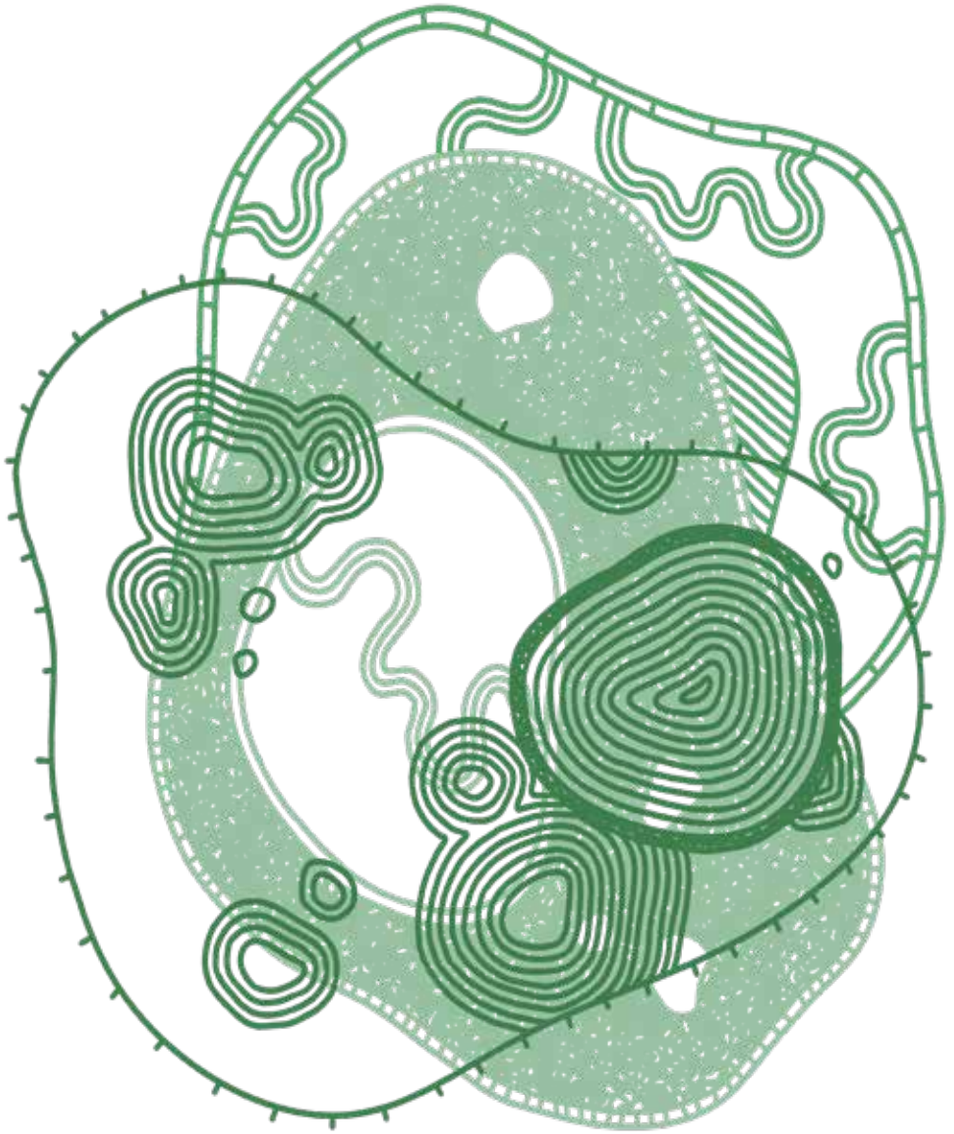
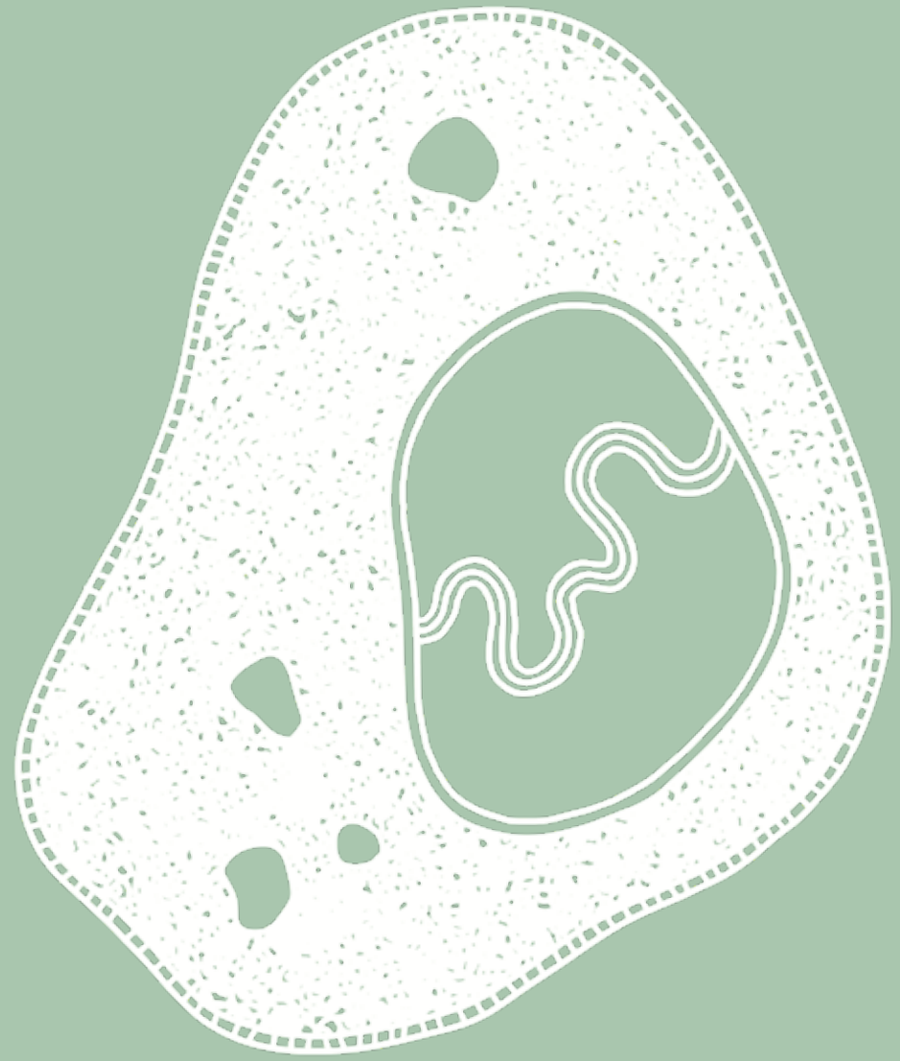
Monitoring immune responsiveness: novel assays to explore immune system dynamics in health and disease

