenyi Biotec).

PHARMACODYNAMIC READOUT MEASURES FOR IMMUNE STATUS

Blood samples for routine hematology and biochemistry were analyzed by the department of clinical chemistry and laboratory medicine in the Leiden University Medical Center (LUMC). At 2 hours post-dose blood samples for measurement of the expression of inhibitory receptors on circulating T and B cell subsets were taken. Red blood cells were lysed and the cells were stained for flow cytometry analysis with the antibodies listed in **Table S1**. Flow cytometry analysis of circulating T and B cell subsets was performed on a MACSQuant 16 analyzer (Miltenyi Biotec).

Measurement of TTV load was analysed as previously described.[19] In short, DNA was extracted from 200 µL EDTA plasma, eluted in 100 µL, of which 10 µL was used as input for a quantitative PCR (qPCR) based on the TTV PCR primers and probe described by Maggi et al.[20] PCR reactions were performed by using a CFX96 real-time detection system (Biorad, Hercules, CA, USA). Analysis of the QPCR data was performed using Bio-Rad CFX Manager version 3.1. Measured TTV loads were log_{10}-transformed, as per general convention for viral load.

DATA ANALYSIS

Analysis of flow cytometry data was performed with Flowlogic software (Inivai Technologies). All data are presented graphically with mean value and standard deviation (SD). Repeated measures correlation (rmcorr) was used to determine the common intra-patient association for repeated PK and PD measures assessed on multiple time points. Repeated measures correlation was conducted using rmcorr R package.[21] For all other correlations, two ratios were calculated. The C_{max}/C_0 ratio represents the maximum *ex vivo* drug effect and was calculated by dividing the PD-effect at the timepoint of maximum tacrolimus concentration by the PD-effect pre-dose (0 h). The Tac_{100}/C_0 ratio represents the maximum *in vitro* drug effect and was calculated by dividing the *in vitro* PD-effect at the maximum tacrolimus concentration (100 ng/mL) by the PD-effect pre-dose. The relationship between *in vitro* PD, *ex vivo* PD and PK profiles was described by Pearson correlation coefficients. For this explorative study, no formal power analysis was performed; hence, no further formal statistical analysis was applied.

Results

PATIENT CHARACTERISTICS

Fourteen patients participated in this study, of which the characteristics are summarized in **Table 1**.

Table 1 Patient characteristics (n = 14).

Subject characteristics	Mean	Range
Age (years)	56	32 – 78
Gender (female/male)	4/10	
BMI (kg/m^2)	26.3	17.4 – 36.7
Transplant type (KTX/KPTX)	12/2	
Time post transplantation (years)	7.7	2.0 – 15.8
Albumin (g/L)	44.4	40.0 – 48.0
Hematocrit (%)	40.0	36.0 – 44.0
Creatinine (µmol/L)	117.0	65.0 – 211.0
Tacrolimus dose (mg/day)	3.9	2.0 – 8.0
MMF dose (mg/day)	1000	500 – 2000
Prednisolone dose (mg/day)	5.7	5.0 – 10.0

BMI – Body Mass Index, KTx – kidney transplant, KPTx – kidney-pancreas transplant

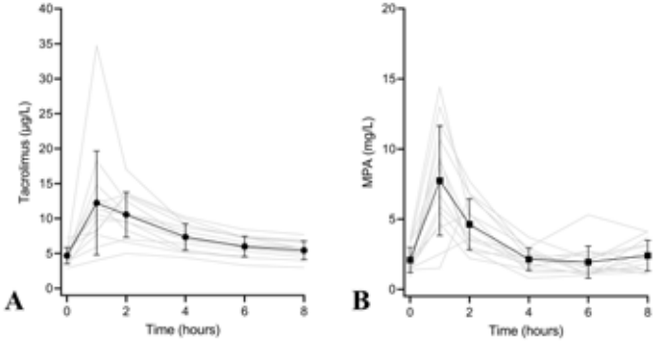
PK OF TACROLIMUS AND MPA

The pharmacokinetic (PK) profiles of tacrolimus and MPA are displayed in **Figure 2** and the PK parameters are summarized in **Table 2**. The highest tacrolimus concentration of 12.2 ± 7.4 µg/L was found at one hour post-dose (C_1) for 9 out of 14 patients. For the other five patients the highest concentration was found at C_2. While the inter-individual variation was limited (3-fold) for tacrolimus trough levels (C_0), the whole blood concentrations were much more variable at C_1 where they varied between 4.0 and 34.7 µg/L (8-fold). For MPA a similar pattern was observed: the highest plasma concentration was found at C_1 for 11 out of 14 patients, with inter-patient variability highest on C_1. The patients with a high peak in tacrolimus levels, however, were not necessarily the same patients for whom a high peak in MPA concentration was measured.

Table 2 Summary of the pharmacokinetic parameters

Pharmacokinetic parameter	Mean	Range
Tacrolimus dose (mg/day)	3.9	2.0 – 8.0
Tacrolimus C_0 (µg/L)	4.7	2.9 – 7.2
Tacrolimus C_1 (µg/L)	12.2	4.0 – 34.7
Tacrolimus C_2 (µg/L)	10.6	5.0 – 17.0
AUC_{0-8} tacrolimus (h*µg/L)	61.7	31.4 – 101
MMF dose (mg/day)	1000	500 – 2000
MPA C_0 (mg/L)	2.1	0.9 – 3.6
MPA C_1 (mg/L)	7.7	1.5 – 14.4
MPA C_2 (mg/L)	4.6	2.2 – 7.7
AUC_{0-8} MPA (h*mg/L)	25.7	15.9 – 44.8

Figure 2 MPA and tacrolimus pharmacokinetics. (A) Whole blood concentrations of tacrolimus and (B) plasma concentrations of MPA over time. In grey, the PK profile is shown for each individual patient. In black, the average drug concentration over time is plotted.

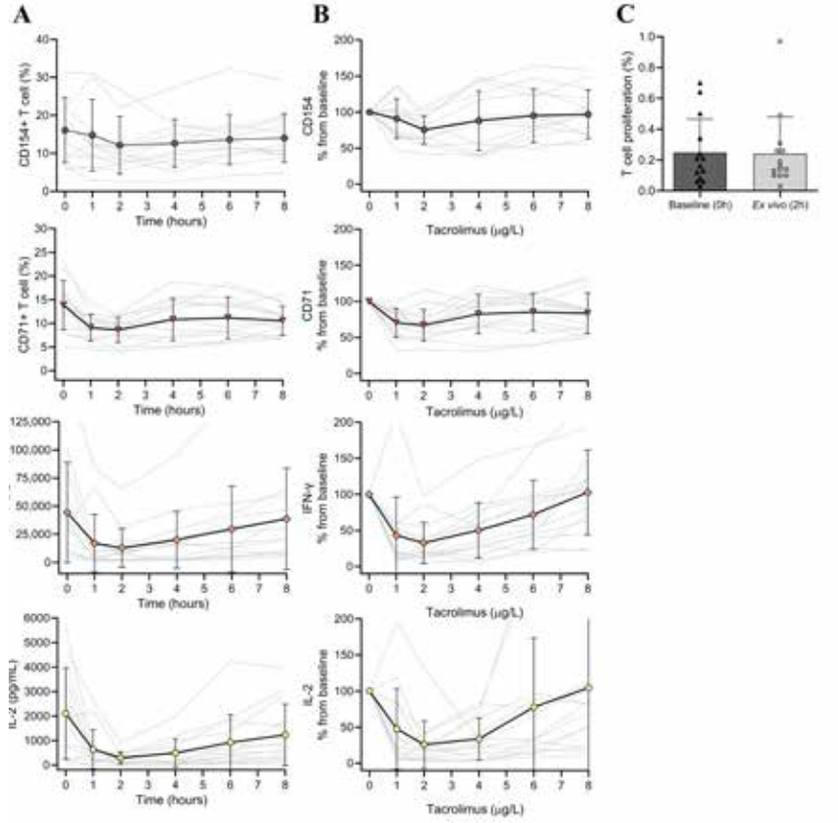


EX VIVO PHARMACOLOGICAL EFFECT ON IMMUNE ASSAYS

The effect of the immunosuppressive treatment regimen in transplantation patients on the pharmacodynamic (PD) endpoints, referred to as *ex vivo drug* effect, is shown in **Figure 3A,B**. The strongest *ex vivo* drug effect on cytokine production and T cell activation marker expression was found at 2 hours post-dose, returning to baseline levels around 8 hours post-dose. Cytokine production was most strongly affected by drug intake. An inhibition of 74% in IL-2 and 70% in IFN-γ production was found at 2 hours post-dose, while CD154 and CD71 expression was reduced with 32% and 25% respectively. Interestingly, CD4+ T cells were most strongly affected in their CD154 expression, whereas CD8+ T cells were most strongly affected in their CD71 expression (**Figure S1**).

We expected very limited levels of T cell proliferation (< 1 %) in renal transplantation patients on MMF therapy, based on previous data in healthy volunteers.[14] T cell proliferation was therefore only measured at two time points: pre-dose (C_0, oh) and 2 hours post-dose (C_2). As expected, at C_0 the percentage of proliferated T cells after stimulation was very low (0.25 ± 0.22 %), and upon drug intake by the patients T cell proliferation was not further suppressed (**Figure 3C**).

Figure 3 Ex vivo drug effect on selected PD markers. (A, B) Activation marker expression (CD154 and CD71) and cytokine production (IFN-γ and IL-2) measured after stimulation with PHA, at several time points after drug intake (0, 1, 2, 4, 6, 8 hours). Data is expressed in (A) absolute values and as (B) percentage from baseline (0 h). (C) PHA-induced T cell proliferation expressed as percentage of total CD3+ T cells, measured before (0 h) and after (2 h) drug intake.



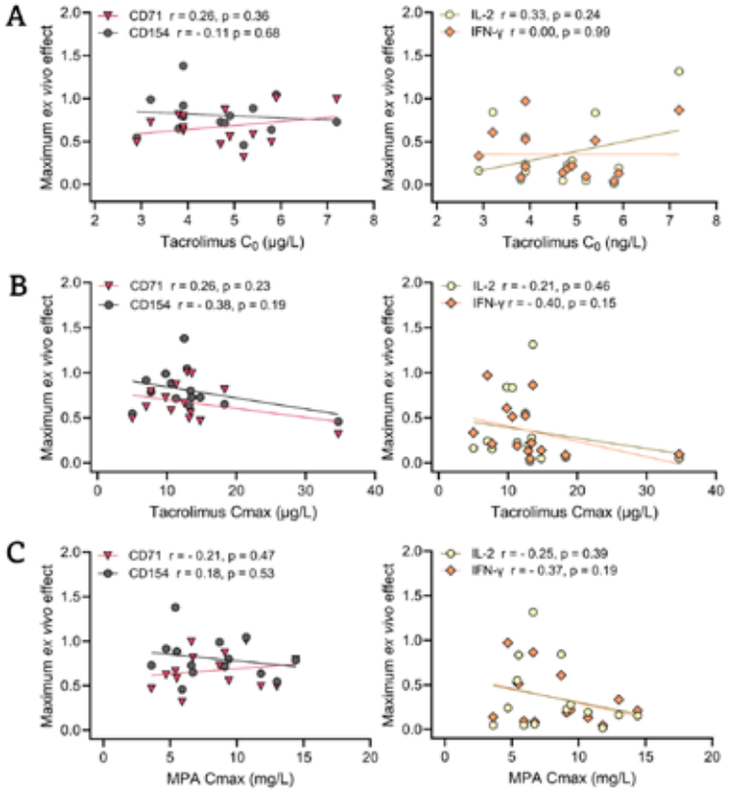
CORRELATIONS WITH PK, AGE, IMMUNE CELL SUBSETS AND TTV LOAD

Figure 3 shows a considerable variability in PD response between patients. While some patients had a substantial inhibition in T cell activity post-drug intake, others exhibited minimal inhibition. Given that renal transplantation patients are typically monitored based on drug concentrations, we aimed to investigate whether this inter-patient variability in PD effect could be explained by MPA or tacrolimus drug concentrations. When comparing the average PK profile depicted in **Figure 2** with the average PD profile in **Figure 3**, there are noticeable similarities between PK and PD. The most pronounced PD effect across all markers occurred at 1 and 2 hours post-dose, aligning with peak concentrations of MPA and tacrolimus at these time points. This finding was confirmed through repeated-measures correlation between the PD markers (CD71, CD154, IFN-γ and IL-2) and tacrolimus whole blood levels (**Figure S2**). On average, higher whole blood tacrolimus concentrations resulted in greater inhibition of PD markers in the patient population studied.

To understand whether this implies that PK parameters can predict PD outcomes at the individual patient level, the PD effect of each patient was standardized as a ratio. This ratio, termed the C_{max}/C_0 ratio, represents the maximum *ex vivo* drug effect for each PD marker and was obtained by dividing the PD outcome at the timepoint of maximum tacrolimus concentration (C_{max}), by the PD outcome at tacrolimus trough level (C_0). For instance, if a patient exhibited a CD71 expression of 15% on T cells prior to drug intake (C_0) and 10% at C_{max}, the resulting ratio would be 0.67, indicating a 33% inhibition in CD71 expression. In **Figure 4** the maximum *ex vivo* PD effect of all immune markers is correlated with three different PK parameters: tacrolimus trough levels (C_0), maximum tacrolimus levels and maximum MPA levels (C_{max}). None of the correlations was significant, indicating that the selected PK parameters were unable to predict the immunosuppressive PD effect at the individual level.

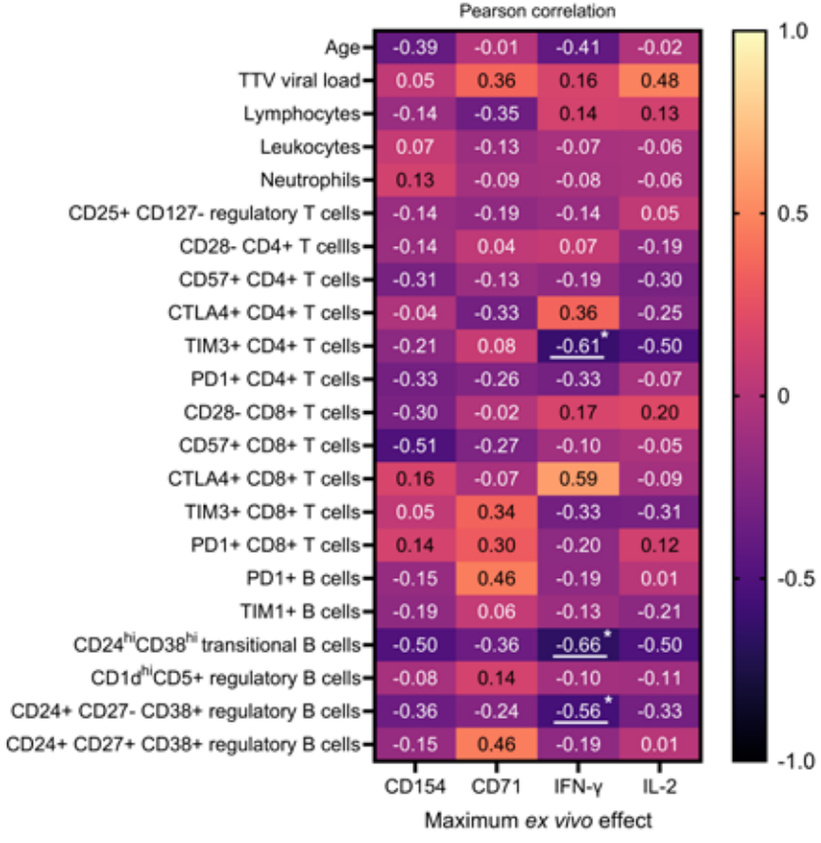
Since the individual PK parameters were not predictive for the *ex vivo* PD response, we searched for alternative endpoints to explain the inter-patient variability in *ex vivo* drug activity. To understand if immunosenescence or T cell exhaustion might influence the PD effect of the immunosuppressive treatment, the expression of inhibitory T cell markers (CD28-, CD57+, PD1+, TIM3+, CTLA4+) and inhibitory B cell markers (PD1+, TIM1+) were correlated with the maximum *ex vivo* PD effect (**Figure 5**). There was no correlation found between the age of the patient and *ex vivo* PD effect for cytokine production and activation marker expression. Besides, for most PD markers there was also no significant correlation found between the maximum PD effect and the number of regulatory or inhibitory T and B cells. Only for IFN-γ production, a larger number of TIM3+CD4+ exhausted T cells, transitional B cells and CD24+CD27-CD38+ regulatory B cells was significantly associated with a stronger inhibition post-dose.

Figure 4 Correlation between the maximum ex vivo drug effect on T cell activation marker expression (CD154, CD71) and cytokine production (IL-2, IFN-γ) and three PK parameters. The *ex vivo* PD effect was correlated with (A) tacrolimus trough levels (tacrolimus C0), maximum tacrolimus whole blood concentration (Tacrolimus C_{max}) and (C) maximum MPA plasma concentration (MPA Cmax).



Finally, the viral load of torque teno virus (TTV) was measured in every patient and correlated with the maximum *ex vivo* PD effect. TTV is a non-pathogenic virus that has been studied as immune biomarker in renal transplantation patients and has shown to be predictive for the occurrence of acute rejection.[19] There was, however, no correlation found between TTV viral load and the level of inhibition in our PD markers.

Figure 5 Heatmap of the Pearson correlation coefficient between C_{max}/C_0 ratio and age, TTV viral load, immune cell counts (lymphocytes, leukocytes and neutrophils), regulatory T and B cell subsets, and the expression of inhibitory receptors on T and B cells. Underlined numbers marked with * indicate significant correlations $p < 0.05$.

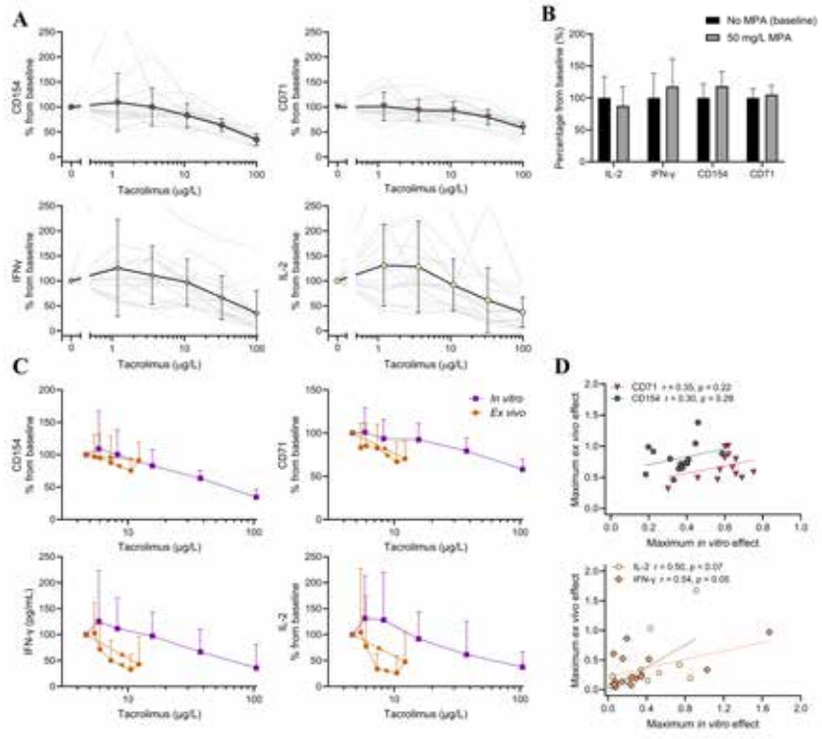


RELATIONSHIP BETWEEN *IN VITRO* AND *EX VIVO* PHARMACOLOGICAL EFFECT

We continued our search for markers that have the potential to explain the *ex vivo* PD effect of the individual patient by investigating the drug effect of tacrolimus and MMF in an *in vitro* setting. For these experiments, a concentration range of tacrolimus (0 – 100 µg/L) and a single concentration of MPA (50 mg/L) was added to the pre-dose whole blood cultures, after which the exact same immune assays were performed as for the monitoring of *ex vivo* drug activity. **Figure 6A** shows that the addition of tacrolimus to the *in vitro* cultures resulted in a dose-dependent reduction in T cell activation marker expression and cytokine production. The largest inhibition found at 100 µg/L tacrolimus, resulting in a mean inhibition of 55%, 34%, 65% and 66% in CD154, CD71, IFN-γ and IL-2 respectively. In contrast to what we found for tacrolimus, the addition of MPA to the *in vitro* cultures did not result in any inhibitory effect on these PD markers (**Figure 6B**). Moreover, Since the T cell proliferation was already very low at C_0 (0.25% proliferation), it was impossible to detect any additional inhibitory *in vitro* effect of MPA on this PD marker (**Figure S3**).

