Pharmacokinetic challenges in renal replacement therapy

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..................................................................................................... III
LIST OF PAPERS ................................................................................................................ IV
ABBREVIATIONS ................................................................................................................ V
ABSTRACT ........................................................................................................................ VII

1 INTRODUCTION .............................................................................................................. 1
  1.1 End-stage renal disease ........................................................................................... 1
  1.2 Renal replacement therapy ...................................................................................... 2
    1.2.1 Dialysis ............................................................................................................ 3
    1.2.2 Renal transplantation ..................................................................................... 3
    1.2.3 Adverse events in immunosuppressive therapy .............................................. 6
  1.3 Pharmacokinetics in patients with ESRD ................................................................. 7
    1.3.1 Absorption ..................................................................................................... 8
    1.3.2 Distribution ................................................................................................. 8
    1.3.3 Elimination .................................................................................................. 10
  1.4 Pharmacokinetics of tacrolimus and belatacept ...................................................... 11
    1.4.1 Pharmacokinetic variability .......................................................................... 12
    1.4.2 Therapeutic drug monitoring ........................................................................ 14

2 AIMS OF PRESENT STUDIES ...................................................................................... 16

3 SUMMARY OF PAPERS ............................................................................................... 17

4 DISCUSSION .................................................................................................................. 20
  4.1 Methodological considerations .............................................................................. 20
  4.2 Pharmacokinetics in ESRD .................................................................................... 22
    4.2.1 CYP3A activity ............................................................................................. 22
    4.2.2 Protein binding ............................................................................................ 24
  4.3 Tacrolimus clearance ............................................................................................. 24
    4.3.1 Tacrolimus clearance and outcomes .............................................................. 25
    4.3.2 Strengths and limitations of the clearance estimate ....................................... 27
  4.4 Belatacept ................................................................................................................. 29
  4.5 Drug dosing strategies in renal replacement therapy .............................................. 29

5 CONCLUSION ................................................................................................................ 31

6 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES ..................................... 32

REFERENCES ..................................................................................................................... 33
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I. Egeland EJ, Witczak B, Zaré HK, Christensen H, Åsberg A, Robertsen I.
Fluctuating, direct inhibition of CYP3A metabolism between hemodialysis sessions in patients with end-stage renal disease
*Manuscript*

High tacrolimus clearance is a risk factor for acute rejection in the early phase after renal transplantation.
*Transplantation. 2017;101: e273-e279*

III. Egeland EJ, Reisæter AV, Robertsen I, Midtvedt K, Strøm EH, Holdaas H, Hartmann A and Åsberg A.
High tacrolimus clearance – a risk factor for development of interstitial fibrosis and tubular atrophy in the transplanted kidney: a retrospective single-center cohort study
*Transplant International. 2019; 32: 257-269*

A fully automated method for the determination of serum belatacept and its application in a pharmacokinetic investigation in renal transplant recipients.
*Therapeutic Drug Monitoring. 2019; 41: 11-18*

*Contributed equally as first authors
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ah</td>
<td>Arteriolar hyalinosis</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
</tr>
<tr>
<td>BPAR</td>
<td>Biopsy proven acute rejection</td>
</tr>
<tr>
<td>C</td>
<td>Concentration</td>
</tr>
<tr>
<td>$C_{\text{elim}}$</td>
<td>Concentration at site of elimination</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>$ci$</td>
<td>Interstitial fibrosis</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>CNI</td>
<td>Calcineurin inhibitor</td>
</tr>
<tr>
<td>$C_p$</td>
<td>Concentration bound to plasma protein</td>
</tr>
<tr>
<td>$C_{\text{rbc}}$</td>
<td>Concentration bound to red blood cells</td>
</tr>
<tr>
<td>$ct$</td>
<td>Tubular atrophy</td>
</tr>
<tr>
<td>$C_{\text{target}}$</td>
<td>Concentration by drug target</td>
</tr>
<tr>
<td>$C_{\text{tissue}}$</td>
<td>Concentration bound to tissue</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic-T-lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>$C_u$</td>
<td>Unbound concentration</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>D</td>
<td>Dose</td>
</tr>
<tr>
<td>dnDSA</td>
<td>De novo donor specific antibodies</td>
</tr>
<tr>
<td>DSA</td>
<td>Donor specific antibodies</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ER</td>
<td>Extended release</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FKB12</td>
<td>FK506 binding protein 12</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>$i$</td>
<td>Interstitial inflammation</td>
</tr>
<tr>
<td>IFTA</td>
<td>Interstitial fibrosis and tubular atrophy</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IR</td>
<td>Immediate-release</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography combined with tandem mass spectrometry</td>
</tr>
<tr>
<td>LCP</td>
<td>Novel-extended release</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>$t$</td>
<td>Tubulitis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>Therapeutic drug monitoring</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time to reach maximum concentration</td>
</tr>
<tr>
<td>$v$</td>
<td>Vasculitis</td>
</tr>
</tbody>
</table>
ABSTRACT

Patients with end-stage renal disease (ESRD) are treated with renal replacement therapy (RRT), which consist of either dialysis or renal transplantation. Pharmacological therapy is essential for these patients, but can be challenged by adverse events. The overall aim of this thesis was to investigate clinical pharmacokinetics in patients receiving RRT to optimize drug therapy and identify risk factors for inferior long-term outcomes.

Loss of renal function in patients with ESRD receiving dialysis affects nonrenal drug elimination. The underlying mechanisms for this are largely unknown. This thesis presents a clinical trial where we observed that increasing degree of uremia between dialysis sessions had a direct inhibitory effect on cytochrome P450 3A (CYP3A) enzyme activity in patients receiving hemodialysis. The reduction was not large enough to be clinically relevant for most CYP3A substrate drugs. However, caution is warranted for drugs with narrow therapeutic range in selected patients.

Tacrolimus is currently the foundation of the immunosuppressive regimen after renal transplantation. There is a twentyfold difference in dose needed to reach target exposure due to large pharmacokinetic variability. This thesis presents two large cohort studies where we found that high estimated tacrolimus clearance was significantly associated with risk of acute rejection, as well as development of interstitial fibrosis and tubular atrophy in the kidney graft. The tacrolimus clearance estimate is a simple marker to detect patients at higher risk of early adverse events that can be detrimental for long-term outcomes.

Belatacept is an immunosuppressive therapeutic protein that can be used as an alternative to tacrolimus. Limited amount of data describing belatacept pharmacokinetics exist, and it is currently unknown if therapeutic monitoring of belatacept can improve clinical outcomes. This thesis presents a clinical feasibility trial where we showed that a novel assay for determination of serum belatacept concentrations performed well. The results signaled that there may be a larger variability in exposure of belatacept than previously described.
INTRODUCTION

1.1 End-stage renal disease

Chronic kidney disease (CKD) is increasingly recognized as a global public health problem, affecting 10% to 15% of all adults, and 15% to 45% of people aged 65 to 74 years. Patients with CKD are at increased risk of morbidity and mortality. CKD is a progressive disease and is categorized in different stages by how severely the renal function, i.e. glomerular filtration rate (GFR), is reduced (Table 1).

<table>
<thead>
<tr>
<th>CKD stage</th>
<th>Description</th>
<th>GFR (mL/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal or increased GFR</td>
<td>≥90</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild reduced GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>3</td>
<td>Moderate reduced GFR</td>
<td>30-59</td>
</tr>
<tr>
<td>4</td>
<td>Severe reduced GFR</td>
<td>15-29</td>
</tr>
<tr>
<td>5</td>
<td>Renal failure / ESRD</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

ESRD, end-stage renal disease, Modified from Levey et al.