Veld, A.E. in 't





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.

REFERENCES

1 KDIGO clinical practice guideline for the care of kidney transplant recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2009, 9 Suppl 3, S1-155.

2 Brunet, M.; van Gelder, T.; Åsberg, A.; Haufroid, V.; Hesselink, D. A.; Langman, L.; Lemaitre, F.; Marquet, P.; Seger, C.; Shipkova, M.; Vinks, A.; Wallemacq, P.; Wieland, E.; Woillard, J. B.; Barten, M. J.; Budde, K.; Colom, H.; Dieterlen, M. T.; Elens, L.; Johnson-Davis, K. L.; Kunicki, P. K.; MacPhee, I.; Masuda, S.; Mathew, B. S.; Millán, O.; Mizuno, T.; Moes, D. A. R.; Monchaud, C.; Noceti, O.; Pawinski, T.; Picard, N.; van Schaik, R.; Sommerer, C.; Vethe, N. T.; de Winter, B.; Christians, U.; Bergan, S., Therapeutic Drug Monitoring of Tacrolimus-Personalized Therapy: Second Consensus Report. Therapeutic drug monitoring 2019, 41 (3), 261-307.

3 Sallustio, B. C., Monitoring Intra-cellular Tacrolimus Concentrations in Solid Organ Transplantation: Use of Peripheral Blood Mononuclear Cells and Graft Biopsy Tissue. Frontiers in pharmacology 2021, 12, 733285.

4 Autissier, P.; Soulas, C.; Burdo, T. H.; Williams, K. C., Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry. Part A : the journal of the International Society for Analytical Cytology 2010, 77 (5), 410-9.