Next, we examined the relationship between the *in vitro* and *ex vivo* drug activity (**Figure 6C**). For all readout measures, a stronger *ex vivo* inhibition was found compared to what could be expected based on the *in vitro* data, as can be seen by the discrepancy between the *in vitro* and *ex vivo* plots. This discrepancy can be explained by the fact that *in vitro* curves only provide information on the PD effect of tacrolimus, while the *ex vivo* PD effect is the result of taking multiple drugs, including tacrolimus, MMF and prednisolone. To better understand the difference between *in vitro* and *ex vivo* drug effect, in **Figure 6D** the maximum *in vitro* PD effect (Tac_{100}/ C_0 ratio) was correlated with the maximum *ex vivo* PD effect (C_{max}/C_0 ratio) for all biomarkers. No significant correlation between the ratios could be found for activation marker expression on T cells (CD154 and CD71), indicating that a strong inhibition upon addition of tacrolimus *in vitro* does not necessarily mean that a strong inhibition also occurs *ex vivo* upon intake of medication. The *in vitro* and *ex vivo* PD effect on cytokine production showed a better correlation ($p = 0.07$ for IL-2 and $p = 0.05$ for IFN-γ), although individual data still showed major differences between *in vitro* and *ex vivo* PD effect.

Figure 6 Relationship between *in vitro* and *ex vivo* drug effect. *In vitro* effect of a concentration range of tacrolimus (A) and a single concentration of MPA (B) on T cell activation marker expression (CD154 and CD71) and cytokine production (IFN-γ and IL-2) after stimulation with PHA. (C) Overlay of the *in vitro* tacrolimus effect (purple square) and *ex vivo* drug effect (orange circle) on cytokine production and T cell activation marker expression. Arrowheads indicate the time course of the *ex vivo* samples (0, 1, 2, 4, 6, 8 hours). (D) Correlation between the maximum *ex vivo* drug effect and maximum *in vitro* tacrolimus effect.



Discussion

We previously investigated several functional immune assays and their suitability in demonstrating drug effect of CNIs and MMF in healthy volunteers.[14-16] In the current study, the selected immune assays were studied in kidney transplant patients. With both male and female patients, ages ranging from 32-78 and the time post transplantation ranging from 2-15.8 years, a heterogenous population of kidney transplantation patients participated in the study. All patients received maintenance immunosuppressive therapy consisting of tacrolimus, MMF and low-dose prednisolone. Moreover, all patients were annually monitored for a tacrolimus trough level (C_0) of 3 – 5 µg/L, which is reflected in the C_0 concentrations that we found in the current study (2.9 – 7.3 µg/L). The peak concentrations of tacrolimus (12.2 ± 7.4 µg/L) and MPA (7.7 ± 3.9 mg/L) and their variation also corresponded to what is described in literature for these patients.[22-23]

To investigate how drug concentrations relate to the patient's immune competence, T cell activation marker expression, T cell proliferation and cytokine production were measured. Based on our previous data on MMF activity in healthy volunteers[14], it was expected that T cell proliferation at trough levels (C_0) would be almost completely suppressed. This was confirmed as the average T cell proliferation was 0.25% before and 0.24% after drug administration, which indicates that there was almost no capacity for T cells to proliferate in response to a stimulus.

While the proliferative capacity was minimal, T cell activation marker expression and cytokine production were still detectable upon stimulation. To understand if monitoring patients using PD markers offers benefits compared to traditional drug level monitoring, we explored the correlation between *ex vivo* drug activity and factors that have been described to play a role in transplantation immunology. The clinical standard for therapeutic monitoring of tacrolimus is based on measurement of tacrolimus trough levels (C_0). In our study, we did not find a significant correlation between tacrolimus C_0 and drug activity. Similar findings were observed for tacrolimus C_{max} and MPA C_{max}, suggesting that patients with comparable drug levels may exhibit varying levels of residual T cell activity. It is important to highlight that all renal transplant patients included in the study had tacrolimus trough levels adjusted to target levels based on TDM. This resulted in a study population with limited variation in PK parameters and a population in which all patients were classified as therapeutically stable according to clinical guidelines. Despite being with the correct TDM range and not requiring any dose adjustments, these patients still showed varying levels of immune suppression in our assays, which indicates a potential additional value of pharmacodynamic monitoring in clinical practice.

We also explored additional factors that could potentially influence or reflect the immune status of transplantation patients. The patient's age and

chronic immune activation by the allograft are known to affect inhibitory T and B cell populations[9, 24-25], making monitoring of these cell subsets an interesting biomarker for immune activity. The number of regulatory T cells, transitional B cells, exhausted T cells, and CD28-CD8+ T cells has previously been associated with a lower rejection-risk after kidney transplantation.[26-28] Moreover, the viral load of TTV, a non-pathogenic DNA virus, has also been described as a marker for immune efficacy and associated with the occurrence of rejection.[19] In our study, we did not find a clear relationship between any of these immune biomarkers and the *ex vivo* PD effect of the patient's immunosuppressive treatment, evaluated by our functional immune assays, with the exception of a correlation between IFN-γ production and the presence of Tim3+ CD4+ T cells and inhibitory B cell subsets. This may not be surprising, since Tim3 expression on CD4 T cells is known to limit IFN-γ production, and inhibitory B cell subsets have been described to inhibit CD4+ T cell activation via IL-10 production.[29-30] Furthermore, low numbers of transitional B cells have previously been correlated with allograft survival[31-32], indicating that monitoring of *ex vivo* tacrolimus activity by PHA-induced IFN-γ production may have clinical relevance.

In our final analysis, we investigated whether the patient's individual *ex vivo* PD responses to immunosuppressive therapy could be predicted based on *in vitro* drug activity, evaluated by incubation of whole blood with a range of drug concentrations. The addition of active metabolite MPA to the *in vitro* incubations had no additional inhibitory effect on these markers, which is in line with previous data[14] and the mechanism of action of MPA as selective inhibitor of lymphocyte proliferation. The addition of tacrolimus to pre-dose samples, on the other hand, resulted in a concentration-dependent decrease in the expression of CD71 and CD154 on T cells, as well as reduced production of IL-2 and IFN-γ. Although the biomarkers that were most strongly affected by the addition of tacrolimus *in vitro* showed a strong *ex vivo* inhibitory effect, the degree of immune suppression observed after pre-dose *in vitro* incubation with tacrolimus didn't align with the suppression levels found *ex vivo* in patient samples. This suggests that the immune effects on these PD markers after drug administration may not be solely caused by tacrolimus but may also involve additional effect of other co-administered immunosuppressants, such as MMF and prednisolone. This is an important difference between the current study and earlier studies in healthy volunteers[15-16], in which the discrepancy between *in vitro* and *ex vivo* immune function was less obvious.

Despite the small sample size, we successfully characterized pharmacodynamic markers for monitoring the immunocompetence of the individual patient over time. The aim was to identify biomarkers reflecting general immune status, for guidance of the dosing strategy of the most frequently used immunosuppressive drugs. However, we only established biomarkers allowing monitoring of tacrolimus effects over time, since MMF-specific endpoints (cell proliferation) were maximally suppressed over the full day, even at trough levels. We currently don't know whether this means that kidney transplant patients could be treated with reduced doses of MMF, or whether alternative biomarkers for monitoring of MMF effects would be required. A critical next step to take is the evaluation of the correlation between pharmacodynamic effect and clinical outcome (graft survival, side effects), in a significantly larger patient population. The future use of these biomarkers in larger patient groups would require automated sample processing because of the labor-intensive nature of these T cell assays.

In summary, our study demonstrates complete suppression of T cell proliferation in kidney transplantation patients undergoing immunosuppressive therapy, while immune function based on other immune biomarkers (PHA-driven T cell activation measured by CD71, CD154, IFN-γ and IL-2) fluctuates over the day. *Ex vivo* tacrolimus effects were highly variable between patients, which could not all be related to *in vitro* drug effects, PK parameters or the presence of regulatory or senescence immune cells. We therefore hypothesize that the PD markers investigated in our study might provide additional insights into the immune status of the transplant patient beyond the traditional PK parameters. To evaluate this hypothesis, we suggest studying the clinical relevance of these biomarkers in an observational study with a longer follow-up period. This would allow for a correlation between immune status and the occurrence of clinical events such as infection or organ rejection.

SUPPLEMENTAL MATERIAL

All mentioned supplementary figures in this chapter can be found on the publisher's website by scanning this QR code.

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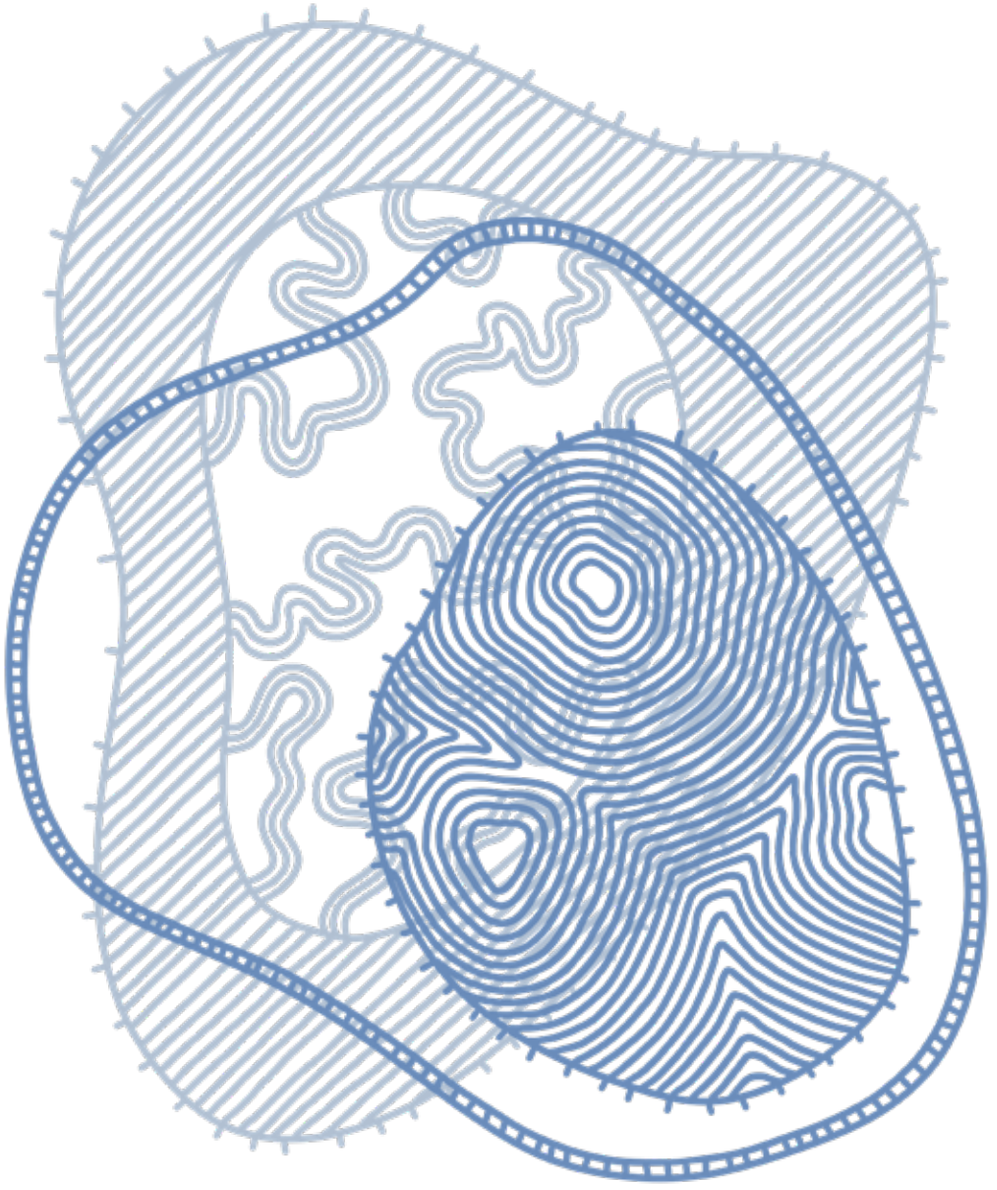
SECTION 2



CHAPTER 6 HYDROXYCHLOROQUINE EFFECTS ON TLR SIGNALLING: UNDEREXPOSED BUT UNNEGLECTABLE IN COVID-19

Journal of Immunology Research. 2021 Mar 9, 2021:6659410. Doi: 10.1155/2021/6659410

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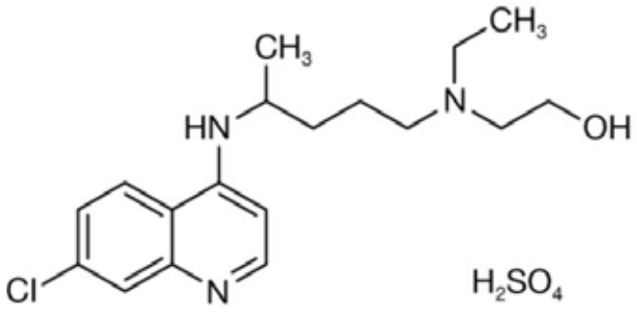
Abstract

The main basis for hydroxychloroquine (HCQ) treatment in HCQ is the compound's ability to inhibit viral replication *in vitro*. HCQ also suppresses immunity, mainly by interference in TLR signalling, but reliable clinical data on the extent and nature of HCQ-induced immunosuppression are lacking. Here we discuss the mechanistic basis for the use of HCQ against HCQ in a prophylactic setting and in a therapeutic setting, at different stages of the disease. We argue that the clinical effect of prophylactic or therapeutic HCQ treatment in HCQ depends on the balance between inhibition of viral replication, immunosuppression, and off-target side effects, and that the outcome is probably dependent on disease stage and disease severity. This is supported by the initial outcomes of the well-designed randomized controlled trials: so far evidence for a beneficial effect of HCQ treatment for HCQ is weak and conflicting.

Introduction

Hydroxychloroquine sulfate (HCQ, **Figure 1**) is a less toxic derivative of the antimalarial drug chloroquine (CQ). Besides the use as antimalarial drug, HCQ is also prescribed for the treatment of several different auto-immune diseases such as rheumatoid arthritis, juvenile idiopathic arthritis, and systemic lupus erythematosus. The compound has been evaluated extensively in an ever-increasing number of clinical trials as treatment modality to fight HCQ infection, also in a prophylactic setting.

Figure 1 Chemical structure of hydroxychloroquine sulfate



The insight we are aiming to provide in this paper is whether the effects of HCQ use on HCQ infection align with the predicted effects of HCQ. Can the molecular activities of the drug, in particular its direct immunosuppressive activities, predict the effect on HCQ infection? We advocate that especially these immunosuppressive effects ultimately determine the clinical outcome, while so far they have remained largely underexposed in clinical trials evaluating HCQ effects on HCQ.

The main reason why HCQ initially emerged as potential treatment in HCQ was because of its *in vitro* antiviral properties against several RNA viruses, including SARS-COV-1 and -2.[1-4] In addition, HCQ possesses immunosuppressive properties that may be beneficial in dampening the derailed immune response in later stages of HCQ infection.[5] Based on these pharmacological activities, HCQ was considered to be a promising drug to combat HCQ, at least until the moment an effective vaccine would become available. In spring 2020, this even urged some governments to recommend prophylactic HCQ use, for example by the Indian Council of Medicinal Research[6] and repeatedly by President Trump in White House briefings. This was remarkable, because at that moment in time conclusive data from large, randomized and well-monitored clinical trials on the preventive or therapeutic efficacy of HCQ in HCQ were pending. The outcomes of initial clinical studies evaluating HCQ effects in HCQ were not convincing, also because many studies suffered from major methodological limitations and decent peer review of study reports was complicated by time constraints. This has been extensively outlined in earlier reviews[7] and was breaking news when two papers in The Lancet and New England Journal of Medicine were retracted.[8-9] Six months later, the results of the first randomized controlled trials have been published, and overall they are disappointing. So far, there is no convincing proof for beneficial effects of HCQ, neither in a therapeutic setting nor in a postexposure prophylactic setting.[10]

A complicating factor for the evaluation of HCQ's effects on HCQ has been the highly variable pathophysiology, within an individual patient over time, but also between patients.[11] HCQ's inhibiting effect on HCQ replication, based on *in vitro* evidence, would be beneficial at any stage of the disease, in any population (being it non-infected subjects, asymptomatic patients, or severe patients). However, this is not equally self-evident for the compound's immunosuppressive effects, as we will outline later in this

manuscript. Importantly, despite extensive mechanistic evidence based on *in vitro* experiments, reliable clinical data on the extent and nature of HCQ-induced immunosuppression are lacking.

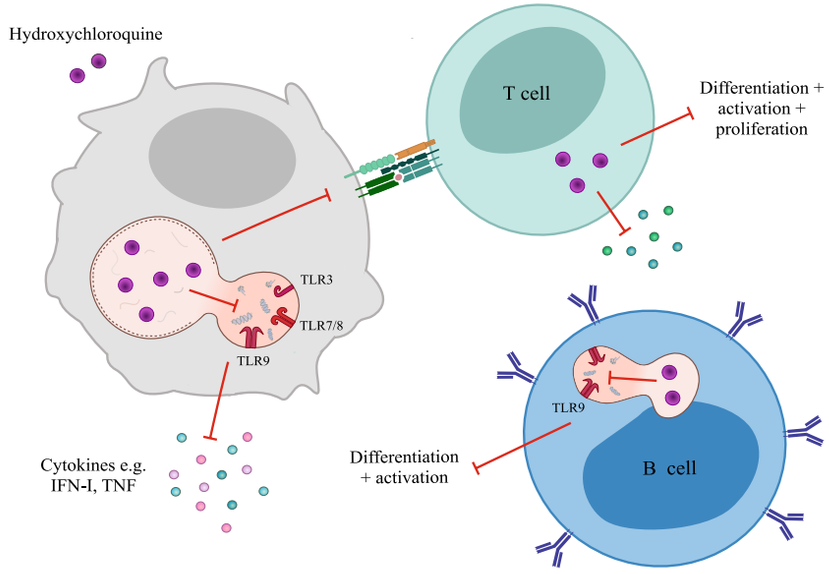
This article discusses the mechanistic basis for the use of HCQ against HCQ in a prophylactic setting and in a therapeutic setting, at different stages of the disease. The focus lies on HCQ's immunosuppressive effects, since we advocate that especially this aspect is largely underexposed in recent clinical trials evaluating HCQ effects on HCQ. A non-systematic review of published literature was performed, mainly PubMed-based, to build this mechanistic basis. This article only discusses HCQ, since this compound suffers less from side effects, drug-drug interactions, and toxicity than its parent compound chloroquine, while their pharmacological activities are well comparable.[12]

Immunosuppressive effects of HCQ

The basis for HCQ's use in autoimmune diseases is its wide range of immunosuppressive properties (**Figure 2**). HCQ accumulates in the lysosomes where it increases the pH and inhibits the enzymatic activity in both lysosomes and autophagosomes. Since these organelles play an important role in antigen processing and MHC class II presentation, a rise in lysosomal pH indirectly inhibits the immune response to both intracellular and extracellular antigens.[13]

Lysosomal accumulation of HCQ does not only result in a pH increase, but also directly affects endosomal TLR signalling triggered by nucleic acids. The endosomal TLRs (i.e. TLR3, TLR7, TLR8 and TLR9) play an important role in the innate immune response by recognizing double-stranded RNA, single-stranded RNA and CpG motifs in viral DNA.[14] HCQ can bind nucleic acids within the endosome, thereby preventing interaction of the endosomal TLRs with their ligands, inhibiting subsequent TLR activation. Downstream innate immune responses are dampened, such as IFN- and TNF production by plasmacytoid dendritic cells.[15-16] In addition, the adaptive immune response is impaired by HCQ effects on B cell differentiation and cytokine production.[17-18] Moreover, HCQ inhibits T cell activation, proliferation and cytokine production by inhibiting intracellular calcium and mobilization and subsequent NFAT signalling[19-20], and apoptosis in CD45RO+ memory and effector T cells by inhibiting autophagy.[21]

Figure 2 Immunosuppressive effects of HCQ. Hydroxychloroquine affects both the innate and adaptive immune system. By accumulating in the lysosome and autophagosome, the PH is increased causing an inhibition of MHC-II antigen presentation and subsequent T cell activation. In addition, HCQ accumulation abrogates viral recognition by endosomal TLRs, resulting in a decrease of the anti-viral innate immune response (i.a. IFN-I production). Moreover, HCQ can also directly affect the adaptive immune system through inhibition of T and B cell differentiation and activation.



The majority of the mechanistic work on HCQ's immunosuppressive activity has been performed in cell lines. Experimental evidence for immune suppression by HCQ in primary human cells is scarce. Some publications are available describing HCQ effects on innate immune responses in human whole blood, peripheral blood mononuclear cells, or T cells, with TLR-mediated cytokine production, or T cell activation and proliferation as endpoint.[22-27] Most experiments used HCQ concentrations largely exceeding expected circulating concentrations *in vivo* after prophylactic or therapeutic dosing. Moreover, with one exception, none of the papers provides a decent HCQ concentrationeffect relationship, so an IC50 for HCQ's immunosuppressive activities cannot be estimated. Interestingly, HCQ's IC50 for inhibition of

HCQ replication (4-17 μM)[28] appears to exceed HCQ concentrations effectively inhibiting TLR responses *in vitro* (3 μM)[24,27], which means that it will be difficult to inhibit viral replication without impairing the immune system.