The kidneys mediate removal of waste products from the blood through urine, and regulate the reabsorption and excretion of water and electrolytes to maintain stable osmolality and volume of body fluids. They are also involved in regulating blood pressure, the acid-base balance and stimulating synthesis of red blood cells. “Uremia” is a term used to describe the unspecific illness that accompanies these more definite pathologies seen in CKD. The illness can be perceived by uremic patients as e.g. itching, nausea and fatigue, and is largely due to accumulation of organic waste products that are normally cleared by the kidney. The presentation of uremic symptoms are not related to a specific CKD stage, but vary significantly between individuals. Although symptoms of uremia are often nonspecific, virtually every organ system in the body is affected by the disruption in metabolic homeostasis associated with advanced CKD. The risk of morbidity and mortality is substantially increased if the patient progresses to CKD stage 5, end-stage renal disease (ESRD). The prevalence of ESRD is increasing in Norway and worldwide (Figure 1). The severely reduced renal function must be treated with renal replacement therapy (RRT).
1.2 Renal replacement therapy

There are two modalities of RRT, dialysis or renal transplantation. The overall survival is better with renal transplantation,\textsuperscript{9,10} but due to an insufficient supply of renal grafts, many patients in Norway and world-wide are treated with chronic dialysis. In Norway, all patients that are considered to benefit from receiving a new kidney are eligible for transplantation, and can enter the waiting list. Of over 5000 prevalent RRT patients in Norway, two thirds are living with a functioning renal graft (Figure 1).\textsuperscript{8} Drug therapy is an important aspect in the management of patients receiving either of the RRT modalities. Patients receiving dialysis have high burden of comorbidities such as hypertension, diabetes, infections, bone disease, electrolyte disturbances and anemia,\textsuperscript{11} which require treatment with several drugs.\textsuperscript{12,13} After transplantation, some of the comorbidities and physiological disturbances seen in dialysis are resolved, but immunosuppressive drugs are crucial to manipulate the immune system and assure long-time graft survival.
1.2.1 Dialysis

Dialysis as a treatment for acute renal failure was introduced at Oslo University Hospital, Rikshospitalet in 1956. Long-term dialysis as a treatment for ESRD was however not introduced before the 1970s. There are mainly two types of dialysis modalities, hemodialysis and peritoneal dialysis. The overall goal of dialysis is to remove uremic substrates and excess total body water. Hemodialysis is an extracorporeal therapy, where blood is pumped through a semi-permeable membrane in a bedside dialyzer, while dialysis fluid (dialysate) flows in the opposite direction around the semi-permeable membrane in the “artificial kidney” to remove waste products from blood. Hemodialysis in the treatment of ESRD is predominantly given in an intermittent fashion with in-center sessions for approximately four hours, three times per week. In peritoneal dialysis, a catheter is implanted in the peritoneal cavity and anchored in the subcutaneous tissues. A buffered dialysate is infused through the catheter into the peritoneum and remains in place for several hours allowing for diffusive solute transport to occur across the peritoneal membrane. Over 1200 patients are treated with hemodialysis, making this the most dominant dialysis modality in Norway (Figure 1).

1.2.2 Renal transplantation

The first renal transplantation was carried out on two monozygotic twins in Boston, USA in 1954. Only two years later, the first renal transplantation in Norway was performed at Ullevål Hospital, Oslo. The patient lived for 30 days with the new kidney, which is impressive with the limited immunosuppressive therapy available at that time. Since 1983, all renal transplantations in Norway have been performed at Oslo University Hospital, Rikshospitalet. The center conducts between 250 to 300 renal transplantations per year, making the center the largest in northern Europe and one of the largest worldwide.

Immunosuppressive therapy

When a renal transplantation is performed, human leukocyte antigens (HLA) from the donor are recognized by the recipient's immune system triggering an alloimmune response. Renal transplant recipients are therefore in need of lifelong immunosuppressive treatment. Immunosuppression is given as a combination of agents with different mechanisms of action. By using a combination of multiple immunosuppressive drugs, the toxicity of each agent can be minimized without compromising the total immunosuppressive effect. The calcineurin inhibitors (CNI), comprising of cyclosporine and tacrolimus, constitute an important part of the
immunosuppressive regimen. Since their introduction, the incidence of acute rejections has been drastically reduced and the short-term outcome significantly improved. In Norway, the current standard immunosuppressive protocol after renal transplantation is based on the Symphony study. It is a quadruple regimen consisting of induction therapy with two doses of 20 mg basiliximab and maintenance therapy of low-dose tacrolimus in combination with mycophenolate mofetil and corticosteroids. Before tacrolimus became the CNI of choice in Norway, patients received cyclosporine. In 2011, the therapeutic protein belatacept was approved as the first therapeutic protein for use in maintenance immunosuppression. In the phase III studies, belatacept was compared with cyclosporine, and showed better renal function and cardiovascular profile. However, belatacept showed numerically more, as well as more severe acute rejections than cyclosporine in the phase III trials. In the one trial were belatacept was compared to tacrolimus, 11 of 20 patients receiving belatacept experienced acute rejection. Belatacept can be used as an alternative to CNI in the quadruple regimen. However, due to a shortage in Europe between March 2017 and March 2019, belatacept could not be prescribed to new patients in this period. As of March 15th 2019, the production situation of belatacept has improved, and prescription restrictions are eased, but full availability is not expected until fall of 2020.

Lymphocytes play a central role in the cell-mediated immune response and are the site of action of the maintenance immunosuppressive drugs (Figure 2). Tacrolimus binds to the immunophilin FK506 binding protein 12 (FKBP12). The tacrolimus-FKBP12 complex inhibits the activity of calcineurin and thereby reduce its phosphatase activity in a dose proportional manner. By inhibiting calcineurin, tacrolimus suppresses the dephosphorylation of nuclear factor of activated T cells (NFAT) and hence prevent the translocation of NFAT into the nucleus where it acts as a transcription factor. This results in an impaired synthesis of interleukin-2 (IL-2) and other important cytokines. IL-2 serves as a cell cycle progression signal for T cells, stimulating both their proliferation and differentiation.
Belatacept is a cytotoxic-T-lymphocyte-associated antigen 4 protein (CTLA-4) connected with the Fc-portion of IgG (CTLA-4-Ig). Belatacept inhibits T cell activation by blocking the co-stimulatory signal from antigen presenting cells (APCs) (Figure 2). Important co-stimulatory molecules are CD80 and CD86 (also named B7-1 and B7-2, respectively) on APCs, which activate T cells by binding to CD28 on the T cell surface. After a normal T cell activation via CD80/86 and CD28, CTLA-4 is up-regulated on the surface of T cells to control this augmented activation. CD80/86 has a higher avidity for CTLA-4 than CD28,\textsuperscript{30} and consequently, T cell activation is dampened through this CD80/86-CTLA-4-pathway. Belatacept binds to CD80/86 on APCs blocking its binding to CD28, leading to T cell anergy.\textsuperscript{31}

Using modern, powerful immunosuppressive drug combination therapy, the incidence of acute rejection is under 20\%, and most centers have one-year graft survival rates of over 90\%.\textsuperscript{32} However, long-term outcomes are still challenged by the adverse events of immunosuppressive
drugs, contributing to late graft failure, cardiovascular morbidity, opportunistic infections and malignancies. Further optimization of immunosuppressive therapies is thus needed.

1.2.3 Adverse events in immunosuppressive therapy

The effects of immunosuppressive drugs can be divided into three groups. The desired effect of suppressing rejection, undesired consequences of immunodeficiency and nonimmune toxicity. Despite the clinical efficacy, tacrolimus therapy is limited by both immunodeficiency toxicities and nonimmune toxicity due to the wide tissue distribution of calcineurin. Calcineurin and NFAT isoform are not T cell specific, and inhibition of this pathway by tacrolimus give rise to nonimmune toxicities such as hypertension, dyslipidemia, neurotoxicity and post-transplant diabetes mellitus. In general, use of immunosuppressive drugs increase susceptibly for infection and cancer. To reduce the risk of these events, it is desirable to keep exposure low. However, too low concentrations increase the risk of acute rejection and development of de novo antibodies to the HLA molecules (donor specific antibodies, dnDSA), which can be detrimental for allograft survival.

