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Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Metabolomics –
New biomarkers for early detection of immunosuppressive-induced nephrotoxicity and chronic rejection after KTX.
A translational analysis from animal model to kidney transplant patients.

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1.0. Abstract

1.1. Abstract (English)

This dissertation investigated biochemical profiles to develop new metabolic biomarkers for early detection of immunosuppressive nephrotoxicity and chronic rejection.
We established an animal trial with 256 healthy Lewis rats and translated the results in a second step to a prospective clinical trial with 48 de novo kidney transplanted patients.
Lewis rats were randomly assigned to treatment groups (n=4/group). All rats were treated with immunosuppressants administered by oral gavage once daily for 28 days.
The study used a control group and three different doses of a calcineurin and mTOR inhibitor in every possible combination (n=4/ dose combination). After 28 days, blood, urine and tissues were collected for analysis.
For clinical trial, after transplantation, patients were started on standard immunosuppressive drugs consisting of a calcineurin inhibitor, steroids, mycophenolate mofetil (MMF) and an induction therapy (Basiliximab/Simulect ®).
14 ml of blood and 10 ml of urine samples were collected from each patient at fixed time-points before and on days 1, 3, 7 and on months 1, 3, 6, 9 and 12 after transplantation.
Renal allograft biopsies were performed as part of the routinely applied diagnostic workup in the case of graft dysfunction (proteinuria and/or an increase in creatinine).

The aim of this dissertation was to win some results by the translational analysis from animal model to transplant patients for developing new specific biomarkers. The overall hypothesis was that kidney transplant function is reflected by metabolite changes in plasma and urine.

To test this hypothesis, I carried out a translational project to evaluate:

A) Effects induced by immunosuppressants in kidney metabolism in the rat as reflected by changes in urine metabolite patterns.
B) Changes induced in kidney metabolism by different immunosuppressants and their combinations (CyA/Tac in combination with Evrl or Srl).

Furthermore the following questions were asked:

1. Can changes in urine metabolite pattern predict changes in kidney function and kidney injury (immunosuppressant nephrotoxicity and/or rejection) with better sensitivity than

the currently established clinical biomarkers typically used to monitor and manage kidney transplant patients such as GFR, serum creatinine concentrations and histological changes in biopsies?

2) Is it possible to predict the clinical outcome in a transplant patient population by assessing these novel biomarkers?

To answer the hypothesis the animal model showed that
A) Urinary metabolites after 28 days of exposure to immunosuppressants were mainly hippurate, creatinine, glucose, succinate, citrate, alpha ketoglutarate, and trimethyl aminooxide (TMAO) Isoprostanes were not changed after 28 days of exposure.
B)When combined with calcineurin inhibitors, everolimus had a less negative effects on urine metabolite patterns compared to sirolimus.
The histology scores showed mild and not statistifically significant alterations in different immunosuppressant treatment doses.

1) There was no association between drug blood concentrations and biopsy-proven alloimmune or immunosuppressant nephrotoxicity.
As already indicated by the rat studies, isoprostanes in urine and plasma seem to be more of an acute marker rather than a marker for long-term monitoring of renal transplant patients.
2) In a next step the human urine metabolites and their immunosuppressant induced changes should be analyzed and put in relation to the animal study results.

Initial clinical data based on this dissertation suggested in the animal model that these biomarkers are more sensitive and predictive than creatinine in serum.
These biomarkers will now have to be qualified in larger prospective clinical trials for the early detection of immunosuppressant-induced nephrotoxicity.

1.2. Abstrakt (Deutsch)

Die vorliegende Arbeit untersuchte biochemische Profile zur Detektierung metabolischer Biomarker, die zur Früherkennung von immunsuppressionsinduzierter Nephrotoxizität und chronischer Rejektion genutzt werden sollen.

Dazu wurde eine Tierstudie mit gesunden Lewis-Ratten etabliert und dessen Ergebnisse in einem zweiten Schritt in einer prospektiven klinischen Studie mit 48 de novo nierentransplantierten Patienten auf den Menschen übertragen.

256 Lewis Ratten wurden willkürlich einer Behandlungsgruppe zugeordnet (n=4). Alle Ratten wurden oral mit Immunsuppressiva einmal am Tag für 28 Tage behandelt. Die Studie benutzte drei verschiedene Dosen der Calcineurin- und mTor-Inhibitoren in jeder möglichen Kombination (n=4/Dosiskombinationen) sowie eine Kontrollgruppe. Nach 28 Tagen wurde Blut, Urin und Gewebe für die Analysen gesammelt.

Für die klinische Studie wurde nach der Transplantation mit der standardisierten Immunsuppressionsgabe begonnen, in Form von einem Calcineurininhibitor, Steroiden, MMF und einer zusätzlichen Induktionstherapie (Basiliximab/Simulect ®).