Mechanistic support for HCQ use in COVID-19

PROPHYLACTIC SETTING

Cell entry by HCQ is thought to be similar to SARS-COV entry, being mediated by spike (S) protein binding to angiotensin-converting enzyme 2 (ACE2).[29-30] *In silico* predictions showed that HCQ prevents the cellular binding and entering of HCQ virus particles, by interfering with sialic acids and surface gangliosides.[31] Based on this pharmacological activity, prophylactic HCQ treatment could theoretically be beneficial and prevent HCQ infection in vulnerable populations or populations professionally exposed to HCQ patients.

Upon cell entry, HCQ is likely recognized by TLR3, TLR4, TLR7, TLR8 and RIG-I[32], resulting in a type I IFN response which is crucial for an efficient adaptive antiviral response.[33] HCQ suppresses parts of the immune system that are essential in fighting infections, including TLR signalling and type I IFN production. In previous SARS-COV and MERS-COV outbreaks, downregulation of IFNs by coronavirus proteins strongly correlated with worse disease progression and increased lethality.[34] Cell and animal models of HCQ infection, and transcriptional and serum profiling of HCQ patients, revealed an imbalanced host response with low levels of type I and III IFNs.[35] Early IFN signalling was protective in SARS-COV-1 infected mice, whereas delayed IFN signalling was detrimental leading to severe disease progression and related lethal pneumonia.[36]

The importance of TLR signalling in viral defence has been well established in SARS-COV-1 mouse models. Both TLR3 and TLR4 deficient mice are more susceptible to SARS-COV-1 infections.[37] Murine MYD88 or TRIF deficiency, which are downstream signalling molecules shared by multiple TLRs, resulted in a mortality rate of over 90% upon experimental infection with SARS-COV-1, which is usually non-lethal in immunocompetent mice.[37-38] HCQ abrogates endosomal acidification thereby reducing endosomal TLR activation[22], but interestingly enough data confirming this HCQ effect on endosomal TLRs in primary human cells are scarce. Since the relationship between HCQ dose/concentration and level of immunosuppression remains largely unexplored in primary human immune cells, it is difficult to estimate the effect of prophylactic HCQ treatment regimens on the innate immune response. If HCQ's immunosuppressive IC50s would fall in the concentration range reached after prophylactic HCQ treatment, endosomal TLR responses, type I IFN production, and T and B cell activation and proliferation could be impaired *in vivo*. Theoretically this could result in an increased viral infection risk, including HCQ infection. On the other hand, HCQ use in rheumatoid arthritis patients is not associated with an increased infection risk.[39-40] So far, prophylactic HCQ studies did not show clinical benefit of HCQ administration.[41-42]

Next to mechanistic arguments, the fact that long-term HCQ use comes with side-effects further fuels doubts about prophylactic use of HCQ. Retinal toxicity, cardiac disease, (reversible) neuromyopathy, dermatological manifestations, gastrointestinal and hematological changes, and hearing abnormalities have been reported upon long-term HCQ treatment, amongst others.[43-45] Such side effects could be avoided by local HCQ administration, for example by inhalation.

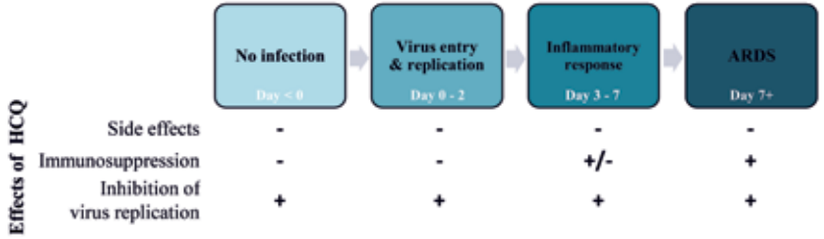
THERAPEUTIC SETTING

Although our understanding of the pathophysiology continues to increase on a daily basis, it is clear that HCQ is a highly heterogenous disease. With increased disease severity, the complexity of the pathophysiology grows.[32,46] Since many excellent reviews are available in the public domain, this manuscript does not revisit HCQ pathophysiology and disease progression. Instead, it discusses the alignment between HCQ's mechanism of action and disease stage: how could specific pharmacological activities of HCQ theoretically affect HCQ's pathophysiology at a particular disease stage? As guidance, the disease progression has been separated into three stages: stage 1 - virus entry and replication in the airway cells (day 0-2), stage 2 - activation of innate immunity in the lung (maladaptive inflammatory response, day 3-7), and stage 3 - acute respiratory distress syndrome (ARDS, >day 7).[47] Obviously, the clinical presentation of HCQ varies between patients from asymptomatic to mild, moderate and severe, and not all patients develop advanced disease stages.

When discussing potential effects of HCQ treatment in a therapeutic setting, most papers focus on the off-target side effects of HCQ, specifically potentially severe cardiac disorders such as QT segment prolongation. However, safety concerns related to the short-term use of HCQ (i.e. regimens of 1 month) are probably limited, as demonstrated by a recently

published (non-peer reviewed) international study in more than 900,000 HCQ-treated patients.[48] We advocate that one of HCQ's pharmacological activities, namely its immunosuppressive effect, is critical when considering HCQ as potential treatment modality for HCQ. Surprisingly, HCQ's exact molecular mechanism of action has remained largely neglected in considerations on therapeutic HCQ use for HCQ. Therefore, we discuss in the next sections how HCQ's pharmacological activities could be beneficial, or detrimental, at different disease stages (stage 1-3, see above) and in different disease severities (asymptomatic, mild, moderate-severe) (**Figure 3**).

Figure 3 Theoretical effects of HCQ at different stages of SARS-COV-2 infection.
Potential HCQ effects on COVID-19 are schematically presented over the course of the disease, ranging from prophylactic use in uninfected subjects to therapeutic use in acute respiratory distress syndrome (ARDS) in severe patients. A beneficial HCQ effect is indicated with '+' and a detrimental HCQ effect with '-'. The stages of SARS-COV-2 infection are indicated in green. Stage 0 – no infection, stage 1 – virus entry and replication in the airway cells, stage 2 – activation of innate and adaptive immune system, stage 3 – ARDS.



For therapeutic treatment, the first stage (day 0-2 of infection) is irrelevant, since patients are asymptomatic and viral titers may be low[49], so patients in this stage of the disease are untreated or fall in the prophylactic treatment category (see previous section). HCQ treatment theoretically could be beneficial in the next stages of the disease (stage 2; day 3-7, and stage 3; >day 7), when the innate immune response in the lungs starts to evolve, and ultimately culminates in respiratory impairment and multi-organ failure. The drug may not only inhibit virus replication, but also suppress TLR-mediated cytokine responses and over-activation and apoptosis of lymphocytes, processes that are observed in severe HCQ.[50-51] Especially prevention of a cytokine storm is critical since this is a major factor driving multi-organ failure, ARDS, disseminated intravascular coagulation, and the resulting high mortality. Taken together, HCQ treatment in progressed HCQ is mechanistically supported by HCQ's pharmacological activities.

Obviously, progressed disease as outlined above (stage 2 and 3) only applies to moderate to severe HCQ patients. The large majority of HCQ patients only suffers from mild disease, or even remains asymptomatic.[52] These patients have a low viral load, develop an efficient type I IFN response, produce virus-neutralizing antibodies, and do not develop a maladaptive inflammatory response.[53] Since it is especially the latter response that could be targeted by HCQ's immunosuppressive activity, the question arises whether HCQ treatment is rational in asymptomatic or mild patients. On one hand, one could argue that HCQ-dependent inhibition of viral replication (though not clinically proven) is important, independent of disease stage. Moreover, HCQ-dependent immunosuppression may prevent mild disease turning into inflammation-driven moderate/severe disease. On the other hand, in the early disease stage it is important that the virus-specific anti-HCQ response is driven by an efficient antiviral innate immune response, and especially this response may be significantly impaired upon HCQ treatment. The net result of HCQ treatment will depend on the balance between these two pharmacological activities. The outcome of therapeutic studies have shown that HCQ treatment overall does not seem to reduce mortality, improve clinical scores, or suppress viral load in moderate to severe HCQ patients.[54-56] However, low dose HCQ treatment (< 2.5 g in total) was associated with a reduced risk of intensive care unit admission and lower mortality rates.[57-58] HCQ's clinical beneficial effects may depend on the inflammatory status of the patient: chronic low-dose HCQ treatment of a large cohort of rheumatic patients coincided with reduced mortality following HCQ infection[59], and another study reported a therapeutic benefit of HCQ treatment in patients with elevated C-reactive protein levels.[60] These reports are mechanistically in line with the immunosuppressive activities of HCQ, as outlined above.

Conclusion

Immunosuppression by HCQ, via interference in endosomal TLR signalling, has remained largely underexposed in the public debate, while it may be a critical factor for the (lack of?) clinical efficacy of HCQ in HCQ. Experimental evidence for immune suppression by HCQ in primary human cells is scarce,

which is surprising for such an old drug. Clinical trials evaluating HCQ as HCQ treatment did not include readout measures to study this immunosuppressive effect of HCQ. As a result, the extent of immunosuppression by HCQ cannot be reliably estimated *in vivo*. If systemic or local HCQ concentrations would be sufficiently high to suppress key components of the innate immune response, this could translate into a clinical benefit. The other side of the coin is that in mild HCQ patients, or in a prophylactic setting, immunosuppression by HCQ could have a detrimental effect, since an efficient virus-specific anti-HCQ response depends on a robust antiviral innate immune response. We argue that ultimately the clinical effect of HCQ treatment in HCQ depends on the balance between inhibition of viral replication, immunosuppression, and off-target side effects (which have been extensively evaluated recently, within and outside the setting of HCQ treatment or prevention[61-62], and are as such not the topic of this article). The outcome of this balance is probably dependent on disease stage and disease severity (**Figure 3**). This is supported by the initial outcomes of the well-designed randomized controlled trials: so far evidence for a beneficial effect of HCQ treatment for HCQ is weak and conflicting.

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CHAPTER 7 IMMUNOSUPPRESSION BY HYDROXYCHLOROQUINE: MECHANISTIC PROOF IN *IN VITRO* EXPERIMENTS BUT LIMITED SYSTEMIC ACTIVITY IN A RANDOMIZED PLACEBO-CONTROLLED CLINICAL PHARMACOLOGY STUDY

Immunologic Research. 2023 Feb 22, 1–11. doi: 10.1007/s12026-02w3-09367-3

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Abstract

Based on its wide range of immunosuppressive properties, hydroxychloroquine (HCQ) is used for the treatment of several autoimmune diseases. Limited literature is available on the relationship between HCQ concentration and its immunosuppressive effect. To gain insight in this relationship we performed *in vitro* experiments in human PBMCs and explored the effect of HCQ on T and B cell proliferation and Toll like receptor (TLR)3/TLR7/TLR9/RIG-I-induced cytokine production. In a placebo-controlled clinical study these same endpoints were evaluated in healthy volunteers that were treated with a cumulative dose of 2400 mg HCQ over 5 days. *In vitro*, HCQ inhibited TLR responses with IC50s >100 ng/mL and reaching 100% inhibition. In the clinical study, maximal HCQ plasma concentrations ranged from 75 to 200 ng/mL. No *ex vivo* HCQ effects were found on RIG-I-mediated cytokine release, but there was significant suppression of TLR7 responses and mild suppression of TLR3 and TLR9 responses. Moreover, HCQ treatment did not affect B cell and T cell proliferation. These investigations show that HCQ has clear immunosuppressive effects on human PBMCs, but the effective concentrations exceed the circulating HCQ concentrations under conventional clinical use. Of note, based on HCQ's physico-chemical properties, tissue drug concentrations may be higher, potentially resulting in significant local immunosuppression.

Introduction

Hydroxychloroquine (HCQ) is a broad immunosuppressive drug, initially developed as an anti-malarial drug. However, due to its anti-inflammatory properties, HCQ is now widely used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA)[1], systemic lupus erythematosus (SLE)[2] and Sjögren's syndrome.[3] The use of HCQ in other diseases has been under investigation, a pilot trial investigating the use of HCQ in patients after myocardial infarction showed a decrease in plasma IL-6 levels compared to placebo, and a larger trial studying the effect on recurrent cardiovascular events is currently ongoing.[4] Furthermore, HCQ was under investigation for use in moderate to severe COVID-19 patients during the COVID-19 pandemic.[5]

The exact mechanisms behind HCQs immunosuppressive functions remain unclear. HCQ accumulates in the lysosomes and inhibits lysosomal function by autophagosome fusion with lysosomes[6], thereby inhibiting antigen presentation.[7-8] In addition, HCQ inhibits pro-inflammatory cytokine production by myeloid cells, possibly via the inhibition of endosomal Toll-like receptor (TLR) signalling.[9] It has been shown that HCQ treatment is associated with decreased interferon (IFN) serum levels in SLE patients.[10] Furthermore, several studies investigating the effect of HCQ on peripheral blood mononuclear cells (PBMCs) or cell lines show that HCQ treatment reduces phorbol 12-myristate 13-acetate (PMA) and ionomycin or lipopolysaccharide induced cytokine production.[11-13]

Besides effects on the innate immune system, HCQ affects the adaptive immune response as well. It has been shown that HCQ inhibits differentiation of class-switched memory B cells into plasmablasts and thereby decreases IgG production in response to TLR9 stimulation or inoculation with inactivated virus.[14-15] HCQ inhibits T cell activation as well, via the inhibition of T cell receptor induced calcium mobilization and dysregulation of mitochondrial superoxide production.[16-18]

However, the concentrations used in such *in vitro* experiments studying the immunomodulatory effects of HCQ largely exceeded obtainable clinical concentrations in patients. A study in cutaneous lupus erythematosus patients receiving HCQ in clinical doses showed that higher HCQ blood levels corresponded with lower *ex vivo* IFN responses after TLR9 stimulation, but not after TLR7/8 stimulation.[13] Moreover, influenza antibody titers after vaccination in Sjögren's syndrome patients receiving HCQ were lower compared to HCQ naïve patients.[15] Unfortunately, little additional literature is available on the *in vivo* immunomodulatory effects of HCQ and comparing it to *in vitro* experiments.

We aimed to assess and quantify the immunomodulatory effects of HCQ on primary human immune cells, both *in vitro* and *ex vivo* in a randomized clinical trial. We assessed the effect of HCQ on cytokine production after endosomal TLR stimulation in isolated PBMCs and on T and B cell proliferation (*in vitro* as well as *ex vivo*). In the clinical trial, healthy subjects were dosed with HCQ in the standard dosing regimen for moderate-to-severe COVID-19 that was advised in the Netherlands when the study was conceived. In the study design, we accounted for a potential age effect on the study outcomes, since general immunocompetence and drug metabolism has been reported to be age-dependent.[19-20] Here we present the outcomes of the *in vitro* experiment and the randomized clinical trial.

Materials and Methods

IN VITRO EXPERIMENTS

Blood was collected by venipuncture using Sodium Heparin vacutainer tubes or Cell Preparation Tubes (CPT, Becton Dickinson, Franklin Lakes, NJ, USA) from healthy volunteers after written informed consent, in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Blood was used for the evaluation of the *in vitro* immunomodulatory activity of hydroxychloroquine (10 - 10,000 ng/mL, Sigma-Aldrich, Deisenhofen, Germany). All experiments were started within one hour after blood withdrawal, and incubations were performed in duplicate. Hydroxychloroquine and stimulant were added simultaneously. Per experiment, blood of 6 donors was used.

CLINICAL STUDY

We conducted a single-blind, randomized, placebo-controlled multiple dose study in forty healthy male volunteers, comprising twenty young (18-30 years) and twenty elderly (65-75 years) subjects. The study was conducted at the Centre for Human Drug Research in Leiden, The Netherlands, between June and September 2020, during the COVID-19 pandemic. All subjects in the clinical trial gave written informed consent according to Declaration of Helsinki recommendations, prior to any study-related activity. The study was approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands) and registered in the Toetsingonline Registry (study number NL73816.056.20), and in the International Clinical Trials Registry Platform (NL8726).

VOLUNTEER SELECTION

To avoid sex-related inter-individual variability in immune responses, only male subjects were included.[21] Subjects were included if they were overtly healthy. The health status of subjects was assessed by medical screening, including medical history, physical examination, vital signs measurements, 12-lead electrocardiography (ECG), urine analysis, drug screen and safety chemistry, coagulation, and hematology blood sampling. BMI of study participants had to be between 18 and 32 kg/m^2. Subjects with a known hypersensitivity reaction to chloroquine, HCQ or other 4-aminoquinolines, abnormalities in the resting ECG (including QTCF-interval >450ms), evidence of any active or chronic disease or condition (including long QT syndrome, retinal disease, G6PD deficiency, autoimmune diseases, diabetes mellitus type I or II, psychiatric disorders) or a positive SARS-COV-2 PCR test were excluded from study participation. Use of concomitant medication was not permitted during the study, and 14 days (or 5 half-lives) prior to the study drug administration, except for paracetamol.

STUDY DESIGN

Subjects were randomized to receive either hydroxychloroquine sulphate (plaquenil®) or placebo tablets, in a 1:1 ratio. Tablets were dispensed by the pharmacy, according to a randomization list generated by a study-independent statistician. Plaquenil® and placebo tablets were packaged in the same way but the tablets were not indistinguishable, study drug administration was therefore performed by dedicated unblinded personnel not involved in any other study tasks. Subjects received HCQ or placebo by a loading dose of 400 mg twice daily (t = 0 h and t = 12 h) followed by a 400 mg once daily dose regimen (t = 24 h, t = 48 h, t = 72 h, and t = 96 h), giving a cumulative dose of 2400 mg. This reflected the standard dosing regimen for moderate-to-severe COVID-19 patients in the Netherlands when the study was conceived (total dose between 2000 and 3800 mg).

PHARMACOKINETIC EVALUATION

For pharmacokinetic (PK) assessments, blood was collected in 3 mL Vacutainer® K2EDTA tubes (Becton Dickinson) on study day 0 (baseline and 3 hours post-dosing), and day 1, 4 and 9 (3 hours post-dosing). Hydroxychloroquine plasma concentrations were measured by Ardena Bioanalytical Laboratory (Assen, the Netherlands) using a validated LC-MS/MS method. The lower limit of quantification (LLOQ) of the analysis was 5 ng/mL.

WHOLE BLOOD STIMULATION

Whole blood was stimulated with 10 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich) for 6 hours and 24 hours. After 6 hours, activation markers on T-cells were measured using CD69-APC (clone: REA824), CD71-FITC (clone: REA902), CD154-VioBlue (REA238) and CD25-PE (clone: 3G10), CD3-VioGreen (REA613), CD4-APC-Vio770 (REA623) and CD8-PE-Vio770 (REA734) antibodies and propidium iodide as viability dye (all Miltenyi Biotec, Bergisch-Gladbach, Germany) using a MACSQuant 16 analyzer (Miltenyi Biotec). After 24 hours, culture supernatants were collected for cytokine analysis.

PBMC ISOLATION AND TLR STIMULATION

PBMCs were isolated from CPT after centrifugation at 1800 x g for 30 minutes, and washed 2x using phosphate buffered saline (PBS, pH 7.2, Gibco, Thermo Fisher, Waltham, MA, USA). PBMCs were stimulated with endosomal TLR ligands poly I:C (TLR3, 50 µg/mL), imiquimod (TLR7, 1 µg/mL), CpG class A (TLR9, oligodeoxynucleotides [ODN] 2.5 µM) and poly I:C/lyovec (RIG-I, 1 µg/mL; all Invivogen, Toulouse, France). Supernatants were collected after 24 hours for cytokine quantification.

PROLIFERATION ASSAY

PBMCs were stained with 2.5µM cell trace violet (CTV, Thermo Fisher) according to user's manual. T cells were stimulated with 5 µg/mL phytohemagglutinin (PHA), and B cells with a monoclonal CD40 antibody (5 µg/mL; clone: G28.5, BioXCell) and CpG class B (2.5 µM; ODN Invivogen). After 5 days of stimulation PBMCs were stained using, CD4-PE (clone: OKT4), CD8-APC (clone: HIT8A), CD19-PE (clone: HIB19, all Biolegend, San Diego, CA, USA) and fixable viability dye eFluor780 (Thermo Fisher) and proliferation was quantified by flow cytometry, using the MACSQuant 16 analyzer.

FLOW CYTOMETRY

Circulating leukocyte subsets were analyzed using flow cytometry. Red blood cell lysis was performed on sodium heparinized blood using RBC lysis buffer (Thermo Fisher Scientific). After washing with PBS (pH 7.2), leukocytes were incubated with fluorochrome-labeled antibodies for 30 minutes on ice. After a final washing step, leukocytes were measured on a MACSQuant 16 analyzer (Miltenyi Biotec). See supplemental **table S1** for a full list of antibodies used.

CYTOKINE MEASUREMENTS

IFNγ and IL-2 were quantified using the Vplex-2 kit (Meso Scale Discovery). IFN and IL-6 were quantified using the pan-specific IFN ELISA^{pro} HRP kit and the IL-6 ELISA^{pro} HRP kit (both Mabtech, Nacka Strand, Sweden).

STATISTICAL ANALYSIS

In vitro data are reported as mean ± standard deviation (SD). The IC50 was calculated using a inhibitory sigmoid E_{max} function where applicable. Analyses were performed using Graphpad Prism version 6.05 (Graphpad, San Diego, CA, USA).