Considering the lack of a clinically available “immunometer” to assess the overall immunosuppression, the intensity of therapy is monitored indirectly by several surrogate markers following transplantation. Plasma creatinine is maybe the most important biomarker used. A rise in plasma creatinine is a signal that an acute rejection episode may have started, and the patient is in need of more intensive immunosuppression. However, increased creatinine can also be a signal of CNI toxicity, requiring a less intense immunosuppressive treatment. In order to verify which process that have induced the rise in plasma creatinine, morphological investigation of renal core biopsies is performed. In addition to performing indication biopsies as a reaction of increase plasma creatinine, many centers use protocol biopsies to monitor the graft status at predefined time points after transplantation. At the transplant center in Norway, protocol biopsies are taken seven weeks, as well as one year after transplantation. Lesions in renal biopsies are scored according to semi-quantitative Banff-classification criteria. The scores have four levels (0, 1, 2, and 3) with increasing degree of severity. These lesions have designated Banff-abbreviations and are shown as lower case letters in italic below.
Acute rejection

The most common form of acute rejection is initiated when donor alloantigens are presented to the recipient’s T lymphocytes by APCs in the lymphoid organs. These T cells differentiate into subgroups and return to the graft where they take part in destroying the transplanted organ. Acute rejections with interstitial inflammation (i) and tubulitis (t) are often reversible with pulse steroid treatment. However, if the rejection is located in the renal arteries, i.e. vasculitis (v), steroid treatment may be ineffective, and may instead require anti-thymocyte globulin therapy. Acute rejection can also be mediated by preexisting DSA before transplantation due to e.g. pregnancy, blood transfusions or previous transplantations, or by dnDSA.

Nephrotoxicity

Treatment with CNIs in renal transplantation is especially hampered by its paradoxical nephrotoxicity. Acute nephrotoxicity may present as an acute oligoanuric syndrome (delayed graft function) or as a rise in serum creatinine. This is largely a hemodynamic effect, which is often reversible by dose reduction. Patients treated with CNI often develop irreversible, chronic structural changes to all compartments of the allograft, including glomeruli, arterioles and tubulo-interstitium. This is also seen in patients receiving in a nonrenal transplant. Although, it should be mentioned that immune-mediated injury also can contribute to chronic nephrotoxicity. Nodular hyaline deposits in the media of afferent arterioles (arteriolar hyalinosis, ah) has previously been regarded as the hallmark of CNI nephrotoxicity, but may be unspecific since it also is related to aging, diabetes and hypertension. Interstitial fibrosis (ci) can be defined as abnormal accumulation of collagens and related molecules in the renal interstitium. Development of tubular atrophy (ct) is usually associated with interstitial fibrosis. Tacrolimus is regarded to be less nephrotoxic than cyclosporine. Chronic nephrotoxicity contributes to the late allograft loss in a substantial proportion of renal transplant recipients.

1.3 Pharmacokinetics in patients with ESRD

Pharmacokinetics refers to the processes of drug absorption, distribution, metabolism and excretion. Numerous physiological and environmental factors influence the fraction of ingested dose reaching the systemic circulation and site of action, to what extent the drug is distributed to different tissues and how efficiently the drug is eliminated from the body.
Detailed knowledge about factors influencing pharmacokinetic processes and drug response is important in order to optimize dosing strategies and drug therapy in the individual patient. Patients with ESRD receiving dialysis are a heterogeneous group, and have a high risk of adverse drug reactions. Changes in pharmacokinetics in patients with ESRD complicates drug therapy.

1.3.1 Absorption
Following oral dosing, the absorption from the intestine is characterized by both the rate of absorption and the total amount of drug absorbed. Several factors in the gastrointestinal tract may influence this process. Patients with ESRD may often exhibit pathophysiological changes in the gastrointestinal tract which may affect drug absorption. Decreased gastrointestinal motility may affect the maximal plasma concentration (Cmax), time required to reach Cmax (Tmax), and oral bioavailability. Alterations in gastric pH that impact drug solubility can affect drug absorption. Patients with ESRD often use phosphate binders such as calcium carbonate, lanthanum carbonate and sevelamer hydrochloride, which may interact with certain drugs in the gastrointestinal tract, and reduce the extent of absorption.

1.3.2 Distribution
Drug distribution refers to the movement of a drug between the blood and various tissues of the body. It is widely accepted that only the unbound drug can be absorbed, distributed and finally give a pharmacological effect. Drug in the body can be distributed to various sites – unbound in plasma, bound to components in plasma such as plasma proteins and erythrocytes, unbound in tissue and bound to, or dissolved in tissue components. Since drug binding in general is rapidly reversible, drug molecules exist in these various states in a type of dynamic equilibrium (Figure 3). This equilibrium is affected by several physiological variables such as protein binding, tissue binding and total-body water, each of which may be altered in ESRD. Changes in tissue binding are rare, but have been reported for selected drugs. Increase in extracellular volume due to fluid retention can lead to increased volume of distribution of hydrophilic drugs with moderate to low volume of distribution. The most common physiological change affecting drug distribution in ESRD is protein binding. The main drug binding proteins in plasma are albumin and α1-acid glycoproteins, lipoproteins and globulins. In general, albumin binds both basic and acidic drugs, while the acute phase α1-acid
glycoprotein binds mainly basic drugs. Several physiologic changes in patients with ESRD are proposed to cause these changes. Altered acid-base homeostasis, hypoalbuminemia, accumulation of endogenous substances (e.g. uremic toxins and free fatty acids) that competitively displace drugs from their binding sites or modulates a conformational change in binding sites. It has been observed that the binding capacity of albumin decreases when the grade of uremia increases, and that hemodialysis may acutely restore the binding capacity in dialysis patients. These changes may affect the distribution of drugs in individual patients with CKD.

**Figure 3.** Schematic relationship between drug binding, elimination and clinical effect. 
$C_{\text{elim}}$, concentration at site of elimination; $C_p$, concentration bound to plasma protein; $C_{\text{rbc}}$, concentration bound to red blood cells (erythrocytes); $C_{\text{target}}$, concentration by drug target; $C_{\text{tissue}}$, concentration bound to tissue; $C_u$, unbound concentration. Modified from references.
1.3.3 Elimination

There are several pathways a drug can be eliminated from the body. The efficacy of drug elimination is described by clearance; the drug elimination rate is described as the product of clearance and drug concentration. Several elimination pathways are known to be reduced or altered in ESRD.

Renal elimination

The kidneys are responsible for the elimination of numerous drugs. Renal clearance is normally considered as the net result of glomerular filtration, tubular secretion and reabsorption. The clinical consequences of reduced renal function on the elimination of renally cleared drugs have been known since Kunin et al published the seminal papers describing the effect of acute renal failure on prolonged half-life of renally cleared antibiotics 60 years ago.84-86 The renal elimination of drugs is reduced in a continuous fashion with increasing severity of renal disease.87 Hence, doses of renally cleared drugs have to be reduced to account for the reduction in renal clearance, especially for drugs with narrow therapeutic range.

Nonrenal elimination

Nonrenal elimination includes all routes of drug elimination apart from the kidneys. There is an increasing awareness that ESRD may affect the nonrenal clearances of drugs eliminated by drug metabolizing enzymes and drug transporters.88-90 Enzyme catalyzed metabolism constitute the primary pathway of nonrenal clearance. Drug metabolizing reactions are classified into phase I and phase II. Phase I reactions involves oxidation, reduction or hydrolysis of the drug, while phase II reactions involve covalently binding of endogenous compound, most often glucuronide acid, glutathione or sulphate, to the phase I metabolite. The most important group of phase I metabolizing enzymes is the cytochrome P450 (CYP) superfamily, contributing to the metabolism of 75% of the most clinically used drugs.91 Fifty-seven CYP isoforms have been identified in humans,92 but only a limited number are found to be important in drug metabolism. These mainly belong to the CYP1, CYP2 and CYP3 families.93 All major CYP subfamilies have substrate drugs with reduced clearance in CKD.65,66

The CYP3A subfamily, consisting mainly of CYP3A4 and CYP3A5 in adults, are estimated to account for 30-50% of all CYP mediated drug metabolism and is therefore considered the most important subfamily of drug metabolizing enzymes.94 Due to the high abundance and variability
INTRODUCTION

of CYP3A enzymes in both liver and gut, these enzymes are contributing determinants of first-pass metabolism, and thus the bioavailability after oral drug administration.\textsuperscript{95,96} CYP3A activity can be phenotyped in individual patients by using probe drugs that are metabolized specifically by CYP3A enzymes. The benzodiazepine drug, midazolam, is considered the gold standard for CYP3A phenotyping.\textsuperscript{97} CYP3A activity in patients with ESRD and healthy controls has been compared, but the results are conflicting. Some studies have shown a significantly reduced CYP3A activity,\textsuperscript{98,99} while other studies have found unaltered activity in patients with ESRD.\textsuperscript{100,101}