5 Udomkarnjananun, S.; Francke, M. I.; Dieterich, M.; van de Velde, D.; Verhoeven, J.; Boer, K.; Clahsen-Van Groningen, M. C.; De Winter, B. C. M.; Baan, C. C.; Hesselink, D. A., Association Between the Intracellular Tacrolimus Concentration in CD3 + T Lymphocytes and CD14 + Monocytes and Acute Kidney Transplant Rejection. Therapeutic drug monitoring 2022, 44 (5), 625-632.

6 Farouk, S. S.; Rein, J. L., The Many Faces of Calcineurin Inhibitor Toxicity-What the FK? Advances in chronic kidney disease 2020, 27 (1), 56-66.

7 Mourad, M.; Malaise, J.; Chaib Eddour, D.; De Meyer, M.; König, J.; Schepers, R.; Squifflet, J. P.; Wallemacq, P., Pharmacokinetic basis for the efficient and safe use of low-dose mycophenolate mofetil in combination with tacrolimus in kidney transplantation. Clinical chemistry 2001, 47 (7), 1241-8.

8 Okour, M.; Jacobson, P. A.; Ahmed, M. A.; Israni, A. K.; Brundage, R. C., Mycophenolic Acid and Its Metabolites in Kidney Transplant Recipients: A Semimechanistic Enterohepatic Circulation Model to Improve Estimating Exposure. Journal of clinical pharmacology 2018, 58 (5), 628-639.

9 Dimitrov, S.; Benedict, C.; Heutling, D.; Westermann, J.; Born, J.; Lange, T., Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. Blood 2009, 113 (21), 5134-43.

10 Budde, K.; Glander, P.; Bauer, S.; Braun, K.; Waiser, J.; Fritsche, L.; Mai, I.; Roots, I.; Neumayer, H. H., Pharmacodynamic monitoring of mycophenolate mofetil. Clinical chemistry and laboratory medicine 2000, 38 (11), 1213-6.

11 Plevris, N.; Lees, C. W., Disease Monitoring in Inflammatory Bowel Disease: Evolving Principles and Possibilities. Gastroenterology 2022, 162 (5), 1456-1475.e1.

12 Wei, K.; Jiang, P.; Zhao, J.; Jin, Y.; Zhang, R.; Chang, C.; Xu, L.; Xu, L.; Shi, Y.; Guo, S.; He, D., Biomarkers to Predict DMARDs Efficacy and Adverse Effect in Rheumatoid Arthritis. Frontiers in immunology 2022, 13, 865267.

13 Prasad, P.; Verma, S.; Surbhi; Ganguly, N. K.; Chaturvedi, V.; Mittal, S. A., Rheumatoid arthritis: advances in treatment strategies. Molecular and cellular biochemistry 2023, 478 (1), 69-88.

14 Szumilas, K.; Wilk, A.; Wiśniewski, P.; Gimpel, A.; Dziedziejko, V.; Kipp, M.; Pawlik, A., Current Status Regarding Immunosuppressive Treatment in Patients after Renal Transplantation. International journal of molecular sciences 2023, 24 (12).

15 O'Flynn, K.; Russul-Saib, M.; Ando, I.; Wallace, D. L.; Beverley, P. C.; Boylston, A. W.; Linch, D. C., Different pathways of human T-cell activation revealed by PHA-P and PHA-M. Immunology 1986, 57 (1), 55-60.

16 Liu, J.; Cao, R.; Xu, M.; Wang, X.; Zhang, H.; Hu, H.; Li, Y.; Hu, Z.; Zhong, W.; Wang, M., Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro. Cell discovery 2020, 6, 16.

17 Wu, S. F.; Chang, C. B.; Hsu, J. M.; Lu, M. C.; Lai, N. S.; Li, C.; Tung, C. H., Hydroxychloroquine inhibits CD154 expression in CD4(+) T lymphocytes of systemic lupus erythematosus through NFAT, but not STAT5, signaling. Arthritis research & therapy 2017, 19 (1), 183.

18 Torigoe, M.; Sakata, K.; Ishii, A.; Iwata, S.; Nakayamada, S.; Tanaka, Y., Hydroxychloroquine efficiently suppresses inflammatory responses of human class-switched memory B cells via Toll-like receptor 9 inhibition. Clinical immunology (Orlando, Fla.) 2018, 195, 1-7.