Repeatedly measured pharmacodynamic data were evaluated with a mixed model analysis of variance with fixed factors treatment, age group, time, treatment by time, age group by time, treatment by age group and treatment by age group by time and a random factor subject and the average pre-value as covariate. If needed, variables were log transformed before analysis. Contrasts between the placebo and HCQ treatment groups were calculated per endpoint. In addition, a potential age-specific HCQ effect was evaluated by comparing the 18-30 years with the 65-75 years age group. For the contrasts, an estimate of the difference (back-transformed in percentage for log transformed parameters), a 95% confidence interval (in percentage for log-transformed parameters), Least Square Means (geometric means for log transformed parameters), and the p-value were calculated. A p-value ≤ 0.05 was considered to be statistically significant. All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA).

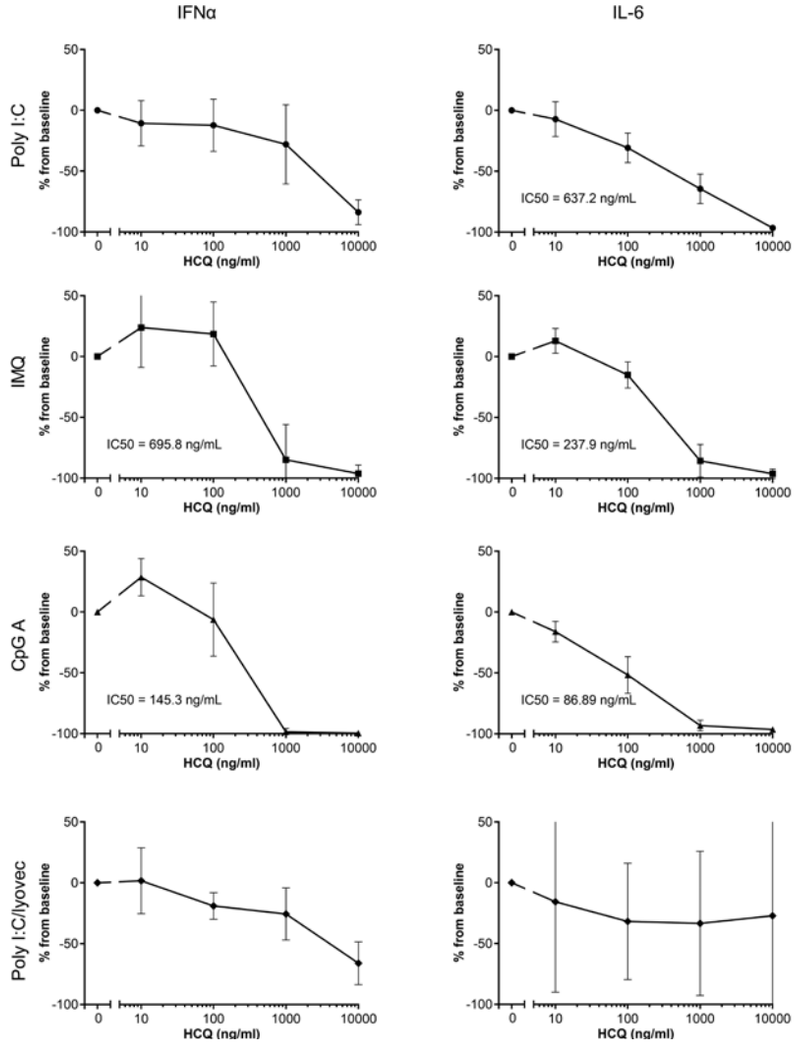
Results

IN VITRO EXPERIMENTS

HYDROXYCHLOROQUINE SUPPRESSED ENDOSOMAL TLR-INDUCED IFNα AND IL-6 RELEASE IN VITRO

PBMCs were stimulated with endosomal TLR ligands in the presence of a dose range of HCQ for 24 hours, and supernatants were analyzed for IRF-mediated IFN and for NFκB-mediated IL-6 secretion. PBMCs were stimulated with different endosomal TLR ligands: poly I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and poly I:C lyovec (RIG-I). HCQ dose-dependently inhibited endosomal TLR-induced IFN and IL-6 secretion (**Figure 1**). Poly I:C-induced IFN and IL-6 release was strongly suppressed at 10.000 ng/mL (IFN: -83.9%, IL-6: -96.6%, IC50 IL-6 = 637.2 ng/mL). Imiquimod (IMQ)-induced cytokine release was completely suppressed at the highest concentration (IFN: -96.3%, IL-6: -96.3%, IC50 IFN: 695.8 ng/mL, IL-6: 237.9 ng/mL). The same was observed for stimulation with CpG class A, IFN was suppressed by 99.6% with an IC50 of 145.3 ng/mL, and IL-6 was suppressed by 96.4%, with an IC50 of 86.9 ng/mL. The RIG-I response to poly I:C/lyovec was less affected by HCQ, while IFN release was suppressed by 66.1% at 10,000 ng/mL HCQ, IL-6 release was not significantly altered.

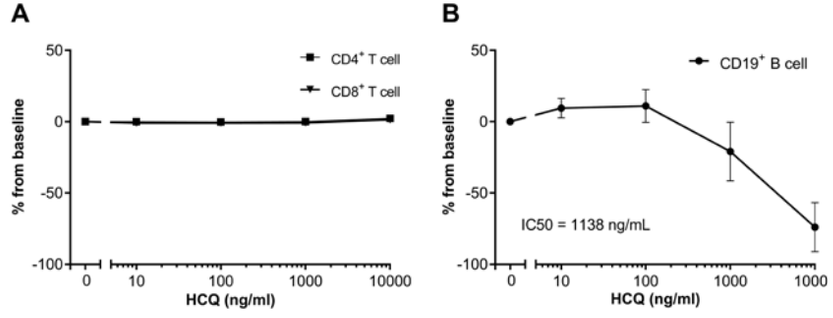
Figure 1 HCQ dose-dependently inhibited endosomal TLR induced IFN and IL-6 release *in vitro*. PBMCs were stimulated with 50 µg/mL POLYI:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5 µM CPG-A (TLR9) or 1 µg/mL POLY I:C/lyovec (RIG-I) for 24 hours in the presence of a dose range of HCQ. IFN and IL-6 release were measured by ELISA. The mean ± SD of the change from baseline of 6 subjects is shown. The IC50 was calculated using a four-parameter non-linear regression fit where applicable.



HCQ INHIBITED B CELL PROLIFERATION BUT NOT T CELL PROLIFERATION IN VITRO

PBMCs were stimulated with phytohaemagglutinin (PHA) or monoclonal anti-CD40 with CpG-B to induce T cell and B cell proliferation respectively, in the presence of a dose range of HCQ. No effect of HCQ was seen on T cell proliferation (**Figure 2A**). Also, no effects were observed on T cell activation markers after PHA stimulation for 6 hours (**Figure S1**). At HCQ concentrations >100 ng/mL, a decrease in B cell proliferation was observed, with an IC50 of 1138 ng/mL (**Figure 2B**).

Figure 2 HCQ dose-dependently inhibited B cell, but not T cell proliferation *in vitro*. PBMCs from 6 healthy donors were stained with CTV and stimulated for 5 days with 5µg/ml PHA for T cell proliferation (A), or 5 µg/mL anti-CD40 MAB + 2.5 µM CPG B for B cell proliferation (B). Proliferation was measured by flow cytometry. The mean ± SD of the change from baseline are shown. The IC50 was calculated using a four-parameter non-linear regression fit where applicable.

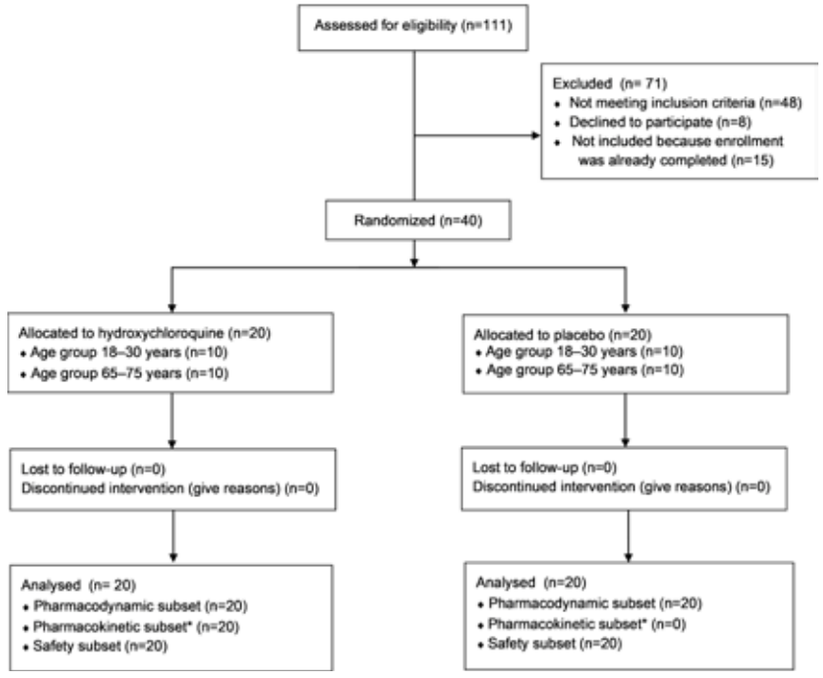


CLINICAL STUDY

DEMOGRAPHICS AND SAFETY

Of the 40 enrolled and randomized healthy subjects, 20 received a cumulative dose of 2400 mg HCQ in 5 days and 20 received placebo (**Figure 3**). The different age groups (18 – 30 and 65 – 75 years) were of equal size. Baseline characteristics are described in **Table 1**. All subjects completed their study treatment. One subject in the 65 – 75 years group erroneously took an additional 400 mg dose of HCQ on study day 2, after which the subject received 400 mg doses (once daily) for two consecutive days to not exceed the cumulative dose of 2400 mg.

Figure 3 Trial flow chart (CONSORT diagram).



** Drug concentrations were only analyzed in the active treatment group.*

Table 1 Baseline characteristics.

	Hydroxychloroquine		Placebo	
	Age group 18-30 yrs (N=10)	Age group 65-75 yrs (N=10)	Age group 18-30 yrs (N=10)	Age group 65-75 yrs (N=10)
Age, median (range)	23 (20-26)	68 (65-70)	23 (18-25)	68 (65-71)
BMI, mean (SD)	21.8 (1.5)	25.8 (2.0)	24.4 (1.9)	24.2 (3.0)
Race or ethnicity*, n (%)				
White	10 (100)	10 (100)	10 (100)	10 (100)
Other	0 (0)	0 (0)	0 (0)	0 (0)

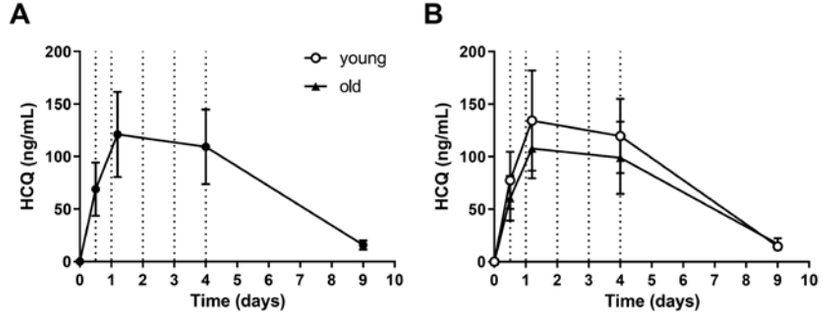
**Self-reported race or ethnicity of subjects. BMI = body mass index; SD = standard deviation.*

Treatment-emergent adverse events were transient, of mild severity and did not lead to study discontinuation. Adverse events were reported more often by subjects in the active treatment arm (50%) compared to placebo (35%). Gastrointestinal complaints (20%) and dizziness (15%) were the most frequently reported adverse events in the active group. There were no findings of clinical concern following assessments of urinalysis, hematology and chemistry laboratory tests, vital signs, physical examination and ECGs.

PHARMACOKINETICS

Mean HCQ concentration time profiles in plasma are depicted in **Figure 4A**. Individual concentration profiles have been published previously.[22] There were no significant differences in HCQ exposures between age groups (**Figure 4B**). Mean concentrations measured 27 hours after starting the treatment course (day 1, 121.0 ± 40.54 ng/mL) were in a similar range to those measured on the last day of the treatment course (day 4, 109.2 ± 35.59 ng/mL).

Figure 4 Pharmacokinetic profile of HCQ. Mean and standard deviation of hydroxychloroquine plasma concentrations for HCQ treatment group (A), split for young and elderly volunteers (B). Dotted vertical lines indicate timing of HCQ dosing (0, 12, 24, 48, 72, 96 hours).



PHARMACODYNAMICS

HYDROXYCHLOROQUINE DID NOT AFFECT CIRCULATING IMMUNE CELLS

The effects of HCQ on different circulating cell populations, both absolute as relative, were evaluated using flow cytometry. No apparent effects were seen on absolute values of total leukocytes, lymphocytes, monocytes or neutrophils (**Table S2**), as well as $CD14^+$ monocytes, $CD19^+$ B cells, $CD3^+$ T cells, $CD4^+$

T cells and CD8$^+$ T cells (**Table S3**). Furthermore, no effects were seen on relative T cell populations (CD3$^+$) in general, nor on subpopulations of T helper cells (CD4+), cytotoxic T cells (CD8$^+$), and regulatory T cells (CD4$^+$CD25$^+$CD127$^-$). Similarly, no apparent treatment effects were observed in natural killer cells (CD56$^+$), B cells (CD19$^+$) and subpopulations of regulatory (CD5$^+$CD1D^{hi}), transitional (CD24^{hi}CD38^{hi}) and antibody secreting B cells (CD27$^+$CD38$^+$). Moreover, also in classical (CD14$^+$), non-classical (CD16$^+$) and intermediate (CD14$^+$CD16$^+$) monocytes and plasmacytoid dendritic cells (pDCs, HLA-DR$^+$CD14$^-$CD16$^-$ CD123$^+$) no differences were found between treatment groups. Also, between both age groups, no evident HCQ effects were observed (**Table S3**).

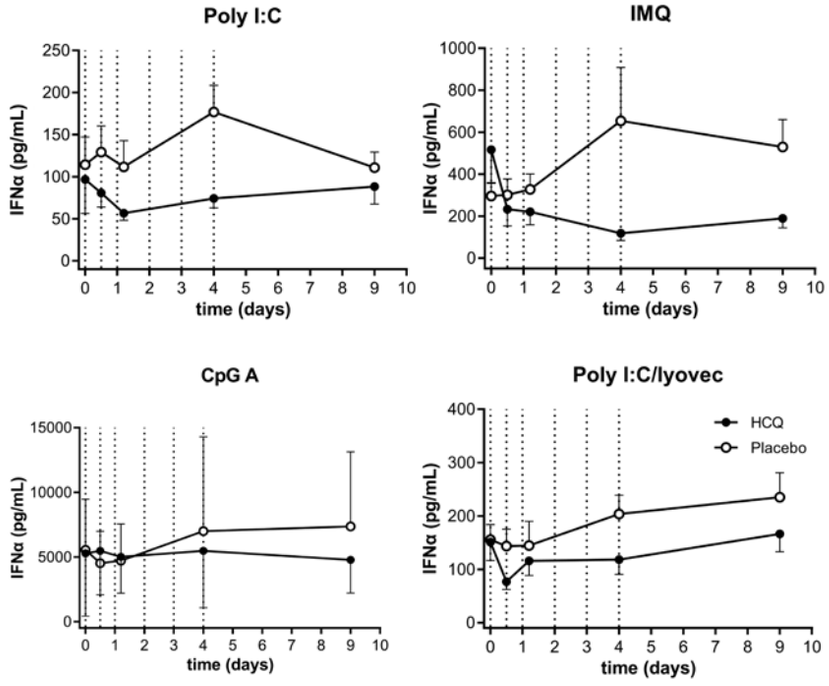
IN VIVO HYDROXYCHLOROQUINE SUPPRESSED IFN SECRETION FOLLOWING TLR7 STIMULATION, BUT NOT AFTER TLR3, TLR9 OR RIG-I-LIKE RECEPTOR STIMULATION

To study the effects of HCQ on TLR/RIG-I-mediated IRF activation, PBMCs were stimulated with different endosomal TLR ligands: poly I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and poly I:C lyovec (RIG-I). Overall, no HCQ effect was observed on IFN responses (**Figure 5**), except for a significant suppression of IMQ-driven IFN production (inhibition of -48.2%, CI95 -72.1% - -4.0%, p = 0.038). Poly I:C-driven IFN release also appeared to be suppressed by HCQ, but not significantly (inhibition -34.2%, CI95 -57.7% - 7.5%, p = 0.091). No differences in HCQ effect on IFN responses were observed between the young and elderly population (**Figure S3**).

IN VIVO HYDROXYCHLOROQUINE SIGNIFICANTLY SUPPRESSED IL-6 SECRETION AFTER TLR7 STIMULATION, BUT NOT FOLLOWING TLR3, TLR9 OR RIG-I-LIKE RECEPTOR STIMULATION

Activation of NFκB signaling via endosomal TLR and RIG-I-like ligands was assessed by measuring downstream IL-6 production (**Figure 6**). HCQ significantly suppressed IMQ-driven IL-6 production (inhibition of -71.3%, CI95 -84.7% – -46.1%, p = 0.0005). No significant HCQ effects were observed on IL-6 production driven by CpG A (TLR9) and poly I:C (TLR3) stimulations (inhibition of -35.9%, CI95 -60. 3% – 3.6%, p = 0.068 and -37.7%, CI95 -62.6% - 3.7%, p = 0.067, respectively). No differences in HCQ effect on IL-6 responses were observed between the young and elderly population (**Figure S3**).

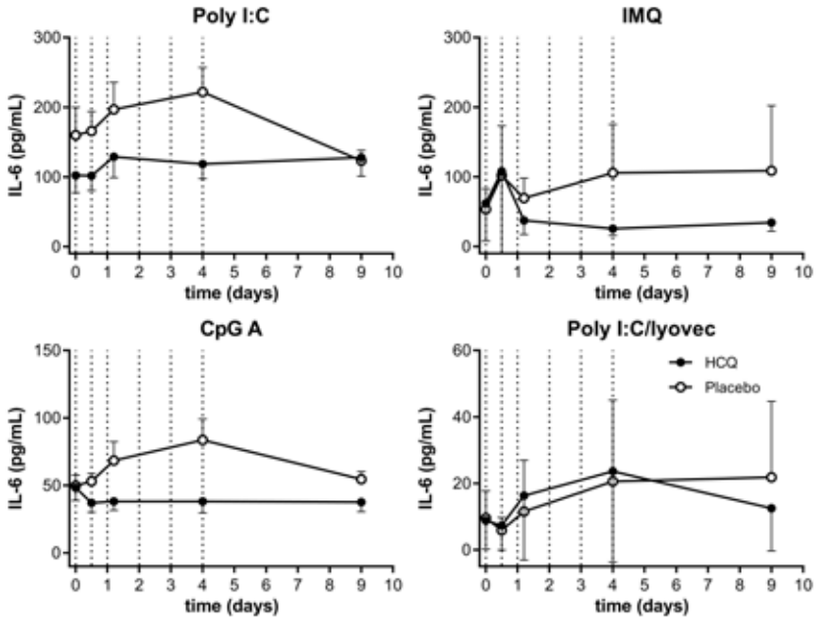
Figure 5 ***In vivo*** **HCQ inhibited IMQ-induced IFN release, but not TLR3, TLR9 and RIG-I.** PBMCs were stimulated with 50 µg/mL POLY I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CPG A (TLR9) or 1 µg/mL POLY I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFN release was measured by ELISA. Data is shown as mean ±SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.



IN VIVO HYDROXYCHLOROQUINE DID NOT ALTER T CELL ACTIVATION

To further investigate the potential immunomodulatory effect of HCQ on T cell activation, whole blood samples were incubated with PHA, which is known to induce a general T cell response.[23] HCQ treatment did not modulate expression of T cell activation markers (CD25, CD69, CD71, CD154) following PHA-stimulation (**Figure S3**). In addition, PHA-induced secretion of IL-2 and IFNγ was assessed, no apparent differences were observed between HCQ and placebo (**Figure S4**).

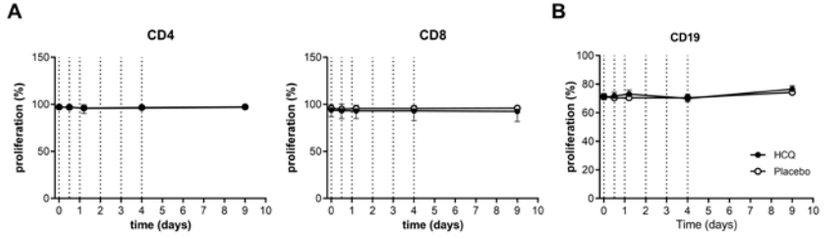
Figure 6 In vivo HCQ inhibited IMQ-induced IL-6 release, but not TLR3, TLR9 and RIG-I. PBMCs were stimulated with 50 µg/mL POLY I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CPG A (TLR9) or 1 µg/mL POLY I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFN release was measured by ELISA. Data is shown as mean ±SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.