The underlying mechanisms of altered metabolic enzyme function in ESRD are complex, and are still not completely understood. The leading hypothesis is that constituents of uremia cause a decrease in protein expression of CYP enzymes, and thus reduced metabolic capacity.\textsuperscript{88-90} Several \textit{ev vivo} and animal models of ESRD have confirmed that intestinal and hepatic CYP protein expression is decreased, leading to an overall reduction in enzymatic activity.\textsuperscript{102-108} There is also a hypothesis that factors in uremia have a direct inhibitory effect on CYP enzymes.\textsuperscript{90} This hypothesis is supported by the findings that uremic toxins acutely reduce CYP3A activity \textit{in vitro} in rat hepatic microsomes, and hemodialysis acutely improve CYP3A activity \textit{in vivo}.\textsuperscript{109,110} These findings support the concept that dialyzable uremic constituents may also directly inhibit CYP3A metabolism in humans.\textsuperscript{90} The direct inhibition hypothesis has been questioned by Decker \textit{et al}, who did not find any difference in midazolam metabolism in human microsomes treated with pre- and postdialytic serum.\textsuperscript{111}

1.4 Pharmacokinetics of tacrolimus and belatacept

The oral absorption of tacrolimus depends on the fat content of the consumed food and the timing of ingestion.\textsuperscript{112,113} After oral dosing, the immediate release formulation of tacrolimus is, in most patients, absorbed rapidly into the bloodstream.\textsuperscript{114} In others, uptake occurs slowly over a prolonged time-period yielding an essentially flat absorption profile.\textsuperscript{115} The oral bioavailability is poor (average 25%) and highly variable (4% to 89%) due to tacrolimus’ lipophilic structure and extensive first-pass metabolism in the intestinal wall.\textsuperscript{116} Belatacept, on the other hand, is a large therapeutic protein (molecular weight \(\approx 100\) kDa), and is therefore given as an intravenous infusion, bypassing the issue with low and variable bioavailability.\textsuperscript{117}

In blood, tacrolimus binds extensively to plasma proteins and is also distributed into blood cells, with a high affinity for erythrocytes. The whole-blood drug concentrations are on average 15
times higher than corresponding plasma concentrations, and is highly variable (range 4-114).\textsuperscript{118} The ratio between whole-blood and plasma concentrations is dependent on the proportion of erythrocytes in blood (hematocrit)\textsuperscript{119} and tacrolimus concentration.\textsuperscript{120} In plasma, tacrolimus is $\sim$99\% bound to plasma proteins.\textsuperscript{118} This excessive binding makes the unbound concentration available to enter T cells and evoke a pharmacological effect only about 0.01\% to 0.1\% of the whole blood concentration. No studies have been performed with radiolabeled belatacept, so the tissue distribution is unknown.\textsuperscript{121} After intravenous dosing belatacept has a low volume of distribution which is equivalent to the volume of vascular and interstitial spaces\textsuperscript{122,123}

Tacrolimus has a predominantly nonrenal elimination via hepatic CYP3A metabolism followed by excretion of metabolites to bile.\textsuperscript{124} Less than 3\% of the dose is excreted in urine, mainly as metabolites.\textsuperscript{124} After infusion of belatacept, the average elimination half-life is 8 days.\textsuperscript{123} Renal function do not affect elimination, and belatacept is not believed to be a substrate for CYP metabolism.\textsuperscript{122,123} The exact mechanism of belatacept elimination has not yet been studied, but is believed to be cleared by macrophages and Kuppfer cells.\textsuperscript{121}

\textbf{1.4.1 Pharmacokinetic variability}

Administering the same dose to all patients will lead to different systemic exposures. Several factors have been identified to cause inter- and intrapatient variability in tacrolimus pharmacokinetics, while the trials conducted by the manufacturer have shown that belatacept may have a less variable pharmacokinetic profile. Intrapatient variability in tacrolimus pharmacokinetics has emerged as a risk factor for several adverse events such as reduced renal function, dnDSA development, acute rejection, histological lesions such as interstitial fibrosis, and graft loss.\textsuperscript{125-130}

Protein expression and activity of drug metabolizing enzymes are thought to be major factors contributing to the variable tacrolimus pharmacokinetics between individuals. The most influential and consistently identified genetic variable for tacrolimus interpatient variability is a single nucleotide polymorphism in the gene encoding CYP3A5 (6986A>G).\textsuperscript{131} Patients carrying hetero- or homozygous \textit{CYP3A5*1} wild-type allele express high levels of functional CYP3A5 protein (i.e. CYP3A5 expressers), while patients carrying the variant \textit{CYP3A5*3} express very low or undetectable levels of functional CYP3A5 protein (i.e. CYP3A5 nonexpressers).\textsuperscript{132} About 15 to 20\% of Caucasians are CYP3A5 expressers.\textsuperscript{119,133,134} These
patients require approximately two times higher doses of tacrolimus compared to nonexpressers due to higher clearance and/or lower bioavailability. Concomitant pharmacological therapy may affect the pharmacokinetics of tacrolimus and contribute to variability in drug response. Potential sites of pharmacokinetic drug-drug interactions include the gastrointestinal tract, drug metabolizing enzymes, drug transporters and biliary excretion. In general, inhibition and induction of the CYP3A mediated metabolism in the intestines and/or liver are regarded as the most common mechanism of tacrolimus drug-drug interactions. Clinically relevant examples of interacting drugs that induce CYP3A are glucocorticoids, rifampicin, St. John’s wort (Hypericum perforatum) and phenytoin which lead to reduced tacrolimus exposure. Examples of inhibitors of CYP3A are ritonavir, grapefruit (Citrus paradisi), azole antifungals and calcium channel blockers, which been demonstrated to increase the exposure of tacrolimus significantly.

Numerous physiological changes occur in older individuals that could potentially affect how they process immunosuppressive drugs. Decline in hepatic and renal blood flow, hepatic mass and renal function are main contributors to decreased clearance of drugs in the elderly. In elderly patients, drug exposure is usually increased and lower doses are often sufficient to achieve therapeutic response compared with younger adults. There are also gender differences in the clearance of tacrolimus. Females often need higher doses to achieve the same exposure as males. Associations between age, gender, race, hepatic function, diabetes or dialysis on belatacept pharmacokinetics has not been reported.

Adherence to immunosuppressive therapy is thought to be reflected in intrapatient variability. The European Society for Patient Adherence, Compliance and Persistence defines medication adherence as “the process by which the patients take their medicines as prescribed”. If a patient take the prescribed tacrolimus dose at a different time than usual, or do not take the dose at all, this will lead to variability in exposure. Adherence to the immunosuppressive drug regimen after renal transplantation is rather poor, which may be detrimental to the graft since periods with suboptimal immunosuppression may induce an immune reaction. Nonadherence to immunosuppressive therapy has been strongly linked to poor long-term outcomes after renal transplantation. There is an increased focus on increasing adherence in the renal transplant population to increase the long time outcomes after renal transplantation.
1.4.2 Therapeutic drug monitoring

Due to the extensive pharmacokinetic variability of tacrolimus, there is a poor correlation between tacrolimus dose and resultant exposure.\textsuperscript{156} Drug concentrations are therefore measured, followed by dose adjustments to achieve exposure within a narrow range to produce an acceptable balance between efficacy and toxicity. This procedure is called therapeutic drug monitoring (TDM).\textsuperscript{157} From a pharmacological perspective, area under the concentration-time curve (AUC) is the best measurement of systemic tacrolimus exposure. However, tacrolimus is usually monitored utilizing trough concentrations, i.e. the concentration in a sample taken immediately prior to dosing. This strategy has been used since the first trials,\textsuperscript{158,159} and is convenient since only one sample is needed. Some trials have shown a satisfying correlation between tacrolimus trough concentrations and $\text{AUC}_{0-12\text{h}}$ ($r^2 > 0.89$).\textsuperscript{160,161} These results has, however, not been confirmed in other trials ($r^2 = 0.34 – 0.54$).\textsuperscript{162-164} Even though TDM is highly recommended for tacrolimus therapy, there is no consensus on the target range.\textsuperscript{165} In the earliest trials, the trough concentrations ranged from 5 to 40 μg/L\textsuperscript{159}, and only a few prospective concentration-controlled trials were conducted to establish target tacrolimus concentrations in relation to clinical outcomes.\textsuperscript{158,166,167} The Symphony trial launched the era of “tacrolimus minimization”. This study showed that renal transplant recipients targeting tacrolimus trough concentration between 3 and 7 μg/L had fewer acute rejections and higher GFR at one year than patients receiving cyclosporine or sirolimus.\textsuperscript{21} In Norway, tacrolimus doses are currently adjusted to achieve trough concentrations between 4 and 7 μg/L in standard immunological risk patients, and 10 to 12 μg/L the first 30 days and 6 to 10 μg/L thereafter in high immunological risk patients.