14ml Blut und 10ml Urin wurden von jedem Patienten an bestimmten Fixpunkten gesammelt: Vor und am Tag der Transplantation, an Tag 1,3,7 sowie in den Monaten 1,3,6,9 und 12 nach der Transplantation. Nierenbiopsien wurden als Teil der Routinediagnostik im Fall der Transplantatdysfunktion (Proteinurie und/oder Kreatininanstieg) durchgeführt.

Das Ziel meiner Dissertation war es durch die Translationsanalyse vom Tier auf den Menschen Erkenntnisse zu gewinnen, die bei der Entwicklung neuer spezifischer Biomarker eine fruchtbare Rolle spielen.

Es wurde die These aufgestellt, dass die Nierentransplantatfunktion bei Mensch und Tier durch spezifische metabolische Veränderungen im Plasma und im Urin zu erkennen sind.

Diese Hypothese wurde anhand des Tiermodels und der klinischen Studie getestet, um

A) die Wirkung der Immunsuppressiva auf den Nierenstoffwechsel der Ratte durch die Veränderungen in den Urinstoffwechselmustern sichtbar zu machen.
B) Unterschiede in den Veränderungen im Nierenstoffwechsel aufzuzeigen, die in Relation zu den verschiedenen Kombinationsmöglichkeiten der Immunsuppressiva stehen (CyA und Tac in Kombination mit Evrl oder Srl).

Außerdem sollten folgende Fragen beantwortet werden:

1) Können die Veränderungen in den Metabolitmustern im Urin herangezogen werden, um die Nierenfunktion bei Nierenerkrankungen (immunsuppressive Nephrotoxizität und/oder Rejektion) mit besserer Sensitivität aufzuzeigen, als die derzeitig etablierten Marker, wie GFR, Serumkreatinin und histologische Biopsie?
2) Ist es möglich durch diese neuen Biomarker das klinische Outcome einer nierentransplantierten Patientenpopulation vorauszusagen?

Aus dem Tierversuch ließ sich folgendes zur Beantwortung der Hypothese ableiten:

A) Die Urinmetabolite, die sich nach 28 Tagen durch die Exposition mit Immunsuppressiva veränderten, waren : Hippurat, Kreatinin, Glukose, Succinat, Citrat, Alpha- Ketoglutarat und TMAO.
Die Isoprostane zeigten sich unverändert nach der Exposition über 28 Tage.

B) In der Kombination mit den Calcineurininhibitoren hatte Everolimus einen deutlich weniger toxischen Effekt als Sirolimus.
Die Histologien zeigten milde und nicht statistisch signifikante Veränderungen in den unterschiedlichen Behandlungsdosen.

1) Die Ergebnisse der klinischen Studie erlaubten keinen signifikanten Rückschluss auf eine Assoziation zwischen den Blutspiegeln und der durch Biopsie geprüften Nephrotoxizität.
Die Isoprostane scheinen aufgrund ihrer vermuteten Anpassungsfähigkeit, wie schon im Tiermodell angedeutet, mehr ein akuter Marker, als für ein Langzeitmonitoring von Nierentransplantierten geeignet zu sein.
2) In einem weiteren Schritt sollten die kompletten humanen Urinmetabolite und deren Veränderung durch die Immunsuppression untersucht und in Relation zur Tierstudie gestellt werden.
Die in einem ersten Versuch in dieser Dissertation durchgeführte Translationsanalyse zeigte im Tiermodell, dass die neuen Biomarker sensitiver und spezifischer sind, als der derzeitige Goldstandard Kreatinin. Die Biomarker sollten nun in größeren prospektiven klinischen Studien weiter untersucht werden um eine bessere Möglichkeit zur Früherkennung von immunsuppressionsinduzierter Nephrotoxizität beim Menschen zu erlangen.