HYDROXYCHLOROQUINE DID NOT ALTER EX VIVO B AND T CELL PROLIFERATION AFTER IN VIVO ADMINISTRATION

Proliferative capability of B cells was assessed by stimulating PBMCs *ex vivo* with anti-CD40 mAbs + CpG B ODNs, a known stimulus for human B cell activation.[24] Following stimulation of PBMCs, the percentage of proliferative B cells in the HCQ-treated group was similar to that of the placebo group (70.47% at day 4 for placebo, 70.03% for HCQ) (**Figure 7**). In addition, PBMCs were stimulated with PHA to induce T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) proliferation. Proliferation of both $CD4^+$ and $CD8^+$ cells was comparable between the HCQ- and placebo-treated group (>95% for both groups for all time points for CD4, >92% for both groups for all time points for CD8). No differences were observed for B and T cell proliferation in the separate age groups (**Figure S5**).

Figure 7 *In vivo* HCQ did not affect T and B cell proliferation. PBMCs were stained with CTV and stimulated for 5 days with 5 µg/ml PHA for T cell proliferation (A), or 5 µg/mL anti-CD40 MAB + 2.5 µM CPG B for B cell proliferation (B). Proliferation was measured by flow cytometry. The mean ±SD are shown. Dotted vertical lines indicate HCQ dosing times.



Discussion

Although HCQ is widely used for the treatment of autoimmune diseases, the exact mechanism behind its immunomodulatory properties remains unclear. In this study we therefore aimed to quantify the immunosuppressive effect of HCQ by studying the endosomal TLR response and lymphocyte proliferation and activation both in *in vitro* experiments and *in vivo* in a randomized placebo-controlled trial in healthy volunteers.

In our *in vitro* experiments, HCQ dose-dependently inhibited TLR3-, 7- and 9-driven IL-6 and IFN production, with profound effects at concentrations >100 ng/mL. These findings are in line with literature on TLR signaling modulation by chloroquine.[9,25] Limited data are available on the immunomodulatory effect of HCQ/chloroquine on RIG-I signaling.[26] RIG-I functions as a cytosolic sensor of nucleic acids, inducing a type I IFN response after activation. HCQ inhibited the IFN responses in THP-1 cells transfected with RIG-I ligands[27], but this effect was not confirmed in cultures of human bronchial smooth muscle and epithelial cells.[28-29] This is in line with the observations in the current study, which shows that HCQ only mildly modulated RIG-I-mediated IFN production in PBMCs, without affecting IL-6 release. Our results suggest that HCQ has a profound effect on endo-lysosomal TLR functioning *in vitro* but affects the cytosolic RIG-I-mediated pathway to a lesser degree. This could be explained by HCQ's excessive affinity to the lysosomal intracellular compartment (expected to be 56,000-fold higher than cytosol).[30]

HCQ did not affect T cell activation *in vitro*. Although a dose-dependent inhibition of T cell proliferation by chloroquine following stimulation with anti-CD3/CD28 has been described[31-33], we did not see any inhibitory effect of HCQ on T cell proliferation or expression of activation markers in our *in vitro* experiments. This may be explained by the fact that a different and more potent stimulus was used in this study (PHA), which might be more difficult to suppress. For B cell proliferation, on the other hand, a dose-dependent HCQ-mediated inhibition was observed *in vitro*, confirming previous research.[34] Although the HCQ-mediated inhibition was not as strong as the inhibition of cytokine production (IC50 of 1138 ng/mL for B cell proliferation vs 145-696 ng/mL for cytokine production), at concentrations > 100 ng/mL a clear HCQ-mediated decrease in B cell proliferation was found.

While HCQ had strong immunosuppressive effects *in vitro*, especially at high concentrations, less pronounced *ex vivo* effects of the compound were observed in our clinical study. Compared to placebo, 5-day HCQ treatment did not significantly suppress B cell proliferation or *ex vivo* TLR-driven IFN and IL-6 secretion in PBMC cultures, except for a suppressive effect on TLR7-driven responses. The most likely explanation for this discrepancy between *in vitro* and *ex vivo* is that there was insufficient drug exposure at the evaluated HCQ dose and regimen in the clinical study. By using a 5-day dose regimen of HCQ (the recommended off-label dose for COVID-19 at the time of study conduct), an average maximum plasma concentration of 121 ng/mL was reached. This concentration is considerably lower than plasma levels found in RA patients receiving HCQ treatment of 200 mg daily for a longer time period, which ranges from 200 – 500 ng/mL.[35-36] Peak exposures of 100-150 ng/mL from the clinical study translate into a maximal inhibitory effect of 20 to 50% in most cellular assays. In combination with the observed variability of the endpoints, such effects remain easily undetected. However, whole blood concentrations are expected to be approximately 2-to-7-fold higher than plasma concentrations due to intracellular uptake in blood components[38-40], which would make the concentrations more in range with the *in vitro* experiments. Also, due to the large volume of distribution[39], and the high HCQ tissue concentrations as compared to plasma[41-42], immunosuppressive effects in specific tissues may be significant. Moreover, HCQ has a gradual onset of action for HCQ, and is biologically active even after drug discontinuation.[8] This would mean that the five-day treatment that was used in the current study is insufficient to detect *ex vivo* drug effects. Other studies, for example investigating HCQ effect in HIV patients[43], showed a discrepancy between plasma levels and drug efficacy.

The widespread use of hydroxychloroquine following the onset of the COVID-19 pandemic was the reason to initiate our experiments. The initial off-label use of HCQ was primarily based on studies that assessed *in vitro* antiviral activity against SARS-CoV-2.[44] However, there is also a long-standing hypothesis that the immunomodulatory properties of chloroquine and HCQ could dampen immunopathology caused by viral infections such as influenza, Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) and COVID-19 by suppressing the host immune response.[45-47] Use of HCQ in COVID-19 patients did not show evident favorable effects for clinical endpoints such as mortality and mechanical ventilation for both prophylaxis and treatment.[48] Our study provides mechanistic insight in the immuno-modulatory effects of a HCQ dosing regimen that was used to treat COVID-19. We found that a 5-day treatment course of HCQ did not have extensive immuno-modulatory effect in healthy individuals. HCQ treatment only significantly inhibited TLR7 responses. In theory, inhibition of the TLR7-mediated innate response to viral agents may be disadvantageous during the initial stages of viral infection.[49-50] However, recent COVID-19 trials did not show an effect of HCQ treatment on disease incidence, and long-term HCQ use in rheumatoid arthritis is not associated with higher incidence of upper respiratory tract infections.[51-52]

In conclusion, we showed extensive and profound immunomodulation by HCQ *in vitro*, however in a clinical study in healthy volunteers, the overall immunomodulatory effects of a 5-day HCQ treatment regimen of 2400 mg were limited. The pharmacological activity of HCQ in autoimmunity remains to be studied in greater detail, based on the assays as presented in our studies and at a therapeutic dose and regimen relevant for the condition of interest.

SUPPLEMENTAL MATERIAL

All mentioned supplementary figures and tables in this chapter can be found on the publisher's website by scanning the QR code.

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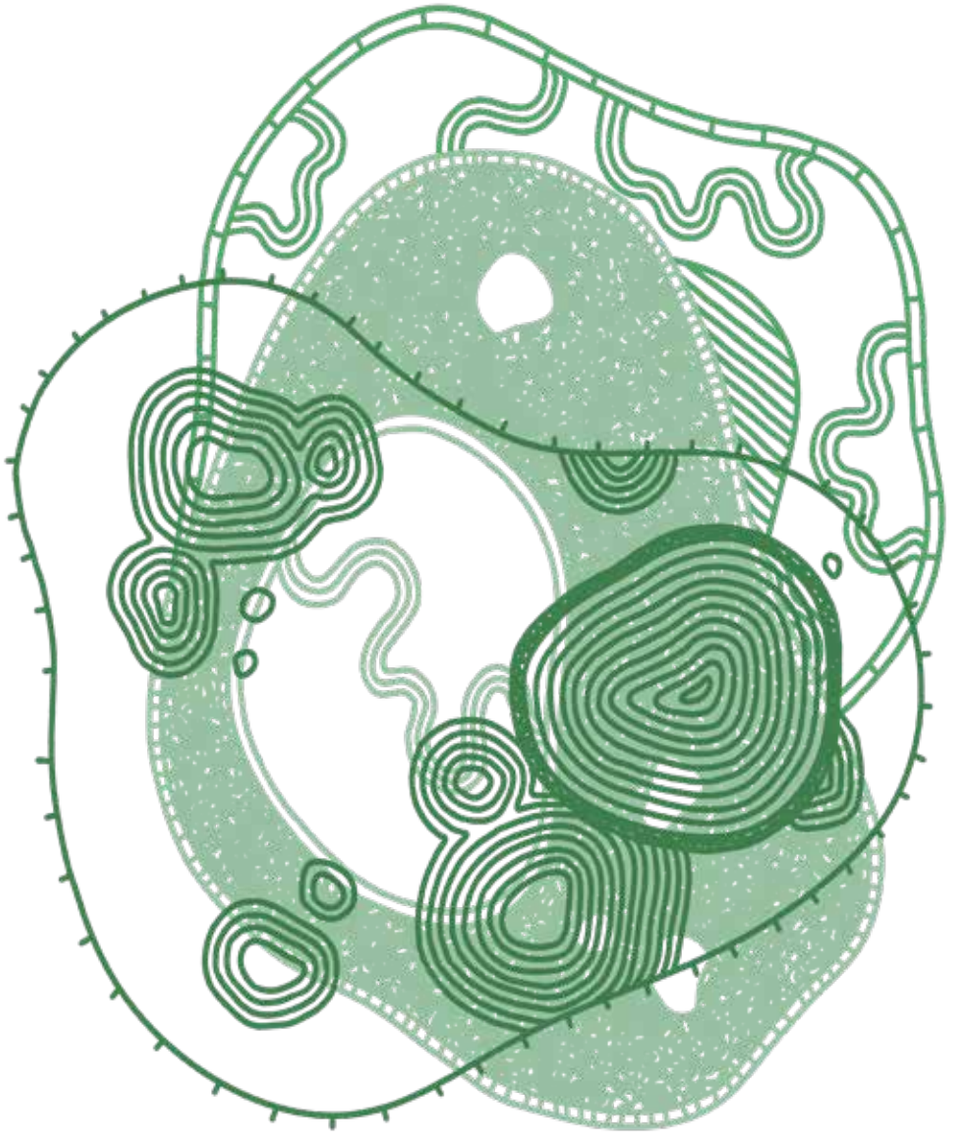

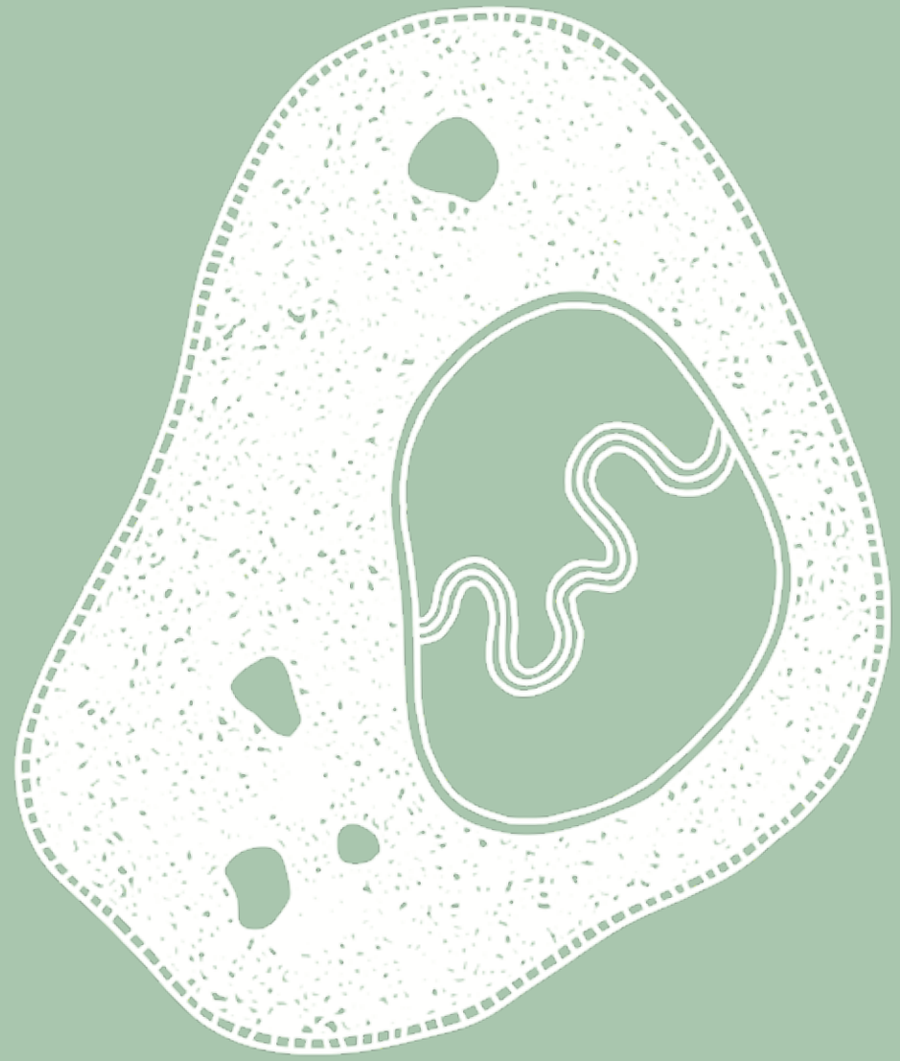

The human immune system is a well-regulated system that is crucial for survival. However, it may experience disturbances caused by diseases (such as allergies, autoimmune conditions, and cancer), pathogens (such as HIV), or the use of medications (like immunosuppressants), leading to over- or underactivity. While an overactive immune system can result in the attack of healthy tissue, an underactive immune response increases the risk for inadequate control of infections or the development cancers. Immunomodulatory drugs can be used to balance such immune system disturbances. However, since these drugs have a significant effect on the immune system, the use of immunomodulatory medication can also result in similar adverse effects, like those observed in immune-related diseases. To prevent immune dysregulation, it is therefore very important to select the right immunomodulatory medication at the right dose for the appropriate indication. This can be a challenge, as the immune system comprises many different cells and molecules. Monitoring of immune functionality, referred to as immunomonitoring, can be a useful approach to monitor the effects of immunomodulatory drugs on their proximal targets.

In this thesis different methods and applications of immunomonitoring are described. **Section I** concentrates on the search for biomarkers to monitor the immune function under treatment for transplant patients. In **section II**, comparable methods of immunomonitoring were used to gain a better insight in the mechanism of action and the dose-effect relationship of the immunosuppressant HCQ.

Section I

The recommended maintenance treatment after kidney transplantation consists of a calcineurin inhibitor (CNI, tacrolimus or cyclosporine A) and an antiproliferative agent (mycophenolate mofetil, MMF) with or without low-dose corticosteroid (prednisolone).[1] To prevent rejection of the transplanted organ, while minimizing toxicity by over-suppression, the exposure to these immunosuppressants is monitored in clinical practice by measuring drug concentration in whole blood or plasma (therapeutic drug monitoring, TDM).[2] While TDM is effective, it does not necessarily correlate with the pharmacological activity of the measured drug. To find the balance between optimal efficacy and minimal toxicity, it might be more informative to monitor patients' immunological status rather than drug concentrations. Section I of this thesis therefore describes the search for biomarkers that inform on the immunosuppressive state of transplantation patients and can improve personalized dosing.

Since T cells are the main mediators transplanted organ rejection, most immunosuppressive therapies aim to inhibit T cell activity. In our search to find biomarkers that reflect the immune status of transplantation patients, we therefore focussed on monitoring T cell activity and function. Activation of the enzyme calcineurin is one of the first steps after T cell activation by an antigen-presenting cell. Calcineurin drives the translocation of NFAT into the nucleus where it induces expression of pro-inflammatory genes that are required for activation and proliferation of the T cell. In **chapter 2** we investigated three T cell function assays in parallel: calcineurin activity, cytokine production (IL-2 and IFN-γ) and T cell activation markers (the expression of CD69, CD25, CD71 and CD154). Calcineurin activity was measured directly from whole blood, while for the measurement of cytokine production and T cell activation marker expression, whole blood was first incubated with a T cell stimulus (PHA) to drive activation of the T cells. Based on the selected T cell function assays, the pharmacological activity of a single dose of the calcineurin inhibitor tacrolimus was evaluated in a clinical study in healthy volunteers. We showed that tacrolimus has a strong inhibitory effect on IL-2 and IFN-γ production, and on the expression of CD71 and CD154, qualifying these markers for monitoring of the pharmacodynamic effects of this calcineurin inhibitor. Whereas IL-2 production was completely inhibited after tacrolimus intake (maximum inhibition of 90%), this was not the case for the other biomarkers, indicating that there was still some remaining T cell activity after dosing. Moreover no drug effect on calcineurin activity was found because of the large inter- and intra-subject variability.

In addition to the evaluation of functional pharmacodynamic (PD) endpoints, this study also included various pharmacokinetic markers identified in the literature as promising.[3] Tacrolimus concentrations were quantified in whole blood as well as within specific target cells, namely peripheral blood mononuclear cells (PBMCs) and T cells. Overall, the pharmacokinetic profiles of all three matrixes were comparable, with a peak concentration at 1.5 hours. The intracellular tacrolimus concentrations, however, were significantly higher in PBMCs compared to T cells, even though the majority of PBMCs consist of T cells (60%).[4] While we were unable to pinpoint which

subset of PBMC cells explained this discrepancy, other studies have shown that monocytes take up more tacrolimus than T cells.[5] Also, the peak concentration in PBMCs demonstrated no correlation with the peak concentration in whole blood. In contrast, a statistically significant correlation was observed between the concentration in T cells and the concentrations in whole blood. We concluded that since T cells are the primary target cells for immunosuppressive therapy, the tacrolimus concentration in whole blood, which is the current method of therapeutic drug monitoring (TDM), serves as a good representation of the concentration within the target cell.

In summary, several immune biomarkers investigated in this healthy volunteer study exhibit potential for demonstrating the immunosuppressive effects of drugs in transplant patients, including IL-2, IFN-γ, CD71, and CD154. However, the biomarker closest to the drug target, namely calcineurin activity, showed no drug effect and was highly variable. We therefore did not include the measurement of calcineurin activity in the subsequent study described in **chapter 3**. In this chapter, the search for biomarkers that inform on the immunosuppressive state of transplantation patients was continued, but with some modifications. Firstly, the experimental setup for assessing the expression of T cell activation markers was optimized by reducing the incubation time of whole blood with the T cell stimulus PHA to 6 hours, as opposed to the previous 48 hours. This optimization resulted in enhanced expression of the selected activation markers, thereby widening the potential window for demonstrating drug effects. Secondly, an additional measure of T cell activity was introduced: the assessment of T cell proliferation. In this immune assay, whole blood was stimulated for 48 hours, and the proliferation of T cells was measured by labelling a nucleoside analogue that is incorporated during DNA synthesis of actively dividing cells. Thirdly, a placebo group was incorporated. While exploring novel biomarkers, including untreated volunteers in the study is important to investigate the inter-subject variability of the chosen immune assays. Lastly, the exclusion of the labour-intensive calcineurin assay enabled the collection and processing of samples at more time points throughout the day, thereby increasing the data points available for analysis.

Among the immunosuppressive drugs prescribed for transplantation patients, calcineurin inhibitors are known for their substantial intra- and interpatient pharmacokinetic variability and narrow therapeutic window.[6] To validate the efficacy of the new immune assays, we conducted a clinical study in healthy volunteers who received a single dose of the calcineurin inhibitor cyclosporine A (CsA). Immune activity was measured *in vitro* and *ex vivo* at three different levels: cytokine production (IL-2 and IFN-γ), the expression of T cell activation markers (CD69, CD25, CD71 and CD154) and T cell proliferation. Similar to what was found for tacrolimus, CsA significantly impacted IL-2, IFN-γ, CD71, and CD154. These biomarkers showed maximal inhibition (approximately 90%) at 2- and 3- hours post-dosing, and returned to baseline levels after 24 hours, aligning with the pharmacokinetic profile of CsA in whole blood. The new readout measure, T cell proliferation, demonstrated a robust 63% inhibition. Overlaying the *in vitro* and *ex vivo* CsA effects on these biomarkers revealed clear similarities, suggesting that the mean *in vitro* dose-response curve serves as a reliable predictor for the *ex vivo* inhibitory CsA effect. Despite the fluctuations in immune markers during the day, which were clearly visible in placebo-treated volunteers, distinction between placebo- and CsA-treated subjects remained possible based on the selected biomarkers. Lastly, we demonstrated that pharmacokinetic profiles of CsA were comparable across whole blood, peripheral blood mononuclear cells (PBMCs), and T cells, underlining the limited additional value of monitoring intracellular CsA concentrations.

Overall, we conclude that IL-2 and IFN-γ production, CD154 and CD71 expression, and T cell proliferation are good biomarkers to monitor the immunosuppressive effect of a calcineurin inhibitor (i.e. tacrolimus and cyclosporine A). For the immunosuppressive treatment after renal transplantation, calcineurin inhibitors are usually combined with an antiproliferative agent, mycophenolate mofetil (MMF). MMF is the pro-drug of mycophenolic acid (MPA), a specific inhibitor of the enzyme IMPDH and thereby blocks lymphocyte proliferation. Before testing the functionality of our immune assays in monitoring the immunosuppressive treatment regimen in transplantation patients, we first investigated the effect of a single dose of MMF on these biomarkers, like for tacrolimus and CsA. In **chapter 4**, a clinical study is described in which healthy volunteers received oral dose of 1000 mg MMF or placebo. Three different pharmacodynamic readout measures were investigated: cytokine production (IL-2 and IFN-γ), T cell proliferation and IMPDH activity.