According to the manufacturer, belatacept should be dosed solely by body-weight.\textsuperscript{168} It is currently unknown if TDM would benefit clinical outcomes in renal transplantation.\textsuperscript{121} A prerequisite for pharmacokinetic TDM is an assay for determination of serum concentrations. The manufacturer used an in-house ELISA assay during drug development, but there is no available assay for clinical belatacept concentration determination.\textsuperscript{169} Of the two dosing regimens were compared in the phase III trials, patients receiving the “more intensive” belatacept regimen had a higher incidence of serious infections and malignancies than patients receiving the “less intensive” regimen.\textsuperscript{170} A pooled efficacy/safety analysis of exposure in relation to efficacy (acute rejection) and toxicity (serious infection) found no association between exposure of belatacept and incidence of acute rejections in the two phase III trials.\textsuperscript{122}
There was however a strong association between higher time-averaged concentrations the first 6 months post-transplant and serious infections.\textsuperscript{122}
2 AIMS OF PRESENT STUDIES

The overall aim was to investigate clinical pharmacokinetics in patients receiving renal replacement therapy to optimize drug therapy and identify risk factors for inferior long-term outcomes.

Specific aims were as follows:

- Study the acute effect of dialysis on CYP3A activity in patients receiving chronic intermittent hemodialysis (paper I)
- Investigate the effect of increased tacrolimus clearance on the risk of acute rejections and development of interstitial fibrosis and tubular atrophy the first year after renal transplantation (Paper II and III)
- Investigate if a novel assay for belatacept concentration determination is applicable for clinical pharmacokinetic studies and therapeutic drug monitoring (paper IV)
3 SUMMARY OF PAPERS

Paper I

*Fluctuating, direct inhibition of CYP3A metabolism between hemodialysis sessions in patients with end-stage renal disease*

The aim of this trial was to investigate the acute effect of hemodialysis on both intestinal and hepatic CYP3A activity in patients with ESRD by determining the absolute bioavailability of midazolam and the extent of midazolam metabolism. Unbound midazolam exposure was utilized to elucidate the mechanism behind eventual changes in CYP3A phenotype. Twelve patients with ESRD receiving chronic intermittent hemodialysis were CYP3A phenotyped using semi-simultaneous oral and intravenous midazolam at a “clean” day (median 18 hours after last dialysis) and “dirty” day (median 75 hours after last dialysis). Unbound midazolam clearance decreased by median (range) 14% (-3 – 41%, P=0.001) from the “clean” to the “dirty” investigation day. The median absolute bioavailability of midazolam was 44% (11% – 95%) on the “clean” day, and 40% (16% – 67%) on the “dirty” day (P=0.18). The unbound fraction of midazolam was significantly increased with median 32% from 1.5% (0.8% – 2.2%) at the “clean” day to 2.0% (1.1% – 3.0%) at the “dirty” day (P=0.002). The increase in unbound fraction masked the inhibiting effect of uremia on CYP3A since it led to unchanged total midazolam exposure, and thus unaltered total midazolam clearance between the investigation days (P=0.23). This trial showed that changes in uremic state between dialysis sessions induced a small, but statistically significant, inhibitory effect on CYP3A activity.

Paper II

*High tacrolimus clearance is a risk factor for acute rejection in the early phase after renal transplantation*

In this study, we aimed to investigate the effect of increased tacrolimus clearance on the incidence of biopsy-proven acute rejection (BPAR) after renal transplantation. All patients receiving a renal transplant in Norway between 2009 and 2013 that received tacrolimus as a part of their immunosuppressive therapy were included in the study. Tacrolimus clearance was estimated by dividing the daily tacrolimus dose by the corresponding trough concentration. In one analysis, patients were stratified into four groups according to their estimated clearance.
The patients in the high clearance group had significantly higher incidence of BPAR (20.6%) with a hazard ratio of 2.39 (95% confidence interval, 1.30-4.40) compared with the low clearance group which had a BPAR incidence of 9.3%. Association between individual, continuous tacrolimus clearance estimates and BPAR was investigated in a multivariable Cox-regression model. Estimated clearance was adjusted for other risk factors such as delayed graft function, HLA-DR mismatches, immunologic high risk, male gender and cold ischemia time. The model showed that a one-unit increase in estimated tacrolimus clearance had a hazard ratio of 2.25 (95% CI, 1.70-2.99, P<0.001) for experiencing BPAR the first 90 days post-transplant. Patients in the low and high clearance groups used 1.4 ± 0.8 and 3.1 ± 4.1 days to reach target trough concentrations. However, there was no difference in neither trough concentrations the first week nor the time to reach target trough concentrations between the patients experiencing and not experiencing BPAR (2.2 ± 2.3 vs 2.2 ± 3.0 days, P=0.95). This study showed that a high estimated tacrolimus clearance lead to a higher risk for BPAR.

**Paper III**

*High tacrolimus clearance – a risk factor for development of interstitial fibrosis and tubular atrophy in the transplanted kidney: a retrospective single-center cohort study*

The aim of this study was to investigate the relationship between estimated tacrolimus clearance and development of interstitial fibrosis and tubular atrophy (IFTA) the first year after renal transplantation. The cohort consisted of the same patients as in **Paper I**, i.e. all renal transplant recipients in Norway transplanted between 2009 and 2013 receiving tacrolimus therapy. The first analyses included all 504 patients with renal core biopsies taken at both 7 weeks and 1 year after transplantation. In these analyses, the outcome variable was increase in IFTA-score (ci+ct) of two or more, regardless of inflammation score. There were significantly more patients in the high tacrolimus clearance group developing this lesion compared to the other clearance groups (P=0.006). This finding was substantiated in a multivariable analysis showing that a one unit increase in estimated tacrolimus clearance had an odds ratio of 1.67 (95% CI, 1.11-2.51, P=0.013) after adjusting for high immunological risk and BPAR between day 90 and 400 post-transplant. In the second analyses, the incidence of IFTA without inflammation (ci+ct ≥ 2 and i+t ≤ 1) at 1 year was investigated in the 233 patients without IFTA (ci+ct ≤ 1) at 7 weeks. Significantly more patients in the high clearance group developed *de novo* IFTA during the first year post-transplantation (P=0.007). A multivariable analysis of the same outcome showed that a one unit increase in estimated tacrolimus clearance had an odds ratio of 2.01 (95% CI, 1.18-
3.50, P=0.010) after adjustment for high immunological risk, donor age and recipient gender. This study shows that an increased estimated tacrolimus clearance is associated with both increased IFTA-development regardless of initial IFTA-score, and increased risk of IFTA at one year post-transplant for patients without any sign of IFTA at 7 weeks.