19 Gardet, A.; Pellerin, A.; McCarl, C. A.; Diwanji, R.; Wang, W.; Donaldson, D.; Franchimont, N.; Werth, V. P.; Rabah, D., Effect of in vivo Hydroxychloroquine and ex vivo Anti-BDCA2 mAb Treatment on pDC IFN Production From Patients Affected With Cutaneous Lupus Erythematosus. Frontiers in immunology 2019, 10, 275.

20 Kashour, Z.; Riaz, M.; Garbati, M. A.; AlDosary, O.; Tlayjeh, H.; Gerberi, D.; Murad, M. H.; Sohail, M. R.; Kashour, T.; Tleyjeh, I. M., Efficacy of chloroquine or hydroxychloroquine in COVID-19 patients: a systematic review and meta-analysis. The Journal of antimicrobial chemotherapy 2021, 76 (1), 30-42.

21 Ou, T.; Mou, H.; Zhang, L.; Ojha, A.; Choe, H.; Farzan, M., Hydroxychloroquine-mediated inhibition of SARS-CoV-2 entry is attenuated by TMPRSS2. PLoS pathogens 2021, 17 (1), e1009212.

22 Samuel, C. E., Antiviral actions of interferons. Clinical microbiology reviews 2001, 14 (4), 778-809, table of contents.

23 Torres Acosta, M. A.; Singer, B. D., Pathogenesis of COVID-19-induced ARDS: implications for an ageing population. The European respiratory journal 2020, 56 (3).

24 Amani, B.; Khanijahani, A.; Amani, B., Hydroxychloroquine plus standard of care compared with standard of care alone in COVID-19: a meta-analysis of randomized controlled trials. Sci Rep 2021, 11 (1), 11974.

25 Derendorf, H., Excessive lysosomal ion-trapping of hydroxychloroquine and azithromycin. International journal of antimicrobial agents 2020, 55 (6), 106007-106007.

26 Walbi, I. A.; Alqhtani, H.; Alqahtani, F.; Alkahtani, S. A.; Mohamed Alshabi, A.; Alali, A. S.; Albarqi, H. A., The relationship between hydroxychloroquine plasma concentration and COVID-19 outcomes in rheumatoid arthritis patients in Saudi Arabia. Saudi Pharmaceutical Journal 2022.

27 Carmichael, S. J.; Charles, B.; Tett, S. E., Population Pharmacokinetics of Hydroxychloroquine in Patients With Rheumatoid Arthritis. Therapeutic Drug Monitoring 2003, 25 (6), 671-681.

28 Tett, S. E.; Day, R. O.; Cutler, D. J., Concentration-effect relationship of hydroxychloroquine in rheumatoid arthritis—a cross sectional study. The Journal of rheumatology 1993, 20 (11), 1874-9.

29 Browning, D. J., Pharmacology of Chloroquine and Hydroxychloroquine. Hydroxychloroquine and Chloroquine Retinopathy. 2014 Apr 4:35-63. doi: 10.1007/978-1-4939-0597-3_2.: 2014.

30 Popert, A. J., Chloroquine: a review. Rheumatology and rehabilitation 1976, 15 (3), 235-8.

31 Liu, Q.; Bi, G.; Chen, G.; Guo, X.; Tu, S.; Tong, X.; Xu, M.; Liu, M.; Wang, B.; Jiang, H.; Wang, J.; Li, H.; Wang, K.; Liu, D.; Song, C., Time-Dependent Distribution of Hydroxychloroquine in Cynomolgus Macaques Using Population Pharmacokinetic Modeling Method. Front Pharmacol 2020, 11, 602880.

32 Rempenault, C.; Combe, B.; Barnetche, T.; Gaujoux-Viala, C.; Lukas, C.; Morel, J.; Hua, C., Metabolic and cardiovascular benefits of hydroxychloroquine in patients with rheumatoid arthritis: a systematic review and meta-analysis. Annals of the rheumatic diseases 2018, 77 (1), 98-103.

33 Jorge, A.; Lu, N.; Choi, H.; Esdaile, J. M.; Lacaille, D.; Avina-Zubieta, J. A., Hydroxychloroquine Use and Cardiovascular Events Among Patients With Systemic Lupus Erythematosus and Rheumatoid Arthritis. Arthritis care & research 2023, 75 (4), 743-748.