The expression of activation markers on T cells was not included since pre-clinical experiments showed that MPA did not have any inhibitory effect on these biomarkers. As expected from an anti-proliferative drug, the

immunosuppressive effect of MPA was best demonstrated in the T cell proliferation assay. Already at 30 minutes after drug intake, proliferation was completely inhibited in the MMF-treated volunteers compared to placebo. The *in vitro* MPA effect varied between subjects, but all volunteers reached maximum inhibition at a concentration of 2 mg/L. In clinical practice, a target AUC_{0-12h} of 30–60 mg*h/L is recommended for transplantation patients, which roughly corresponds to a trough concentration (C_0) of ~2 mg/L.[7-8] At this concentration, both our *in vitro* and *ex vivo* data showed maximum suppression of T cell proliferation, indicating that T cell proliferation in renal transplantation patients is most likely always completely suppressed in MMF-treated transplant patients. IL-2 is the cytokine that mainly drives T cell proliferation, and in the *in vitro* incubations with MPA a concentration-dependent inhibition of IL-2 was found. No difference, however, was found between active and placebo-treated subjects after dosing, potentially caused by the diurnal rhythmicity in circulating T cell numbers.[9]

Besides cytokine production and T cell proliferation, the enzymatic activity of IMPDH was also studied. Since IMPDH is the direct target of MPA, it does not provide information about activity of the overall immune response but is an interesting biomarker to demonstrate direct MPA effects. Although this biomarker has been described to successfully demonstrate IMPDH activity in transplantation patients[10], no substantial *ex vivo* effect from MMF treatment on IMPDH activity was observed in our study, possibly because of the large intrasubject variation caused by both technical and biological variability. Finally, as in the previous studies, pharmacokinetics of MPA were studied in three different matrixes: plasma, PBMCs and T cells. A strong correlation between plasma concentrations and the MPA concentrations inside the target cell (e.g., PBMCs and T cells) was found, indicating that there was no added value in measuring intracellular MPA concentrations rather than plasma concentrations. Interestingly, concentrations in the T cells were higher than those in PBMCs, which would be beneficial as T cells are the targeted population for post-transplant immunosuppressants.

After demonstrating that the selected immune assays (cytokine production, T cell proliferation, and T cell activation marker expression) were suitable to demonstrate immunosuppressive effects of CNIs (tacrolimus and cyclosporine A) and MMF in healthy volunteers, we continued to study the potential value of these functional biomarkers in transplantation patients. A small patient population of stable kidney transplantation patients, treated with the standard triple immunosuppressive therapy (tacrolimus, MMF and low-dose prednisolone), was included in the study described in **chapter 5**. We measured drug concentrations of MMF and tacrolimus and investigated how these concentrations relate to the patient's immune competence by measuring cytokine production, T cell proliferation, and T cell activation marker expression over one day. Overall, the results of this patient study confirmed what we had previously shown in the healthy volunteer studies. T cell proliferative capacity in transplantation patients was completely suppressed, with proliferation not exceeding 0.5%. Despite this, cytokine production (IL-2 and IFN-γ) and the expression of activation markers on T cells (CD154 and CD71) remained detectable and showed fluctuations throughout the day. The peak concentration of tacrolimus in the blood occurred between 2- and 3-hours after drug intake and resulted in the most pronounced inhibition of T cell immune activity. On average, the maximal inhibition of IL-2, IFN-γ, CD154, and CD71 *in vitro* was similar to the maximum inhibition *ex vivo*, observed 2-3 hours after drug administration. The individual patient analysis, however, revealed no significant correlation between *in vitro* and *ex vivo* immunosuppressive effects. This is probably because MMF and prednisolone, which are co-administered with tacrolimus, were not added to the *in vitro* cultures. Although these drugs have only shown a very minimal immunosuppressive effect on the selected biomarkers in pre-clinical experiments, long-term use of these compounds may have a more profound effect in kidney transplantation patients. Furthermore, we explored the correlation between *ex vivo* drug activity and other factors that have been described to play a role in transplantation immunology, including age, tacrolimus trough levels (C_0), tacrolimus peak levels (C_{max}), the presence of inhibitory T and B cell populations, and the viral load of TTV. We did not find a clear relationship between most of these factors and the immunosuppressive effect evaluated by our functional immune assays.

Measurement of T cell activity after activation with PHA is a method to broadly evaluate the general responsiveness of the immune system. The immune assays described in this section of the thesis were not developed to specifically mimic a rejection immune response, nor were they designed to demonstrate drug-specific effects. The aim of the immune assays was to function as biomarkers that can be used to monitor the general immune status of transplantation treated with a combination of immunosuppressive drugs. In the clinical studies described in chapter 2, 3, and 4 we identified

the three immune assays that have the potential to monitor under- or over-immune suppression: production of IL-2 and IFN-γ, T cell proliferation and T cell expression of CD71 and CD154. In chapter 5, we demonstrated that two out of three immune assays (cytokine production, and T cell activation marker expression) were successful in demonstrating drug-effect in kidney transplant patients who have been treated with immunosuppressive therapy for more than 2 years. Interestingly, the most effective biomarker in demonstrating the immunosuppressive effect of MMF in healthy volunteers, T cell proliferation, was completely suppressed in all kidney transplantation patients. This means that either it is necessary to completely inhibit T cell proliferation to prevent rejection, or the patients are over-suppressed and the dose of MMF can be lowered. Because we have not investigated how our PD markers relate to clinical outcome, we cannot conclude which one of the two statements is true.

Section I of this thesis focused on two main objectives. Firstly, we explored whether monitoring patients with immune assays offers benefits compared to traditional drug level monitoring. Our findings revealed that immune responsiveness fluctuates throughout the day in transplantation patients, varying from maximal suppression post-medication to limited suppression at trough drug levels, just before the next dose. These fluctuations varied significantly among transplant patients. Although there was some overlap between drug levels and immune biomarkers on a general scale, individual patient analysis showed that drug levels, including C_0 and C_{max}, couldn't reliably predict immune suppression. Consequently, we conclude that functional immunomonitoring offers additional insights compared to drug level monitoring, but its added value does require further investigation in a larger prospective study. In the patient study described in this thesis, we solely investigated the level of immunosuppression throughout the day. We did not explore whether the degree of immunosuppression, as measured by the selected biomarkers, correlates with organ rejection or the occurrence of side effects. It is therefore currently not possible to make dosing decisions based on our biomarkers. As a next step, we would propose to conduct a study in which patients are followed longitudinally for an extended period, while their immune status is regularly assessed using the biomarkers described in this section. This would provide a better understanding of assay variability over time and would give insights in the relationship between our biomarkers and clinical outcomes such as toxicity, side effects, and rejection. Moreover, we suggest including patients undergoing immunosuppressant dose modifications during the study period, to investigate the impact of such adjustments on the level of immunosuppression. For the second main objective, we explored whether individual responses to immunosuppressive therapy, measured by our PD markers, can be predicted based on *in vitro* incubation with a concentration range of the drug. The degree of immune suppression observed after pre-dose *in vitro* incubation with tacrolimus, however, did not with the suppression found *ex vivo* in patient samples. This suggests that the immune effects on these PD markers after dosing is not solely caused by tacrolimus but may also involve other immunosuppressants that are co-administered, like MMF and prednisolone. To bridge the gap between *in vitro* and *ex vivo* drug activity, and to assess the predictive value of *in vitro* experiments, an extensive pharmacometric modeling approach would be desired.

The selected immune markers (included CD154, CD71, IL-2, IFN-γ and T cell proliferation) were exclusively studied in healthy volunteers and stable transplant patients undergoing the most used and well-known immunosuppressive therapy. However, we deliberately chose markers providing a broad view of the immune system's status for wider applicability. Although not explored in this thesis, the immune assays could be relevant for other immunosuppressive drugs and other types of patients. Rheumatoid arthritis (RA) or inflammatory bowel disease (IBD), for example, are two auto-immune diseases where T cells play an important role in the immune response underlying the disease. Every patient responds differently to the available immunosuppressive therapies[11-13], making it an interesting population to investigate whether immune monitoring has any additional value in treatment-decision making. Moreover, in addition to the standard immunosuppressants for transplantation discussed in this thesis, there are also other drugs to prevent long-term organ rejection. Immunosuppressants including MTOR inhibitors (i.e. rapamycin) or the selective T cell co-stimulation blocker belatacept (CTLA4 inhibitor) are currently used in transplantation patients where the standard triple immunosuppressive treatment regimen does not have the desired effect.[14] Our assays are based on PHA-induced T cell stimulation which offers robust immune activation[15], making it suitable to monitor the drugs with a broad immunosuppressive effect. Given rapamycin's potent inhibition of lymphocyte proliferation and belatacept's direct impact on T cell activation, we believe that our chosen

PD markers have the potential for effective immunomonitoring of these drugs. However, if we also want to use the biomarkers for novel and more specific immunomodulatory drugs, such as janus kinase (JAK) inhibitors or TNF blockers that are prescribed in autoimmune diseases, our T cell assays may not be suitable to demonstrate their subtle effects on the immune response. For these immunosuppressive drugs it would be interesting to explore alternative readout measures that are more target-related, such as JAK- or TNF-mediated cytokine production instead of the NFAT-mediated cytokine production that was described previously. Overall, we can conclude that the biomarkers described in this thesis provide us with a broad overview of T cell function, making them potentially applicable to other conditions and drugs that have a strong effect on the immune response.

Section II

Hydroxychloroquine (HCQ) is an antimalarial drug that, because of its immunosuppressive properties, is also prescribed for autoimmune disease such as rheumatoid arthritis and systemic lupus erythematosus. During the COVID-19 pandemic it was discovered that HCQ also has anti-viral activity against SARS-COV-2[16], which led to the start of multiple clinical trials investigating HCQ treatment in COVID-19 patients or in a prophylactic setting. It was hypothesised that the antiviral properties of HCQ could prevent SARS-COV-2 infection, and that its immunosuppressive properties would help dampening the overactive immune response in critically ill patients with COVID-19. Interestingly, while HCQ is a drug that is on the market for over 20 years, reliable clinical data on its immunosuppressive and antiviral properties were lacking. Most of HCQ's mechanistic evidence was based on *in vitro* experiments, with HCQ concentrations largely exceeding the concentrations measured in patients.[17-19] When the outcomes of initial clinical studies evaluating HCQ effects IN COVID-19 were not convincing[20], we wanted to have a better understanding on the mechanistic basis for the use of HCQ against SARS-COV-2. This resulted in a literature review on the potential role of HCQ at different stages of the disease is therefore described in **chapter 6**.

HCQ exerts its immunosuppressive effects by several different mechanisms. First, upon entering the cell, HCQ accumulates in the lysosomes and endosomes where it increases the pH. As a result, the enzymatic activity in these organelles decrease, and antigen processing, MHC class II presentation and subsequent immune activation are inhibited. Besides its effect on pH, HCQ can also directly inhibit the interaction between endosomal TLRs and their ligands (nucleic acids), and HCQ can inhibit intracellular calcium mobilization and subsequent NFAT-activity, which is important in T cell activation. The main reason for the use of HCQ in COVID-19 was because of its capability to prevent the cellular binding and entering of SARS-COV-2 virus particles into the cell.[21] The prevention of SARS-COV-2 cell entry makes HCQ very interesting in a prophylactic setting, especially in people with a high risk of infection. However, HCQ is also an inhibitor of the endosomal TLR response. Endosomal TLRs (TLR3, TLR4, TLR7, TLR8) are the receptors that are responsible for recognizing bacterial and viral nucleic acids, including SARS-COV-2, and start the innate immune response by inducing production of type 1 interferons (IFN). These cytokines are essential in the anti-viral immune response.[22] Theoretically, using HCQ as a prophylactic treatment could inhibit this type 1 IFN response and result in an increased viral infection risk, including SARS-COV-2 infection. However, the relationship between HCQ dose and level of immunosuppression is largely unexplored in primary human immune cells, making it difficult to estimate the actual effect of prophylactic use of HCQ treatment on the innate immune response.

While the use of HCQ in a prophylactic setting is debatable, in a therapeutic setting the immunosuppressive effects of HCQ could be more of interest. In short, the pathogenesis of COVID-19 can be split into three phases. In the first days (stage 1, day 0-2), the virus enters and replicates in the airway epithelial cells. In following days (stage 2, day 3-7) the innate immune system in the lung is activated and induces the adaptive immune response to clear the infection. In severe cases, the virus cannot be cleared by the immune system leading to a dysregulated immune response (cytokine storm), respiratory impairment and multi-organ failure (stage 3, > 7 days).[23] HCQ treatment in progressed COVID-19 patients is mechanistically supported by HCQ's pharmacological activities. By inhibiting virus replication, suppressing the TLR-mediated cytokine response and over-activation of lymphocytes, HCQ could prevent a cytokine storm and subsequent organ-failure. Most COVID-19 patients, however, only suffers from mild disease. These patients have a low viral load, develop an efficient type I IFN response, produce virus-neutralizing antibodies, and do not develop a maladaptive inflammatory response. On one hand, HCQ-dependent immunosuppression

could prevent mild disease turning into inflammation-driven severe disease in these patients. On the other hand, it is important that the virus-specific anti-SARS-COV-2 response is driven by an efficient antiviral innate type 1 IFN immune response in the early stages of disease, which may be significantly impaired upon HCQ treatment. We therefore conclude that the net result of HCQ treatment will probably depend on the balance between inhibition of viral replication, immunosuppression, and off-target side effects, as well as disease stage and disease severity.

Now the COVID-19 pandemic has come to an end, all clinical data on the use of HCQ in COVID-19 patients can be reviewed. Interestingly, the population of patients theoretically would benefit most from the pharmacological effects of HCQ, severe COVID-19 patients, showed no beneficial effect of HCQ treatment in comparison to the standard care[24], further underlining the incomplete understanding of the compounds pharmacology. As previously indicated, most of the immunosuppressive effects of HCQ have never been properly investigated in primary human cells or in a clinical setting. In **chapter 7**, we therefore aimed to assess and quantify the immunomodulatory effects of HCQ on primary human immune cells, both *in vitro* and *ex vivo* in a randomized clinical trial. Healthy volunteers were dosed with HCQ or placebo in the standard dosing regimen for moderate-to-severe COVID-19 that was advised in the Netherlands. The *ex vivo* effect of HCQ on the innate immune response, by measuring cytokine production after endosomal TLR or RIG-I stimulation, and on the adaptive immune response, by measuring T and B cell proliferation, was investigated. Moreover, the dose-response relationship of HCQ on these readout measures was also studied *in vitro* by adding a concentration range of HCQ to freshly isolated primary human cell.

The results of our *in vitro* experiments suggest that HCQ has a strong inhibitory effect on endo-lysosomal TLR functioning but that the cytosolic RIG-I-mediated pathway is affected to a lesser degree. Where TLR3-, TLR7- and TLR9-mediated IL-6 and IFN production was inhibited at HCQ concentrations >100 ng/mL, RIG-I-mediated IFN production was only mildly affected by HCQ. This could be explained by HCQ's excessive affinity to the lysosomal intracellular compartment, which is expected to be 56,000-fold higher than cytosol.[25] HCQs effect on the adaptive immune response was studied by measuring T and B cell proliferation, T cell-mediated cytokine production, and T cell activation. While HCQ did not affect any of the T cell functions, we did find a clear HCQ-mediated decrease in B cell proliferation *in vitro* at concentrations > 100 ng/mL. Interestingly, the immune assays where HCQ had strong *in vitro* immunosuppressive effects, especially at high concentrations, showed limited *ex vivo* HCQ effects in the clinical study. Compared to placebo, 5-day HCQ treatment did not significantly suppress B cell proliferation or TLR-driven IFN and IL-6 secretion in PBMC cultures, except for a suppressive effect on TLR7-driven responses.

The most likely reason for the discrepancy between *in vitro* and *ex vivo* HCQ effect is that the recommended off-label dose for COVID-19 at the time of study conduct resulted in insufficient HCQ exposure to exert immunosuppressive effects. In our study, an average maximum plasma concentration of 121 ng/mL was reached, which is considerably lower than plasma levels found in RA patients receiving HCQ treatment, ranging from 200 – 500 ng/mL.[26-28] Moreover, because of HCQs large volume of distribution due to extensive storage of the drug in tissues, it usually takes 3-6 months to reach steady state concentrations and therapeutic effect in auto-immune patients treated with HCQ.[29] This would mean that the five-day treatment that was used in our clinical study was insufficient to detect *ex vivo* drug effects. Furthermore, because HCQ tissue concentrations are significantly higher compared to plasma concentrations[30-31], there is a possibility that systemic pharmacodynamic monitoring underestimates HCQ's activity in specific peripheral tissues (e.g. lungs, liver, kidney). Additionally, the beneficial effect of HCQ in diseases such as RA and SLE is not solely based on its immunosuppressive effects, but on its protective effects on cardiovascular diseases and the development of diabetes. The de-acidification of lysosomes by HCQ reduces insulin degradation and inhibits cholesterol synthesis. HCQ increases LDL receptor levels in the liver, leading to lower cholesterol levels and thereby preventing cardiovascular diseases, which are major causes of mortality in RA.[32-33]

In conclusion, our study showed substantial immunomodulatory effects of HCQ *in vitro*. Nonetheless, the *ex vivo* immunomodulatory response to a 5-day HCQ treatment regimen with usual clinical doses was limited. The pharmacological activity of HCQ in autoimmunity remains to be studied in greater detail, based on the assays as presented in our studies and at a therapeutic dose and regimen relevant for the specific condition of interest.

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NEDERLANDSE SAMENVATTING

Een aantal belangrijke begrippen die in de Nederlandse samenvatting voorkomen worden hieronder toegelicht.

Aangeboren immuunsysteem → Het deel van het immuunsysteem dat bestaat uit cellen en moleculen die direct op een ziekteverwekker kunnen reageren en deze opruimen.

Aangeboren immuuncellen → Immuuncellen van het aangeboren immuunsysteem, zoals antigeen-presenterende cellen, neutrofielen en granulocyten

Activatiemarker → Een molecuul dat zich op de buitenkant van de cel bevindt wanneer een cel is geactiveerd. De activatiemarkers die in dit proefschrift worden beschreven zijn CD25, CD69, CD71 en CD154.

Antigeen presenterende cel (APC) → Een aangeboren immuuncel die een ziekteverwekker kan opnemen, in stukjes knippen en deze vervolgens aan de buitenkant van de cel presenteert. Het presenteren van het antigen is cruciaal voor de activatie van het verworven immuunsysteem.

Biomarker → Meetbare indicator van een biologische toestand. In dit proefschrift wordt met biomarker altijd een biologische marker voor immuunactiviteit bedoeld.

Bloedplasma → Bloedplasma is de vloeistof van het bloed, zonder de bloedcellen en de bloedplaatjes.

CD25 → IL-2 receptor. Dit molecuul komt tot expressie op verschillende celtypes, waaronder T-cellen. De IL-2 receptor speelt een belangrijke rol bij T-celproliferatie. Een verhoging in de hoeveelheid CD25 op de cel is een kenmerk van T-celactivatie.

CD69 → Een lectine receptor. Dit eiwit wordt vrijwel direct na activatie van de T-cel aan de buitenkant van de cel tot expressie gebracht.

CD71 → Transferrine receptor 1. Een eiwit op de cel dat zorgt voor het ijzertransport. Verhoging van de hoeveelheid CD71 op de cel is een kenmerk van T-celactivatie.

CD154 → CD40 ligand. Een molecuul op de T-cel dat betrokken is bij de activatie van de T-cel receptor en daarnaast ook assisteert bij het activeren van B-cellen.

COVID-19 → Coronavirus disease 2019. Een infectie van de luchtwegen die wordt veroorzaakt door het SARS-COV-2 virus.

Cyclosporine A (CsA) → Een geneesmiddel dat aan transplantatiepatienten wordt gegeven om het immuunsysteem te onderdrukken. Cyclosporine A remt het enzym calcineurine en blokkeert daarbij de expressie van genen die voor immuun-activatie zorgen. Cyclosporine A heeft hetzelfde werkingsmechanisme als tacrolimus.

Cytokine → Een eiwit dat door een cel wordt uitgescheiden om andere cellen te signaleren, bijvoorbeeld om andere immuuncellen te activeren of te remmen.

Doseren → Het innemen/toedienen van een geneesmiddel

Ex vivo → Een experiment waarbij het bloed van een proefpersoon of patiënt wordt afgenomen nadat deze persoon een geneesmiddel heeft ingenomen. Dit bloed wordt in het lab onderzocht, waar het eventueel eerst nog geincubeerd wordt met een stimulans.

Farmacodynamiek (PD) → Een begrip uit de farmacologie. Het omschrijft het effect dat een geneesmiddel heeft op de cellen van het lichaam. Elk geneesmiddel heeft zijn eigen werkingsmechanisme, dus voor elk geneesmiddel zijn er andere manieren om de PD te monitoren. In dit proefschrift worden immuunsuppressiva onderzocht, dus meten we de PD door naar de activiteit van immuuncellen te kijken.