**Paper IV**

*A fully automated method for the determination of serum belatacept and its application in a pharmacokinetic investigation in renal transplant recipients*

In this paper a validation and application of an analyte-capture assay to measure serum concentration of belatacept is presented. The assay was successfully validated according to the European Medicine Agency guideline on bioanalytical method validation. The assay was applied in a small pharmacokinetic feasibility study including five stable renal transplant patients receiving belatacept as part of an ongoing clinical trial. Samples were collected at several occasions for each of the five patients, and belatacept concentrations were determined in a total of 203 serum samples. The concentrations of belatacept ranged from 0.9 to 127 mg/l, which was within the linear range of the assay. Trough concentrations ranged from 0.9 to 16.2 mg/L, with the highest concentrations during two-week interval dosing. Using a population pharmacokinetic model, the mean ± SD of volume of distribution in the central compartment and elimination rate constant was 3.5 ± 0.6 L and 0.013 ± 0.002 h⁻¹, which give a clearance and elimination half-life of 0.045 ± 0.009 L/h and 54 ± 7 hours, respectively. The results also gave indications that the lightest and heaviest patients may risk under- and over exposure since the current dosing recommendation is only according to body weight. This can now be investigated in future clinical trials with the assay presented in this paper.
4 DISCUSSION

Individualized drug therapy to obtain optimal balance between efficacy and toxicity is a goal in pharmacological treatment. Knowledge of pharmacokinetic challenges may guide clinicians to attain better long-term outcomes when treating patients in RRT.

4.1 Methodological considerations

Bioanalytical

Unbound midazolam concentrations were determined in Paper I. A challenge when determining unbound concentration in plasma samples is that the true unbound concentration is unknown, and the delicate equilibrium in plasma (Figure 3) may be disturbed during sample preparation. All samples were thawed and kept at 37°C during sample preparation in Paper I to attain physiological conditions and not disturb the equilibrium between midazolam and plasma proteins. In Paper I, unbound midazolam was determined using ultrafiltration, which is a more rapid method than equilibrium dialysis since the ultrafiltrate may be analyzed directly. Nonspecific adsorption of the analyte to the filtration device can be an issue in ultrafiltration, but less than 14% of midazolam was adsorbed, assessed by a mass-balance approach.

All tacrolimus whole-blood concentrations used to estimate the tacrolimus clearance in Paper II and III were measured with the chemiluminescent microparticle immunoassay method. In the immunoassay, tacrolimus concentrations in the entire concentration range are on average 18% higher compared to tacrolimus concentrations determined using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), which is considered the gold standard. This is mainly due to cross-reactivity with tacrolimus metabolites. This overestimation of tacrolimus concentrations is not likely to affect the results because the immunoassay was consistently applied through the entire study period (2009 to 2013).

The pharmacokinetic results presented in Paper IV were calculated from serum concentrations determined with a novel assay. Five percent of patients have been shown to develop anti-belatacept antibodies. Belatacept molecules bound to any neutralizing antidrug antibodies will not be able to bind to CD80 in the assay, and will be removed during the wash step. Only pharmacologically active drug was thus measured.
**Population pharmacokinetics**

A nonparametric population pharmacokinetic model was built from the belatacept serum concentrations in **Paper IV**. The data was primarily handled by population modeling to give a broad overview of the pharmacokinetics of belatacept. Considering the limited data available, no firm conclusion should be drawn from the results, but the visualization of the concentration-time profiles should be relatively representative.

**Epidemiological**

**Paper II** and **III** present retrospective studies conducted in the same patient cohort, transplanted in Norway between 2009 and 2013, which received tacrolimus as a part of their immunosuppressive therapy. The major strength of the cohort is its large size, and that all patients had a uniform follow-up at the same transplant center. In addition, the patients were treated with the most frequently prescribed immunosuppressive protocol currently used around the world. Data from the cohort was continuously reported to the Norwegian Renal Registry for research purposes. Since the studies in **Paper II** and **III** were designed after data was collected, the information bias is limited. External validity is limited by selection bias occurring when specific patients are systematically excluded from the study. In the period from 2009 to 2011, patients older than 50 years, patients with body mass index over 28 kg/m² or known impaired glucose tolerance, received cyclosporine instead of tacrolimus. These patients were not included in **Paper II** and **III**. Due to the stratification criteria, it is likely that these patients were systematically different from the included patients. Seventy-nine patients were transferred to their local hospitals before the in-depth investigational day at eight weeks, and were therefore not included in the cohorts. Transferred patients are often more frail, and in need of more care. This may lead to better adherence. This may have introduced bias, but the decision to transfer patients is not based on any known factor that directly affect risk of acute rejection or nephrotoxicity. In **Paper III**, patients not undergoing renal core biopsy at 1 year were not included in the main analyses. This conditioning on a future event could lead to bias. Therefore, sensitivity analyses were conducted to assess the impact of this selection, and it did not affect the overall results. The cohort in **Paper II** and **III** consisted predominantly of Caucasian patients, which may limit the relevance of the results to centers with different ethnic compositions.
Statistical

The patients in Paper I were investigated at two separate occasions and each patient served as its own control. The sample size in Paper I was calculated to find an anticipated clinically relevant change in midazolam absolute oral bioavailability of 25% between the “clean” and “dirty” investigation days. The study was therefore not powered to infer associations between degree of uremia and CYP3A activity. Unlike Paper I, the large number of included patients Paper II and III made it possible to build statistical models to quantify the risk of outcomes (BPAR and IFTA) according to exposure (estimated tacrolimus apparent oral clearance) in the cohort. The multivariable Cox-regression model in Paper II, and the binominal logistic model in Paper III, were built with a priori knowledge of risk factors for BPAR and IFTA, respectively. There were also several characteristics such as deceased donor status and recipient age that were significantly different between the clearance groups, which could have confounded the results in Paper III. These characteristics were included in the initial models, and eliminated in a stepwise fashion using augmented backwards elimination.175 This method excluded variables that had P-values over 0.2 and did not cause a change-in-estimate (i.e. the other odds ratios) more than 0.05.175 The size of the effect estimates from the final models in Paper III should be interpreted with care, as fewer adjustment variables may lead to larger odds ratios. A limitation to the findings in Paper II and III is that the causality of the associations is not possible to infer due to the retrospective nature of the studies.176 However, the complete follow-up of all patients and very few missing values further strengthened the findings in Paper II and III.

4.2 Pharmacokinetics in ESRD

Patients with ESRD receiving dialysis often receive a plethora of drugs to treat several comorbidities, and especially the significant risk of cardiovascular disease. Nonrenal clearance is believed to be reduced in patients receiving hemodialysis, but there is a paucity of clinical trials investigating what the physiological changes of uremia does to pharmacokinetic processes in this patient population.

4.2.1 CYP3A activity

The expression of CYP enzymes is reduced in models of uremia.102-108 In Paper I, the effect of varying degree of uremia on the activity of CYP3A enzymes was assessed. Changes in uremic
milieu between the dialysis sessions induced a small, but statistically significant, decrease in the CYP3A activity, substantiating the hypothesis that fluctuating levels of uremic toxins may directly inhibit drug metabolism and transport. In Paper I, the oral intrinsic clearance estimated from unbound midazolam concentrations was reduced from the “clean” to the “dirty” day. With the assumption that the fraction of midazolam absorbed into the portal vein ($F_{abs} \cdot F_G$) is unaltered, the estimated oral intrinsic clearance, i.e. CYP3A activity, was reduced by 14% at the “dirty” day compared to the “clean” day. Five of twelve patients showed a more than 20% reduction in CYP3A activity which corresponds to a 1.25 increase in unbound exposure. Furthermore, the variability in unbound oral exposure was almost fivefold. We did not quantify any specific uremic toxins in Paper I. Dialyzable uremic toxins such as indoxyl sulphate, hippuric acid, and p-cresol have been linked to reduced CYP3A activity in vitro, and may be the culprits behind the reduction in CYP3A activity.

Hemodialysis has previously been shown to acutely increase hepatic CYP3A activity by using the erythromycin breath test. This is in line with the findings in Paper I. Erythromycin is however considered to be a less selective CYP3A probe than midazolam, since it also is a substrate for drug transporters such as organic anion transporting polypeptides and P-glycoprotein, and is dependent on active transport into hepatocytes for CYP3A metabolism to take place. The altered erythromycin metabolism may thus also to some extent be explained by a change in transporter activity, which has previously been shown to be reduced in patients with ESRD by using the transporter probe fexofenadine.