Farmacokinetiek (PK) → Een begrip uit de farmacologie. Het omschrijft hoe een geneesmiddel zich in het lichaam gedraagt; o.a. hoe het geneesmiddel wordt opgenomen, hoe snel het geneesmiddel zich door het lichaam verdeelt en hoe het geneesmiddel door het lichaam wordt afgebroken. PK kan worden onderzocht door de concentratie van het geneesmiddel en zijn afbraakproducten in het lichaam te meten op verschillende tijdspunten.

IL-2 → Een cytokine dat voornamelijk door T-cellen wordt gemaakt. Het speelt een belangrijke rol in T-celactivatie en T-celdeling.

IFN-γ → Een cytokine dat voornamelijk door T-cellen, maar ook door andere immuuncellen gemaakt kan worden. IFN-γ zorgt o.a. voor activatie van aangeboren immuuncellen die vervolgens ziekteverwekkers en geïnfecteerde cellen aanvallen.

Immuunsuppressief → Het onderdrukken/remmen van het immuunsysteem

Immuunsuppressiva → Een geneesmiddel dat het immuunsysteem onderdrukt/remt.

Incubatie → Het kweken van cellen buiten het lichaam. Bijvoorbeeld in combinatie met een stimulans of een geneesmiddel.

In vitro → Een experiment waarbij het bloed van een proefpersoon of patiënt wordt afgenomen, en in het lab wordt geïncubeerd met een stimulans en geneesmiddel

Klinische studie → Een onderzoek waarbij een behandeling wordt onderzocht in menselijke proefpersonen. Dit kunnen gezonde vrijwilligers of patiënten zijn.

Lysosoom → Kleine blaasjes in de cel die afvalstoffen afbreken met behulp van enzymen. Deze afvalstoffen kunnen vervolgens opnieuw gebruikt worden of worden uitgescheiden door de cel.

MMF → Het geneesmiddel mycofenolaatmofetil. MMF wordt aan transplantatie patiënten wordt gegeven om het immuunsysteem te onderdrukken. Het remt de celdeling van T- en B-cellen. De werkzame stof van MMF is MPA.

MPA → Mycofenolzuur, de werkzame stof van het geneesmiddel MMF.

Perifere bloedmononucleaire cellen (PBMCs) → Een groep immuuncellen die zich in het bloed bevinden. Het bestaat onder andere uit T-cellen, B-cellen, antigeen presenterende cellen en andere aangeboren immuuncellen.

Piekconcentratie → De hoogste geneesmiddel concentratie die na doseren in de patiënt/proefpersoon wordt gevonden.

Placebo → Een geneesmiddel zonder werkzame stof. Ook wel nep-geneesmiddel genoemd.

Prednisolone → De werkzame stof van prednison. Prednisolon is een geneesmiddel dat het immuunsysteem onderdrukt. Het lijkt sterk op het lichaamseigen bijnierschorshormoon dat de activiteit van het immuunsysteem reguleert, bijvoorbeeld tijdens stress.

Proliferatie → Celdeling. Het vermeerderen van cellen.

SARS-COV-2 → Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2) is het virus dat de luchtwegen kan infecteren en de ziekte COVID-19 veroorzaakt. Dit virus veroorzaakte in 2020 een pandemie.

Stimulatie → Het activeren van cellen met behulp van een stimulans.

Stimulans → Een molecuul dat gebruikt wordt om een cel te activeren.

Tacrolimus → Een geneesmiddel wat aan transplantatiepatiënten wordt gegeven om het immuunsysteem te onderdrukken. Tacrolimus remt het enzym calcineurine en blokkeert daarbij de expressie van genen die voor immuun-activatie zorgen. Tacrolimus heeft hetzelfde werkingsmechanisme als cyclosporine A.

Toll-like Receptor (TLR) → Een sensor op de cel die onderdelen van ziekteverwekkers kan herkennen, en zorgt dat het aangeboren immuunsysteem wordt geactiveerd. Deze sensor zit aan de buitenkant van de cel, maar kan ook in de cel zitten. Er zijn verschillende type TLRs die verschillende type ziekteverwekkers kunnen herkennen.

Transplantatie → Transplantatie is een behandeling waarbij een slecht werkend orgaan van een patiënt wordt vervangen door het orgaan van een donor.

Verworven immuunsysteem → Het deel van het immuunsysteem dat bestaat uit cellen en moleculen die voor een immuunreactie zorgen die specifiek op de ziekteverwekker is gericht. Het verworven immuunsysteem is ook belangrijk in het vormen van het immuungeheugen.

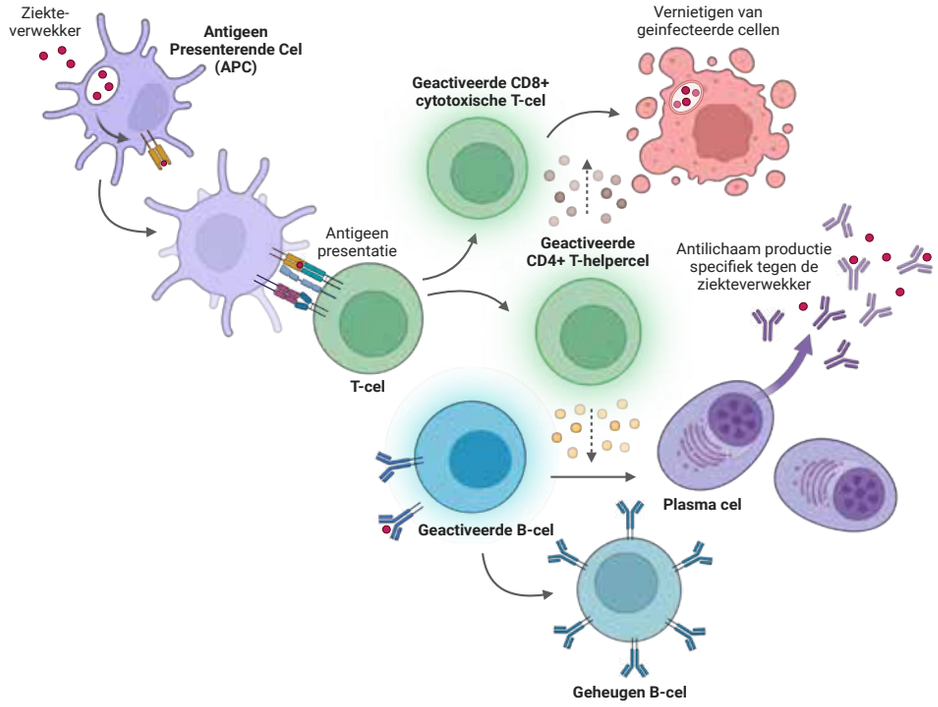
Verworven immuuncellen → Immuuncellen van het verworven immuunsysteem, zoals T-cellen en B-cellen.

Het menselijke immuunsysteem is een ingewikkeld systeem gebaseerd op de interactie tussen verschillende soorten immuuncellen en moleculen. Het kan grofweg worden ingedeeld in twee categorieën: het aangeboren immuunsysteem en het verworven immuunsysteem. Het aangeboren immuunsysteem bestaat uit verschillende celtypen die het vermogen hebben om ziekteverwekkers te herkennen en hierop te reageren door extra immuuncellen aan te trekken en de ziekteverwekkers te elimineren. Antigeen-presenterende cellen (APC's) zijn aangeboren immuuncellen met een belangrijke taak. Ze nemen de ziekteverwekkers in zich op, verwerken deze tot antigenen en presenteren stukjes van de ziekteverwekker (antigenen genoemd) aan de buitenkant van de cel. Deze presentatie van antigenen is cruciaal om het verworven immuunsysteem (T-cellen en B-cellen) te activeren. T-cellen kunnen specifiek de ziekteverwekker herkennen, en gaan zich vermenigvuldigen. De geactiveerde T-cellen kunnen verschillende functies uitoefenen. Cytotoxische T-cellen (ook wel CD8+ T-cellen genoemd) herkennen en vernietigen geïnfecteerde cellen, terwijl T-helpercellen (ook wel CD4+ T-cellen genoemd) andere imuuncellen ondersteunen en helpen bij de activatie van B-cellen. Geactiveerde B-cellen maken antilichamen aan, waardoor het immuungeheugen wordt gevormd. De rol van de verschillende type immuuncellen is weergegeven in **figuur 1**.

Hoewel het immuunsysteem over het algemeen goed is gereguleerd, zijn er gevallen waarin de interactie tussen de verschillende immuuncellen is verstoord. Ziekten (zoals bijv. allergieën, auto-immuunziekte, kanker), ziekteverwekkers (zoals bijv. HIV) of het gebruik van medicijnen (bijv. immuunsuppressiva) kunnen het immuunsysteem beïnvloeden. Dit kan ervoor zorgen dat het immuunsysteem overactief of onderactief wordt. Een overactief immuunsysteem zorgt voor afbraak van gezonde cellen en weefsels, terwijl een onderdrukt immuunsysteem ervoor kan zorgen dat infecties of kanker zich kunnen ontwikkelen. Om patiënten met een verstoord immuunsysteem te behandelen, kunnen geneesmiddelen worden gebruikt die het immuunsysteem veranderen (immuun-modulerende geneesmiddelen). Maar net zoals ziekten die het immuunsysteem verstoren, kunnen immuun-modulerende medicijnen dezelfde gevolgen hebben als ze verkeerd worden gebruikt. Het kiezen van de juiste medicijnen voor de juiste diagnose in de juiste dosis is dus erg belangrijk. Maar omdat het immuunsysteem uit zoveel verschillende cellen en moleculen bestaat, kan dit een uitdaging zijn. Om te begrijpen hoe immuun-modulerende medicijnen werken en welke effecten ze hebben, is het belangrijk om het immuunsysteem nauwlettend in de gaten te houden. Dit wordt ook wel immuun-monitoren genoemd.

In dit proefschrift worden verschillende methoden en toepassingen van immuun-monitoring beschreven. **Deel I** beschrijft de zoektocht naar methodes van immuun-monitoring die kunnen bijdragen aan het personaliseren van de immuunsuppressieve behandeling bij transplantatiepatiënten. In **deel II** worden vergelijkbare methodes van immuun-monitoring gebruikt om een beter inzicht te krijgen in het werkingsmechanisme van het geneesmiddel hydroxychloroquine (HCQ).

Figuur 1 Overzicht van de interacties tussen de verschillende immuuncellen van het verworven imuunsysteem.



DEEL I

Voor patiënten met ernstig nierfalen is een niertransplantatie vaak de beste behandelingsoptie. Nadat de nier van de orgaandonor is getransplanteerd zullen de immuuncellen van de ontvanger echter direct een immuunreactie tegen het getransplanteerde orgaan opwekken. De antigeen-presenterende

cellen van de ontvanger herkennen het getransplanteerde orgaan namelijk als lichaamsvreemd en activeren de T-cellen van de ontvanger. Deze T-cellen vermenigvuldigen zich en starten een immuunreactie om het getransplanteerde orgaan te vernietigen. Om te voorkomen dat het orgaan wordt beschadigd en afgestoten door het immuunsysteem, moet deze immuunreactie na transplantatie worden onderdrukt. De standaardbehandeling om het immuunsysteem te onderdrukken na transplantatie bestaat uit een combinatie van drie immuunsuppressiva. Een calcineurineremmer (CNI, tacrolimus of cyclosporine A), een middel dat celdeling remt (mycofenolaatmofetil, MMF), en een lage dosis corticosteroïde (prednisolon). Aan de ene kant is het belangrijk dat deze geneesmiddelen het immuunsysteem voldoende onderdrukken om te voorkomen dat het getransplanteerde orgaan wordt afgestoten. Aan de andere kant is het ook belangrijk dat de patiënt zo min mogelijk bijwerkingen van deze immuunsuppressiva ervaart. Om dit in balans te houden, word de dosering van deze geneesmiddelen bij patiënten regelmatig gecontroleerd in het ziekenhuis, door middel van het meten van geneesmiddelconcentraties in het bloed. Dit wordt ook wel 'Therapeutic Drug Monitoring' (TDM) genoemd.

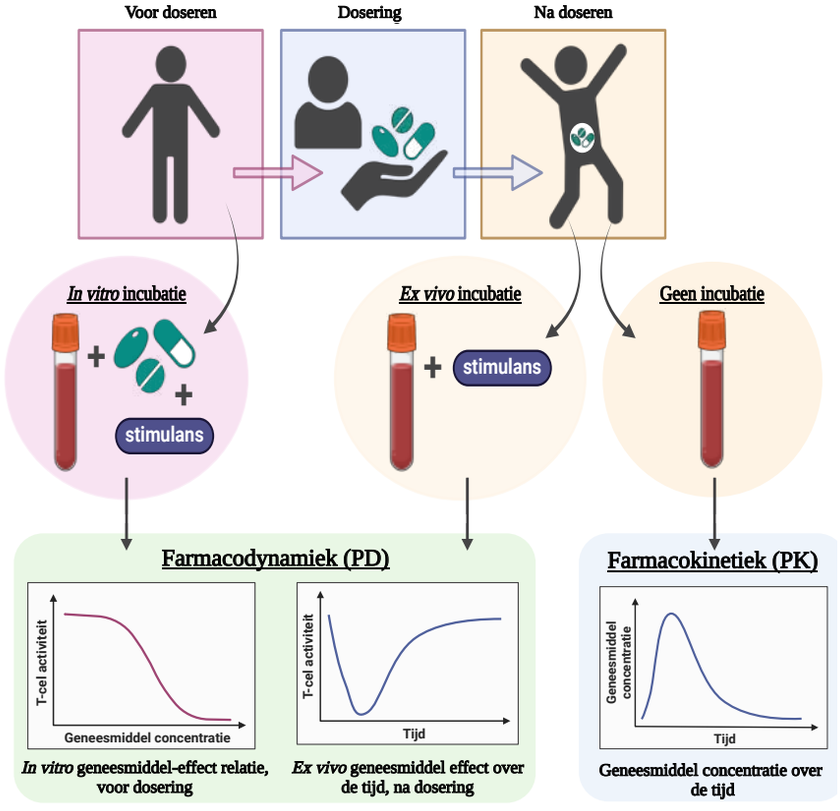
Hoewel het meten van geneesmiddelconcentraties in het bloed in de loop der jaren heeft bijgedragen aan het voorkomen van afstoting en betere overlevingskansen van patiënten, gaat langdurige immuunsuppressieve behandeling nog steeds gepaard met een breed scala aan bijwerkingen, zoals diabetes, kanker, hart- en vaatziekten, infecties en afstoting van het getransplanteerde orgaan. Dit komt mogelijk omdat de geneesmiddelconcentraties die bij TDM gemeten worden, niet altijd correleren met de activiteit van het gemeten geneesmiddel. Om de immuunsuppressieve behandeling beter op de individuele patiënt af te stemmen, en bijwerkingen als gevolg van langdurige immuunsuppressie te voorkomen, is het daarom misschien beter om het effect van de geneesmiddelen op het immuunsysteem te monitoren, in plaats van medicijn concentraties. In dit proefschrift zijn we op zoek gegaan naar indicatoren (ook wel biomarker genoemd) die inzicht geven in de status van het immuunsysteem van de patiënt, waardoor het effect van geneesmiddelen op het immuunsysteem van de patiënt kan worden bestudeerd.

T-cellen zijn een van de belangrijkste cellen in het opwekken van de immuunreactie tegen het getransplanteerde orgaan. De meeste immuunsuppressieve geneesmiddelen die worden gebruikt na transplantatie zijn

er daarom op gericht om de activiteit van T-cellen te remmen. In de zoektocht naar biomarkers die informatie geven over het immuunsysteem van transplantatiepatiënten, hebben we ons daarom gericht op het monitoren van T-celactiviteit. Dit hebben we gedaan door het bloed van een patiënt of gezonde vrijwilliger af te nemen, en dit bloedmonster vervolgens in het laboratorium te stimuleren met een stimulans die specifiek T-cellen activeert. Nadat het bloedmonster een aantal uur met de T-cel stimulans is geincubeerd, kan de activiteit van de T-cellen in het bloedmonster gemeten worden met behulp van verschillende uitleesmaten: signaal moleculen die door de T-cel worden uitgescheiden (ook wel cytokines genoemd), de aanwezigheid van moleculen op de buitenkant van de T-cel (ook wel expressie van activatie-markers genoemd) en de celdeling van de T-cellen (ook wel T-celproliferatie genoemd). Het monitoren van het effect van immuunsuppressieve geneesmiddelen op de T-celactiviteit is op twee verschillende niveaus gedaan: *in vitro* en *ex vivo*. Voor het monitoren van het *in vitro* geneesmiddel-effect werden behalve de T-cel stimulans ook verschillende concentraties van het onderzochte geneesmiddel aan het bloedmonster toegevoegd. Op deze manier kon het effect van de verschillende concentraties geneesmiddel op de T-cel bestudeerd worden. Voor het monitoren van het *ex vivo* geneesmiddel effect werden proefpersonen gevraagd om het geneesmiddel in te nemen. Vervolgens werden er op verschillende tijdstippen na medicatie-inname bloedmonsters afgenomen die geïncubeerd werden met een T-cel stimulans. Doordat de concentratie van het geneesmiddel in het bloed van de proefpersoon vlak na inname hoog is en een paar uur na inname weer omlaag gaat, kan op deze manier ook het effect van verschillende concentraties geneesmiddel op het immuunsysteem worden bestudeerd. Het verschil tussen *in vitro* en *ex vivo* geneesmiddel-effect staat ook uitgelegd in **figuur 2**. Hierin wordt ook het verschil tussen farmacodynamiek (het effect van het geneesmiddel op het immuunsysteem) en farmacokinetiek (de concentraties van het geneesmiddel in het lichaam) toegelicht.

Om te onderzoeken of de biomarkers inderdaad inzicht geven in het effect van geneesmiddelen op het immuunsysteem van de patiënt hebben we een aantal klinische studies uitgevoerd. In **hoofdstuk 2** wordt de eerste klinische studie beschreven waarin drie functionele T-cel uitleesmaten werden onderzocht: activiteit van het enzym calcineurine, hoeveelheid cytokineproductie (IL-2 en IFN-γ) en de expressie van activatiemarkers op de T-cel (CD69, CD25, CD71 en CD154). Deze T-cel biomarkers werden onderzocht in

Figuur 2 Overzicht van de opzet van de klinische studies. Overzicht van de opzet van de klinische studies. Het *in vitro* geneesmiddel-effect werd onderzocht door incubatie van volbloed met een T-cel stimulans in combinatie met verschillende concentraties van het immunosuppressivum. Dit werd gedaan voordat het geneesmiddel aan de proefpersoon werd toegediend. Het *ex vivo* geneesmiddel-effect werd onderzocht door incubatie van bloedmonsters die zijn verkregen van proefpersonen die gedoseerd waren met het immunosuppressivum. Deze bloedmonsters werden op meerdere tijdstippen na inname van het geneesmiddel genomen en gestimuleerd met exact dezelfde T-cel stimulans als de *in vitro* bloedmonsters. Voor zowel *in vitro* als ex vivo incubaties werd het effect van het immunosuppressivum op de T-celactiviteit gemeten. Dit effect wordt farmacodynamiek (PD) genoemd. Op dezelfde tijdstippen als de *ex vivo* farmacodynamische metingen, werd ook de concentraties van het immunosuppressivum in het bloed van de deelnemers aan het onderzoek gemeten om de farmacokinetiek (PK) te onderzoeken.



gezonde vrijwilligers die een enkele dosering van het immuunsuppressieve geneesmiddel tacrolimus kregen. Hier zagen we dat tacrolimus een sterk remmend effect had op de productie van IL-2 en IFN-γ en op de expressie van CD71 en CD154. De metingen van calcineurine enzymactiviteit waren echter zeer variabel en lieten geen significant geneesmiddel-effect zien. Naast de evaluatie van de farmacodynamische (PD) eindpunten werden er in deze studie ook verschillende farmacokinetische (PK) biomarkers onderzocht, waaronder tacrolimus concentraties in het bloed en in immuuncellen. Om de concentratie van tacrolimus in immuuncellen te bepalen werden twee groepen immuuncellen uit het bloed geisoleerd: perifeer bloed mononucleaire cellen (PBMCs) en T-cellen. PBMCs is een groep van verschillende immuuncellen, waaronder T-cellen, B-cellen, antigeen-presenterende cellen en andere aangeboren immuuncellen. Over het algemeen zagen de farmacokinetische profielen voor bloed, PBMCs en T-cellen er vergelijkbaar uit. Daarnaast werd een duidelijke correlatie tussen de tacrolimus concentratie in T-cellen en de concentratie in bloed gevonden. Aangezien T-cellen de belangrijkste cellen zijn voor immuunsuppressieve therapie, concludeerde we dat de tacrolimus concentratie in het bloed, de huidige methode voor monitoring van patiënten in het ziekenhuis, een goede weergave is van de concentratie in de T-cel.