To our knowledge, Paper I presents the first study to investigate midazolam absolute oral bioavailability in patients with ESRD. The absolute oral bioavailability ranged from 11% to 95%. This large variability shows that patients with ESRD receiving hemodialysis are a heterogeneous group, making the exposure following oral drug administration difficult to predict. Furthermore, the median midazolam absolute bioavailability was over 40%, which is somewhat higher than the 25% to 30% assessed by semi-simultaneous midazolam dosing in healthy volunteers. This finding may support the hypothesis that patients with ESRD have altered drug metabolism due to reduced expression CYP3A enzymes. As a direct inhibitory effect on CYP3A enzymes by the fluctuating degree of uremia was observed, it may be proposed that it is likely a combination of the two mechanisms (direct inhibition and reduced expression) that explain the alterations in drug metabolism observed in patients with ESRD. However, this theory warrants further investigations both in experimental and clinical studies.
4.2.2 Protein binding

Changes in protein binding capacity and thus unbound drug fractions are common in patients with ESRD. Unbound exposure of highly protein bound probe drugs should be measured to draw valid conclusions regarding the underlying mechanisms of altered pharmacokinetics in this patient population. Alterations in unbound fraction, regardless of any changes in metabolic enzyme activity will directly affect total (bound plus unbound) drug exposure. In Paper I, increased degree of uremia at the “dirty” day led to an increase in unbound fraction by 30%. This is similar to findings with warfarin and diazepam. The increased unbound fraction was relatively larger than the reduction in CYP3A activity, which was reflected in the unchanged total midazolam exposure between the investigation days. Without determining the unbound midazolam exposure, the observation of a reduced CYP3A activity would not have been recognized. Midazolam has been reported to have 67% higher unbound fraction in patients with ESRD (6.5%) compared to healthy controls (3.9%). Other studies comparing midazolam pharmacokinetics between patients with ESRD and healthy controls have not determined the unbound midazolam exposure. It is therefore unknown if the findings of increased and unaltered total midazolam exposure is due to effects on CYP3A activity, unbound fraction, or both.

4.3 Tacrolimus clearance

Estimated apparent oral tacrolimus clearance is a simple way to estimate the crude metabolic capacity in an individual patient from easy accessible clinical data. For simplicity, this estimate is referred to as “clearance” in this discussion. It is estimated as the ratio between the daily tacrolimus dose and the corresponding trough concentration. If a patient needs a higher dose (D) to achieve the same trough concentration (C) as another patient, the patient with the higher dose has the highest estimated clearance. This estimate has been given different names in the literature such as “metabolism rate” and “dosage requirement”. Most studies estimating tacrolimus clearance report concentration-dose ratio (C/D). This is counterintuitive since a decreasing C/D ratio in these studies infer a higher clearance. Because of this, the clearance has been estimated as a D/C ratio in Paper II and Paper III, i.e. increasing estimate for increasing clearance. This does not affect the results, but eases the interpretation.
4.3.1 Tacrolimus clearance and outcomes

Even though the short term outcomes after renal transplantation are significantly better with modern immunosuppression, there is a need to detect patients at risk for outcomes such as acute rejection and nephrotoxicity the first year post-transplant.\textsuperscript{188} This is important since these adverse outcomes has been shown to affect long-term graft survival.\textsuperscript{43,44,60-62}

**Acute rejection**

The major finding in Paper II was that renal transplant recipients with high tacrolimus clearance had a higher risk of experiencing an acute rejection episode in the early phase after renal transplantation. The quartile of patients with the highest clearance had a more than twofold higher risk of BPAR compared to the low clearance group. A patient with high clearance would theoretically have increased risk of under immunosuppression in case of a delayed or skipped dose compared to a patient with lower clearance.\textsuperscript{189} The median time to rejection was eight days. In this period, all patients were hospitalized in the surgical ward with an anticipated adherence close to 100\%. This make it unlikely that adherence was the main cause for the effect on BPARs seen in Paper II, but it cannot be ruled out. The time to reach the target trough concentrations was different between the clearance groups. Patients in the low clearance group reached the target trough concentration range faster than the patients in the other clearance groups. This could have been an explanation for the difference in risk of BPAR observed between the groups. However, there was no significant difference in time to reach the target concentrations between patients experiencing, and not experiencing BPAR. A previous study failed to find an association between lower trough concentrations after transplantation and acute rejection.\textsuperscript{190} It is likely that the potent induction therapy including basiliximab was sufficient to cover the initial small difference in target achievement the initial period after transplantation. There is no obvious explanation for the increased risk of acute rejection, but it may be that tacrolimus concentrations within the lymphocytes are lower in patients with high tacrolimus clearance.

**Nephrotoxicity**

The main finding in Paper III, was that renal transplant recipients with a phenotype of high tacrolimus clearance showed an increased risk of developing IFTA during the first year post-engraftment. This was shown in two different analyses investigating changes in protocol biopsies from 7 weeks to 1-year post-engraftment. The first analysis included all tacrolimus
treated patients with paired biopsies from 7 weeks and 1 year, where more patients in the high clearance group developed an increase in $ci + ct \geq 2$ score during the first year post-transplant. The other analysis included patients with kidneys not showing any signs of IFTA at 7 weeks. More than twice as many patients in the highest quartile of tacrolimus clearance developed de novo IFTA during the first post-transplant year compared to the lowest quartile. Of note, there was no association with increasing tacrolimus clearance and the development of $ci + ct \geq 1$ score. Implying that this biomarker is not sensitive enough for predicting small changes in IFTA-score in kidneys already exposed to some degree of IFTA. No association was found between high estimated tacrolimus clearance and development of arteriolar hyalinosis. This finding is contradictory to a previous study by Kuypers et al which found that patients with arteriolar hyalinosis had significantly higher clearance than patients not developing arteriolar hyalinosis.\footnote{Kuypers et al.\textsuperscript{187}} The present data support that arteriolar hyalinosis is not a specific marker for tacrolimus nephrotoxicity.

The mechanisms behind tacrolimus induced nephrotoxicity seem to be complex, and need further elucidation. The increased risk of under immunosuppression in case of nonadherence in high clearance patients may still be present in the period following 7-weeks post-transplant. Such intermittent underexposure may lead to alloimmune activation, which has been shown to drive fibrosis.\footnote{Thölking et al.\textsuperscript{184,185}} Furthermore, the patients with high clearance need higher doses to achieve similar tacrolimus trough concentrations as patients with lower clearance. This may lead to higher tacrolimus peak concentrations ($C_{\text{max}}$) after each dosing. The findings in \textbf{Paper III} cannot discriminate if it is the high peak concentrations and/or transient under-immunosuppressive episodes that drives the IFTA development. Another potential explanation for the present findings is higher levels of tacrolimus metabolites in patients with high clearance. To our knowledge, no studies comparing demethylated metabolite concentrations and histological lesions in transplanted kidneys have been conducted. Zegarska \textit{et al} found a significant negative correlation between the 15-O-demethyl tacrolimus (15-DMT, also named M-III) concentration and estimated glomerular filtration rate, which indirectly may support this hypothesis.\footnote{Zegarska \textit{et al.}}

The patients in the high clearance group in \textbf{Paper III} had lower eGFR at one year compared to the other groups (Figure 4). This is in concordance with the findings of Thölking \textit{et al} who previously have reported an association between high tacrolimus clearance and low eGFR after renal- and liver transplantation.\footnote{Thölking et al.\textsuperscript{184,185}}
Figure 4. Violin plot of estimated glomerular filtration rate (eGFR) at one year post-transplant calculated with MDRD formula. Groups are compared with ANOVA, black dot is the mean of the group, and colored dots are the eGFR for individual patients.