Samengevat, werd in hoofdstuk 2 aangetoond dat de immuun-biomarkers IL-2, IFN-γ, CD71 en CD154 mogelijk geschikt zijn om immuunsuppressieve effecten van geneesmiddelen bij transplantatiepatiënten te bepalen. In **hoofdstuk 3** werd de zoektocht naar de geschiktheid van deze biomarkers voortgezet, maar met enkele wijzigingen ten opzichte van de vorige studie. Ten eerste werd het meten van de expressie van activatiemarkers op de T-cel geoptimaliseerd door de incubatietijd van de bloedmonsters met de stimulans te verkorten naar 6 uur, in tegenstelling tot de 48 uur in de vorige studie werd gebruikt. Deze optimalisatie zorgde voor een verhoging in de expressie van activatie markers, waardoor het makkelijker werd om geneesmiddel-effecten aan te tonen. Ten tweede werd een extra uitleesmaat voor T-celactiviteit onderzocht: T-celproliferatie. Voor deze biomarker werden bloedmonsters gedurende 48 uur gestimuleerd waarna de proliferatie van T-cellen werd gemeten door een DNA-label toe te voegen dat alleen in de cel wordt opgenomen tijdens celdeling. Ten slotte werd een placebogroep in de studie opgenomen, vrijwilligers die geen echt geneesmiddel kregen toegediend tijdens het onderzoek, maar een nep-geneesmiddel

zonder werkzame stof. Met behulp van deze placebogroep kunnen we onderzoeken hoe de biomarkers zich over de tijd gedragen, zonder dat het geneesmiddel-effect hier een rol in speelt. Met deze aanpassingen werd de effectiviteit van de geselecteerd biomarkers opnieuw in een klinische studie onderzocht. Dit keer met behulp van het geneesmiddel Cyclosporine A (CsA). CsA is de voorganger van tacrolimus, en werkt op dezelfde manier door het enzym calcineurine te remmen. In deze klinische studie ontvingen twaalf gezonde vrijwilligers een dosering van CsA, waarna de *in vitro* en *ex vivo* immuunactiviteit werd gemeten met behulp van drie verschillende uitleesmaten: cytokineproductie (IL-2 en IFN-γ), de expressie van activatiemarkers op de T-cel (CD69, CD25, CD71 en CD154) en T-celproliferatie.

Net als tacrolimus, had CsA een groot remmend effect op IL-2, IFN-γ, CD71 en CD154. Deze biomarkers vertoonden maximale remming 2 en 3 uur na inname van CsA, en waren na 24 uur weer terug op het beginniveau. Dit komt precies overeen met het farmacokinetische profiel van CsA, waar de hoogste concentratie in het bloed ook werd gevonden op 2 en 3 uur. Daarnaast liet de biomarker die we nog niet eerder hadden onderzocht, T-celproliferatie, een duidelijke remming zien na inname van CsA. In de placebogroep werd duidelijk dat de immuun-markers gedurende de dag veel varieerden. Ondanks deze variatie was het nog steeds mogelijk om onderscheid te maken tussen de placebo- en CsA-behandelde proefpersonen op basis van deze immuun-biomarkers. Daarnaast werd aangetoond dat het *in vitro* en *ex vivo* effect van CsA vergelijkbaar was. Dit suggereert dat het geneesmiddel-effect van CsA dat *in vitro* te zien is mogelijk een voorspeller is voor het geneesmiddel-effect van CsA dat na dosering *ex vivo* werd gevonden. Tot slot werd in deze studie aangetoond dat de farmacokinetische profielen van CsA vergelijkbaar waren in volbloed, PBMCs en T-cellen.

Op basis van hoofdstuk 2 en 3 concludeerden we dat productie van IL-2 en IFN-γ, expressie van CD154 en CD71, en T-celproliferatie goede biomarkers zijn om het immuunsuppressieve effect van een calcineurineremmer (d.w.z. tacrolimus en cyclosporine A) te monitoren. In de behandeling van transplantatiepatienten worden calcineurineremmers echter vaak gecombineerd met een ander geneesmiddel: mycofenolaatmofetil (MMF). Wanneer MMF door het lichaam wordt opgenomen komt de werkzame stof mycofenolzuur (MPA) vrij. Dit is een specifieke remmer van celdeling van B- en T-cellen. In **hoofdstuk 4** wordt een klinische studie beschreven waarin wordt onderzocht wat het effect is van MPA op de geselecteerde biomarkers uit hoofdstuk 2 en 3. In deze studie kregen zestien gezonde vrijwilligers een enkele dosering van MMF of placebo, waarna drie verschillende farmacodynamische metingen werden onderzocht: cytokineproductie (IL-2 en IFN-γ), T-celproliferatie en IMPDH-activiteit, een enzym dat belangrijk is voor de celdeling van T-cellen. De resultaten van de studie lieten zien dat het immuunsuppressieve effect van MPA het best kon worden aangetoond met T-celproliferatie. Al 30 minuten na inname van het geneesmiddel werd de proliferatie van T-cellen volledig geremd in de MMF-behandelde proefpersonen. Het *in vitro* en *ex vivo* effect van MPA verschilde per proefpersoon, maar alle proefpersonen bereikten maximale remming in T-celproliferatie bij een concentratie van 2 mg/L MPA. In de kliniek hebben transplantatie patiënten vrijwel altijd een concentratie van minimaal 2 mg/L in het bloedplasma, wat aangeeft dat T-celproliferatie bij deze patiënten hoogstwaarschijnlijk altijd volledig onderdrukt is. Hoewel het leek of MMF een licht remmend effect had op de cytokineproductie van IL-2 en IFN-γ, werd er geen significant verschil gevonden tussen MMF en placebo-behandelde proefpersonen. Ook het meten van de activiteit van IMPDH, het enzym dat door MPA geremd wordt, liet geen effect van MMF-behandeling zien. Tot slot werd, net als in de eerdere studies, de farmacokinetiek van MPA bestudeerd in drie verschillende matrices: bloedplasma, PBMCs en T-cellen. Er werd een sterke correlatie gevonden tussen plasmaconcentraties en de MPA-concentraties in de immuuncel (PBMCs en T-cellen), wat aangeeft dat het meten van MPA-concentraties in de cel in plaats van in bloedplasma geen toegevoegde waarde heeft.

Nadat in hoofdstuk 2, 3 en 4 was aangetoond dat de geselecteerde immuun-biomarkers (cytokineproductie, T-celproliferatie en expressie van activatiemarkers op de T-cel) geschikt waren om immuunsuppressieve effecten van CNI's (tacrolimus en cyclosporine A) en MMF aan te tonen bij gezonde vrijwilligers, was het doel om te onderzoeken of deze functionele biomarkers ook geschikt zijn voor gebruik in transplantatiepatiënten. Hiervoor werd een kleine groep niertransplantatiepatiënten onderzocht in een studie die in **hoofdstuk 5** staat beschreven. Veertien patiënten die langer dan 2 jaar geleden een niertransplantatie hadden ondergaan en werden behandeld met de standaard immuunsuppressieve therapie (tacrolimus, MMF en prednisolon) werden in deze studie geïncludeerd. Over het algemeen bevestigden de resultaten van deze patiëntenstudie wat eerder werd aangetoond in de studies met gezonde vrijwilligers. Zoals verwacht,

was de T-celproliferatie in transplantatiepatiënten gedurende de hele dag volledig onderdrukt, zowel voor als na inname van medicatie. De cytokineproductie (IL-2 en IFN-γ) en de expressie van activeringsmarkers op T-cellen (CD154 en CD71) waren daarentegen nog steeds detecteerbaar en fluctueerden gedurende de dag. Gemiddeld was de maximale remming van IL-2, IFN-γ, CD154 en CD71 *in vitro* vergelijkbaar met de maximale remming *ex vivo.* De analyse van individuele patiënten liet echter geen significante correlatie zien tussen *in vitro* en *ex vivo* immuunsuppressieve effecten. Bovendien onderzochten we de relatie tussen het immuunsuppressieve medicijneffect en andere factoren waarvan in de literatuur is beschreven dat ze een rol spelen bij transplantatie-immunologie, zoals leeftijd, tacrolimus concentraties, de aanwezigheid van remmende T- en B-celpopulaties en de aanwezigheid van het TTV virus. We vonden echter geen duidelijke relatie tussen deze factoren en onze functionele immuuntests.

Samengevat werden in deel I van dit proefschrift twee hoofdvragen beantwoord. Als eerste werd onderzocht of het monitoren van patiënten met behulp van immuun-biomarkers voordelen biedt in vergelijking met het monitoren van geneesmiddel concentraties. Onze studieresultaten toonden aan dat de activiteit van het immuunsysteem van transplantatiepatienten fluctueert gedurende de dag. Hoewel er enige overlap was tussen de geneesmiddel concentraties en de immuun-biomarkers, toonde onze analyses aan dat de geneesmiddel concentraties de mate van immuunsuppressie niet betrouwbaar konden voorspellen voor de individuele patiënt. We concluderen daarom dat de functionele immuun-biomarkers die in dit proefschrift staan beschreven mogelijk extra inzichten biedt in vergelijking met geneesmiddel concentraties in het bloed of bloedplasma. De echte toegevoegde waarde van deze biomarkers moet echter beter worden onderzocht in een grotere studie waarin patiënten gedurende een langere tijd gevolgd worden. Dit zou inzicht geven in de relatie tussen onze biomarkers en klinische uitkomsten, zoals het ontstaan van bijwerkingen en afstoting. Ten tweede werd onderzocht of het immuunsuppressieve effect van geneesmiddelen op onze biomarkers voorspeld kon worden aan de hand van *in vitro* incubaties met het onderzochte geneesmiddel. De mate van immuunsuppressie die werd waargenomen na *in vitro* incubatie met tacrolimus vóódat de patiënten hun medicatie innamen, kwam echter niet overeen met de suppressie die *ex vivo* werd gevonden nadat de patiënten hun medicatie hadden ingenomen. Dit suggereert dat de immuuneffecten die na toediening van medicatie werden gevonden niet alleen veroorzaakt werden door tacrolimus, maar ook door andere immuunsuppressiva die gelijktijdig worden toegediend, zoals MMF en prednisolon.

DEEL II

Hydroxychloroquine (HCQ) is een geneesmiddel dat wordt gebruikt voor de behandeling en het voorkomen van malaria. Behalve dat HCQ werkzaam is als anti-malaria middel, heeft HCQ ook immuunsuppressieve eigenschappen. HCQ kan het immuunsysteem remmen, en is daardoor ook een geschikt geneesmiddel om auto-immuunziekten zoals reumatoïde artritis en systemische lupus erythematosus te behandelen. Tijdens de COVID-19 pandemie werd aangetoond dat HCQ mogelijk ook geschikt was om het corona-virus (SARS-COV-2) te remmen. Deze ontdekking leidde ertoe dat er een groot aantal studies werden gestart om te onderzoeken of COVID-19-patiënten met HCQ konden worden behandeld. Er werd gedacht dat de antivirale eigenschappen van HCQ een infectie met SARS-COV-2 zouden kunnen voorkomen, en dat daarnaast de immuunsuppressieve eigenschappen van HCQ het overactieve immuunsysteem van ernstig zieke COVID-19-patiënten zou kunnen verminderen. Hoewel HCQ een geneesmiddel is dat al meer dan 20 jaar op de markt is, was er tot die tijd niet veel onderzoek gedaan naar de immuunsuppressieve en antivirale eigenschappen van HCQ in mensen. Het meeste bewijs voor het antivirale effect van HCQ was gebaseerd op experimenten in gekweekte cellen, waarbij de HCQ-concentraties veel hoger waren dan de concentraties die bij patiënten werden gemeten. Toen de resultaten van de eerste onderzoeken naar de effecten van HCQ in COVID-19-patiënten werden gepubliceerd, bleek al snel dat HCQ geen overtuigend positief effect had op het herstel van de patiënt. Om beter te begrijpen wat het mechanisme is waardoor HCQ mogelijk geschikt leek te zijn als middel voor COVID-19, startte we een literatuuronderzoek dat in **hoofdstuk 6** staat beschreven. In dit hoofdstuk wordt de mogelijke rol van HCQ in verschillende stadia van de ziekte COVID-19 beschreven.

Er zijn verschillende manieren waarop HCQ het immuunsysteem kan remmen. Allereerst doordat wanneer HCQ in de cel komt, het zich ophoopt

in de lysosomen. Dit zijn kleine blaasjes in de cel die afvalstoffen afbreken met behulp van enzymen. Op deze manier kunnen de afgebroken afvalstoffen opnieuw worden gebruikt, of kunnen deze door de cel kunnen worden uitgescheiden. Omdat de enzymen in de lysosomen het beste werken in een zure omgeving, is de pH in de lysosomen laag. Wanneer HCQ zich in de lysosomen ophoopt wordt de pH verhoogd waardoor de enzymen hun werk niet goed meer kunnen doen. Een van de gevolgen hiervan is dat het afbreken van ziekteverwekkers door antigeen-presenterende cellen in haar lysosomen, zoals ook in figuur 1 is weergegeven, niet goed meer werkt waardoor het immuunsysteem minder snel geactiveerd wordt. Daarnaast heeft HCQ ook effect op de sensoren van de immuuncel die ziekteverwekkers herkennen, ook wel toll-like receptors (TLRs) genoemd. Deze sensoren zitten zowel in de lysosomen als aan de buitenkant van de cel, waar ze verschillende soorten ziekteverwekkers kunnen herkennen. Na het herkennen van een ziekteverwekker zorgen TLRs dat de immuuncel wordt geactiveerd en signaalmoleculen aanmaakt (cytokines) om ervoor te zorgen dat het immuunsysteem geactiveerd wordt. Wanneer HCQ zich echter in de lysosomen van een immuuncel bevindt, kunnen de TLRs minder goed ziekteverwekkers herkennen en wordt de immuunrespons geremd.

De belangrijkste reden voor het gebruik van HCQ tijdens de coronapandemie was dat HCQ het binnendringen van SARS-COV-2 virusdeeltjes in de cel kon voorkomen. Omdat HCQ mogelijk een infectie van het virus kan voorkomen, werd het gezien als een potentieel interessant geneesmiddel om aan mensen te geven die nog niet besmet waren met het coronavirus maar wel een hoog risico hadden om besmet te worden (zoals bijvoorbeeld ziekenhuismedewerkers). Het probleem van het werkingsmechanisme van HCQ is echter dat HCQ ook een onderdeel van het immuunsysteem onderdrukt dat een belangrijke rol speelt in het bestrijden van een virusinfectie. De sensoren die belangrijk zijn voor het herkennen van SARS-COV-2, de TLRs, worden namelijk door HCQ geremd. In theorie zou het gebruik van HCQ in gezonde mensen er dus voor kunnen zorgen dat ze een minder sterke virus-specifieke immuunreactie tegen het SARS-COV-2 virus hebben, wat juist voor een verhoogd risico op COVID-19 zou zorgen. De concentratie waarbij HCQ deze immuunsuppressieve effecten laat zien is echter niet goed onderzocht, waardoor het moeilijk is om in te schatten of het gebruik van HCQ in gezonde mensen een risico is.

Hoewel op basis van theoretische gronden het gebruik van HCQ in een gezonde mensen in twijfel kan worden getrokken, zouden de immuunsuppressieve effecten van HCQ in COVID-19-patiënten juist wel gunstig kunnen uitpakken. Het ziekteverloop van COVID-19 kan worden opgesplitst in drie verschillende fases. In de eerste dagen na infectie (dag 0-2) dringt het virus het lichaam binnen en vermenigvuldigt zich in de longen. In de volgende dagen (dag 3-7) wordt het aangeboren immuunsysteem in de longen geactiveerd en zorgen immuuncellen ervoor dat het adaptieve immuunrespons de infectie opruimt. In ernstige gevallen kan het virus niet worden opgeruimd door het immuunsysteem, wat leidt tot een over actief immuunsysteem, ademhalingsstoornissen en uitvallen van meerdere organen (> 7 dagen). Gebaseerd op het werkingsmechanisme van HCQ zouden ernstige COVID-19-patiënten gebaat zijn bij de eigenschappen van HCQ. Behalve dat HCQ het vermenigvuldigen van het virus remt, zorgt het er ook voor dat het immuunsysteem niet overactief wordt. Bij patiënten met milde klachten in de eerste fase van COVID-19 is het echter twijfelachtig of de immuunsuppressieve eigenschappen van HCQ gunstig uitpakken. Aan de ene kant zorgen de antivirale effecten van HCQ er mogelijk voor dat de ziekte niet ernstiger wordt, aan de andere kant zorgt het remmen van de virus-specifieke immuunreactie er mogelijk juist voor dat risico op ernstige ziekte wordt vergroot.

Aan het einde van de pandemie kon alle data over het gebruik van HCQ bij COVID-19-patiënten worden samengevoegd en goed worden geanalyseerd. Interessant genoeg werd er geconcludeerd dat er in de patiëntenpopulatie die theoretisch het meest profijt zou hebben van de HCQ-behandeling, de ernstige COVID-19-patiënten, geen gunstig effect van HCQ-behandeling te zien was in vergelijking met de standaardbehandeling. Dit maakt duidelijk dat het werkingsmechanisme van HCQ nog niet voldoende is onderzocht in mensen. Om de kennis over de immuunsuppressieve effecten van HCQ te verbeteren startte we een onderzoek naar de effecten van HCQ op het menselijke immuunsysteem. In dit onderzoek, dat in **hoofdstuk 7** staat beschreven, kregen een aantal gezonde vrijwilligers dezelfde dosis HCQ toegediend als de ernstige COVID-19-patiënten. Het effect van HCQ op het immuunsysteem werd onderzocht door bloed van deze gezonde vrijwilligers af te nemen, en de immuuncellen te kweken met een stimulans die de sensoren van de immuuncellen, de TLRs, activeren. Net zoals in deel I van dit proefschrift werden de immuuncellen zowel *in vitro* als *ex vivo* onderzocht.

Voor het *in vitro* onderzoek werden de immuuncellen van gezonde vrijwilligers geïncubeerd met een TLR-stimulans en verschillende concentraties HCQ. Hieruit bleek dat HCQ een sterk remmend effect heeft op de TLRs die zich in de lysosomen bevinden. Zowel de cytokineproductie die belangrijk is voor de antivirale immuunreactie, als de B-celdeling was sterk geremd bij hoge concentraties HCQ. Voor het *ex vivo* onderzoek werd er bloed afgenomen op verschillende momenten nadat de proefpersonen HCQ hadden ingenomen. Hier vonden we echter beperkte *ex vivo* HCQ-effecten. Vergeleken met placebo behandeling zorgde de 5-daagse HCQ-behandeling voor geen enkel remmend effect op de cytokines en B-celproliferatie waar eerder *in vitro* wel remming op werd gezien.

De meest waarschijnlijke reden voor het verschil tussen het *in vitro* en *ex vivo* HCQ-effect is dat de dosering die in de studie werd gebruikt voor onvoldoende blootstelling aan HCQ zorgde om de immuunsuppressieve effecten in het bloed te kunnen meten. De maximale HCQ-concentraties die in het bloedplasma van de gezonde vrijwilligers werd gevonden, waren aanzienlijk lager dan de *in vitro* HCQ-concentraties waarbij een remmend effect op de TLRs werd aangetoond. Dit werd bevestigd door het feit dat auto-immuun patiënten die met HCQ worden behandeld gemiddeld 2x tot 5x hogere HCQ-concentraties in hun bloedplasma hebben dan de HCQ-concentraties die in deze studie werden gemeten. Daarnaast weten we dat de HCQ concentraties in de weefsels (zoals longen, lever en nieren) veel hoger zijn dan in het bloed. Mogelijk was er geen immuunsuppressief HCQ effect meetbaar in het bloed, maar waren deze effecten wel aanwezig in de weefsels. Ten slotte is het gunstige effect van HCQ bij auto-immuun ziekten zoals reumatoïde artritis en systemische lupus erythematodes niet alleen gebaseerd op de immuunsuppressieve effecten, maar ook op de beschermende effecten van HCQ op hart- en vaatziekten en de ontwikkeling van diabetes. Kortom, er is aangetoond dat HCQ immuunsuppressieve eigenschappen heeft *in vitro,* maar dat dit bij concentraties gebeurt die niet worden bereikt bij een 5-daagse behandeling met HCQ. Een vervolgstudie met proefpersonen die voor een langere tijd of met een hogere dosering behandeld worden met HCQ en met dezelfde uitleesmaten worden onderzocht, zou een beter inzicht kunnen geven in de immuunsuppressieve HCQ-effecten die bij de behandeling van auto-immuunziekte een rol spelen.

CURICULUM VITAE

Aliede Eveline in 't Veld was born on 19 January 1993, in Alphen aan den Rijn, the Netherlands. She graduated from secondary school at Scala College in Alphen aan den Rijn in 2011. That same year, she began her studies in Biomedical Sciences at Leiden University. In 2014, she obtained her Bachelor of Science degree, which she partially pursued at the Karolinska Institutet in Stockholm, Sweden. Eveline continued her education with a Master of Science in Biomedical Sciences, during which she completed internships at the Department of Surgery at Leiden University Medical Center (LUMC), the Department of Translational Immunology at the German Cancer Research Center (DKFZ), and the Institute of Biology Leiden (IBL). After obtaining her master's degree in 2016, Eveline began her career as a clinical scientist and PhD student at the Centre for Human Drug Research (CHDR) in 2017. During her PhD, she was supervised by Dr. Matthijs Moerland and Prof. Dr. Koos Burggraaf. At CHDR, she combined her role as a project leader in the Immuno-Cardiovascular research group with her work as scientist in the Research & Development (R&D) laboratory. Eveline in 't Veld currently lives in Voorschoten with her husband Coen Mulders.

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