4.3.2 Strengths and limitations of the clearance estimate

It is well known that patients expressing functional CYP3A5 on average need approximately twice the dose to achieve the same trough concentration as a CYP3A5 nonexpressers.\textsuperscript{135} Therefore it is likely a larger proportion of patients expressing CYP3A5 in the high clearance group. A limitation to the findings in Paper II and III is that CYP3A genotype was not assessed. Associations between CYP3A5-expression and incidence of BPAR and various histological lesions has earlier been investigated in renal transplanted patients with inconsistent findings. With regards to BPAR, studies some have found,\textsuperscript{194,195} while others have not found\textsuperscript{196-199} associations between CYP3A expression and acute rejection. The same inconsistency is seen for CYP3A5 expression and nephrotoxicity. Some studies have observed a significant associations,\textsuperscript{187,200} while others have not.\textsuperscript{195,196,198,201} One study even found an association
between the nonexpressing genotype and nephrotoxicity, supporting that other factors most probably also are involved. However, due to the many factors affecting the pharmacokinetic variability of tacrolimus, not all CYP3A5 expressers need as high doses as others to achieve target tacrolimus trough concentrations. The clearance estimate may encompass many factors that affect tacrolimus variability, and thus be more sensitive parameter than CYP3A genotype to assess the overall tacrolimus disposition. This was substantiated in multivariable analyses in both Paper II and III including continuous tacrolimus clearance estimates for all patients. Higher clearance estimate, regardless of initial value, was significantly associated with both BPAR and IFTA development.

The tacrolimus clearance was estimated by doses and several concentrations over a relatively short time-period. For patients experiencing BPAR the first 90 days, the estimate was determined before the initiation of acute rejection therapy. For all other patients, clearance was estimated at eight weeks. This was done because BPAR treatment consisted of pulses of high dose glucocorticoids, which are known to induce CYP3A enzymes, and lead to higher tacrolimus apparent oral clearance. However, patients received high doses of corticosteroids at the time of transplantation which were tapered over time. Nevertheless, it may take days for induction to become clinically evident, and even longer to reach its maximum effect.

It is well established that intrapatient tacrolimus pharmacokinetics may change within the first year post-transplant. There is a progressive decline in the dose needed to achieve target trough concentrations the first six months after transplantation. Most studies investigating intrapatient variability and outcomes estimate the variability from whole-blood concentrations obtained between six months and one year due to this effect. An estimate determined in an early period featuring large changes in pharmacokinetics is not as robust as these estimates from a more stable period. However, an issue with calculating variability at later time-points, is that acute rejections and nephrotoxicity may have already developed. Estimating tacrolimus clearance early after renal transplantation may detect patients at risk for adverse outcomes early after transplantation. It may be beneficial to reassess the clearance estimates over time as a part of the clinical follow-up.
4.4 Belatacept

TDM is a powerful tool to avoid adverse events when dosing immunosuppressive drugs and is applied to all patients in Norway receiving tacrolimus therapy after renal transplantation. It is currently unknown if belatacept may benefit from TDM. In Paper IV an assay to determine belatacept serum concentrations was validated and applied in a pharmacokinetic feasibility study in five renal transplant recipients. Belatacept concentrations determined within a dosing interval has only been presented from 24 renal transplanted adults at steady-state, and 9 renal transplanted adolescents after a single dose. Even though we only were able to include five patients, the study signaled that there might be a larger interpatient variability than previously communicated. Two of the patients had very different body compositions. Both were male and weighed 63 and 109 kg, and the absolute dose was thus almost twofold different. When using weight adjusted dosing it is anticipated that the systemic exposure should be comparable between patients. The heavier patient showed more than twice as high trough level, and almost twofold higher peak concentration than the lighter patient. The nonlinearity between body size and blood volume may explain the higher peak serum concentrations in the heavier patients as shown in this study. We did not report any clinical outcomes from the feasibility study, but the increase in exposure may potentially increase the risk of infection and malignancy in heavier patients. In addition, it should be determined whether lighter patients have an increased risk of rejection due of the lower exposure.

The population pharmacokinetic model indicated that belatacept also had a low central volume of distribution, in line with previously published data on belatacept. The mean half-life of approximately 50 hours observed in this study is shorter than previously reported in non-compartmental analyses. The results from the pharmacokinetic estimates in Paper IV must be interpreted with great care since it is based on data from only five renal transplant recipients, but they underline the importance of performing more pharmacokinetic investigations on belatacept in this population.

4.5 Drug dosing strategies in renal replacement therapy

The results from Paper I shows that there is a large variability in hepatic CYP3A activity in patients with ESRD receiving chronic intermittent hemodialysis. Administering the same dose of a CYP3A substrate may give large differences in unbound exposure, which again may lead to treatment failure or risk of toxicities. When initiating pharmacological therapy with CYP3A
substrates in this population, the patients should start with a low dose to avoid overexposure. Stable drug dosing regimens with most CYP3A substrates do not have to be modified according to dialysis days. However, doses of CYP3A substrates with narrow therapeutic range, such as anticancer- and immunosuppressive drugs, may have to be reduced when closing in on the next dialysis session in selected patients.

After oral dosing of drugs with hepatic metabolism, or intravenous dosing of low extraction ratio drugs, changes in unbound fractions do not alter the unbound exposure of a drug and have minor clinical relevance. However, therapeutic drug monitoring utilizing total concentrations of highly protein bound drugs, such as valproate and phenytoin, is affected by changes in protein binding. Protein binding of other drugs may vary during a hemodialysis week in the same manner as midazolam, and that this should be taken into account when applying therapeutic drug monitoring in patients with ESRD receiving hemodialysis.

Patients identified with high tacrolimus clearance may benefit from switching to extended release formulations. Three main tacrolimus formulations are available on the Norwegian and the international market; immediate-release (IR-tacrolimus, dosed twice daily, e.g. Prograf®), extended-release (ER-tacrolimus, dosed once daily, e.g. Advagraf®), and a novel extended-release (LCP-tacrolimus, dosed once daily, Envarsus®). Switching to a prolonged release formulation may be beneficial since the newer LCP-tacrolimus formulation have shown to have lower peak-trough fluctuation than both IR-tacrolimus and ER-tacrolimus. However, a previous study comparing IR- and ER-tacrolimus did not show any differences in biopsy-findings obtained 14 days and 6–12 months after transplantation. Histologic lesions has, to our knowledge, not yet been compared between patients receiving LCP-tacrolimus and the other tacrolimus formulations.

Belatacept is administered in fixed time-intervals even though the elimination half-life ranges 3 to 15 days. Timing of infusions may thus be adjusted to be more frequent in patients with a rapid elimination, and less frequent in patients with slower elimination. However, this hypothesis must be investigated in future prospective trials.
5 CONCLUSION

Changes in uremic milieu between dialysis sessions induced a small, but statistically significant, direct inhibitory effect on CYP3A activity in patients with ESRD receiving intermittent hemodialysis. The simultaneous change in unbound fraction of midazolam increased the total clearance of midazolam, concealing the inhibitory effect.

High estimated tacrolimus clearance was associated with development of acute rejection the first 90 days after renal transplantation. Elevated tacrolimus clearance was also associated with development of interstitial fibrosis and tubular atrophy in the renal graft the first year after renal transplantation.

A novel assay for belatacept serum concentration determination is applicable for further pharmacokinetic studies and eventual therapeutic drug monitoring in renal transplant recipients. Patients with high and low body-weight seem to have different exposure when weight adjusted belatacept dosing is utilized.
6 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Findings in Paper I showed that stable drug regimens of most CYP3A substrates do not have to be altered during a hemodialysis week. However, caution is warranted for CYP3A substrates with narrow therapeutic range in patients with severe uremia. Future studies should investigate the absolute bioavailability of midazolam in patients with ESRD compared to healthy controls to elucidate if dosing of CYP3A substrates should be adjusted. Trials using highly protein bound probes, such as midazolam, should be encouraged to determine the unbound exposure due to the alterations in protein binding seen in ESRD.

All transplant centers utilizing therapeutic drug monitoring can readily estimate tacrolimus clearance from Paper II and III in individual renal transplant recipients. This may identify patients at increased risk for early adverse events, which may affect long-term outcomes. Extended release formulations may be beneficial for these patients, but this hypothesis must be confirmed future trials.

The BelataSlow study is a randomized, controlled trial with the primary objective to investigate the short term effect belatacept versus tacrolimus on renal function in patients experiencing delayed graft function. The trial was planned to start the winter 2015/2016. Due to initial difficulties with financing, and later shortage of belatacept, the study was stopped in its tracks. It was planned to give a thorough description of belatacept pharmacokinetics from the 33 patients planned to receive belatacept in the study. With the recent lifting of prescribing restrictions in Europe (March 15th 2019), the planning of this trial can restart, with a validated assay available for belatacept serum concentration determination (Paper IV).
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