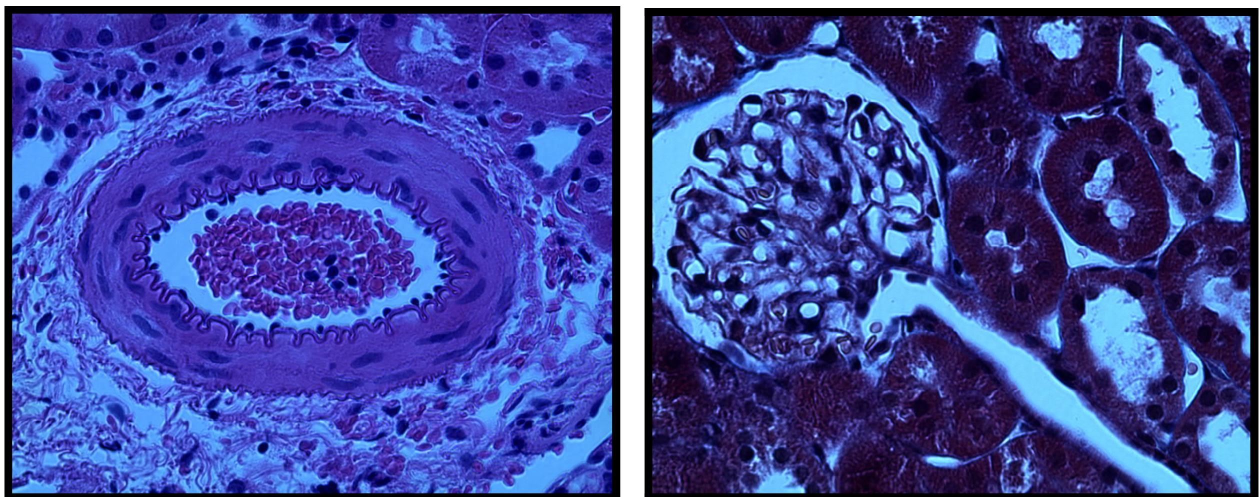
2. Introduction

The first successful kidney transplantation was performed more than 50 years ago (1).

In the intervening period, kidney transplantation has become the most successful and widespread form of organ transplantation performed today. However, without carefully controlled immunosuppression, this life-saving and life-transforming surgery would not be possible.

Over the past three decades, immunosuppressants, in particular calcineurin inhibitors such as cyclosporine and tacrolimus have been successful in dramatically decreasing acute rejection episodes (2, 3).

Although one-year survival has markedly increased over the last 30 years and has achieved a rate of approximately 95% in most transplant centers, there has been less progress in terms of long-term graft survival after kidney transplantation (4). One reason is the toxicity of calcineurin inhibitors. In addition to other negative effects, they are nephro- and neurotoxic and thus damage the very organs they are supposed to protect (3, 5-7). This has always been recognized, but was tolerated due to cyclosporine's effective ability to improve short-term outcomes after transplantation (3).



Figures 1A and B. *10x40-fold magnification of a healthy vessel (1A, TC stain) and healthy glomerulus (1B, HE stain) of a kidney by light microscope, N.Brunner 2008.*

One strategy to reduce the side effects of calcineurin inhibitors is to decrease their dose and to combine them with other immunosuppressive drugs such as sirolimus or mycophenolate mofetil (2, 8-13).

In sirolimus (rapamycin, Rapa), a mammalian target of rapamycin (mTOR) inhibitor, an almost equally potent immunosuppressant that lacks the most serious side effects of calcineurin inhibitors, has become available. (5, 14)

The combination of sirolimus or everolimus with cyclosporine or tacrolimus is attractive since it results in synergistic immunosuppressive activity and allows for decreasing doses the two combination partners (2, 15).

Also, the combination of calcineurin inhibitors with mycophenolate mofetil, the morpholine ethylester prodrug of mycophenolic acid, is commonly used and allows for a marked reduction of exposure to the nephrotoxic calcineurin inhibitors. (16)

Other important factors contributing to the development of nephropathy are acute and chronic rejections (17). This creates a dilemma since immunosuppressant-mediated nephrotoxicity and rejection require opposite clinical measures: rejection indicates the need for an increase while nephrotoxicity requires a reduction of the doses of immunosuppressive drugs (13, 18, 19).

2.1. Theoretical and Practical Background

Today, clinical diagnosis of acute kidney injury continues to be primarily based on an elevation of serum creatinine and the detection of oliguria. A kidney biopsy remains the gold standard to obtain an accurate histological assessment of the pathology behind the deteriorating kidney function. The established clinical markers not only lack sensitivity and describe events already defining a symptomatic disease process usually associated with irreversible kidney damage, but they also lack specificity and in many cases are unable to differentiate whether immunosuppressant nephrotoxicity or chronic rejection are the primary cause of kidney dysfunction observed in a patient.

"Serum creatinine is a poor marker of early renal dysfunction, because serum concentration is greatly influenced by numerous non-renal factors (such as body weight, race, age, gender, total body volume, drugs, muscle metabolism, and protein intake)"(20) Increases in serum creatinine concentrations can be delayed by up to several days after the injury occurs (20). By the time creatinine serum levels increase by 20%, a threshold considered clinically significant, often 50% of the kidney function is already lost (21). Therefore, interventions may occur too late. Significant renal disease can exist with minimal or no change in creatinine because of renal reserve, enhanced tubular secretion of creatinine, or other factors (4) .

Pharmacokinetic therapeutic drug monitoring, routinely implemented after transplantation, utilizes the concentrations of immunosuppressants as a surrogate marker for drug activity. The

therapeutic target concentrations vary among transplant centers and have been validated to avoid acute rejection and acute immunosuppressant toxicity (4).

A huge problem is that pharmacokinetic drug monitoring usually does not take into account genetic inter-individual variability, additional diseases and additive or synergistic pharmacodynamic drug-drug interactions, and as current experience shows, it has failed to prevent chronic long-term toxicity.

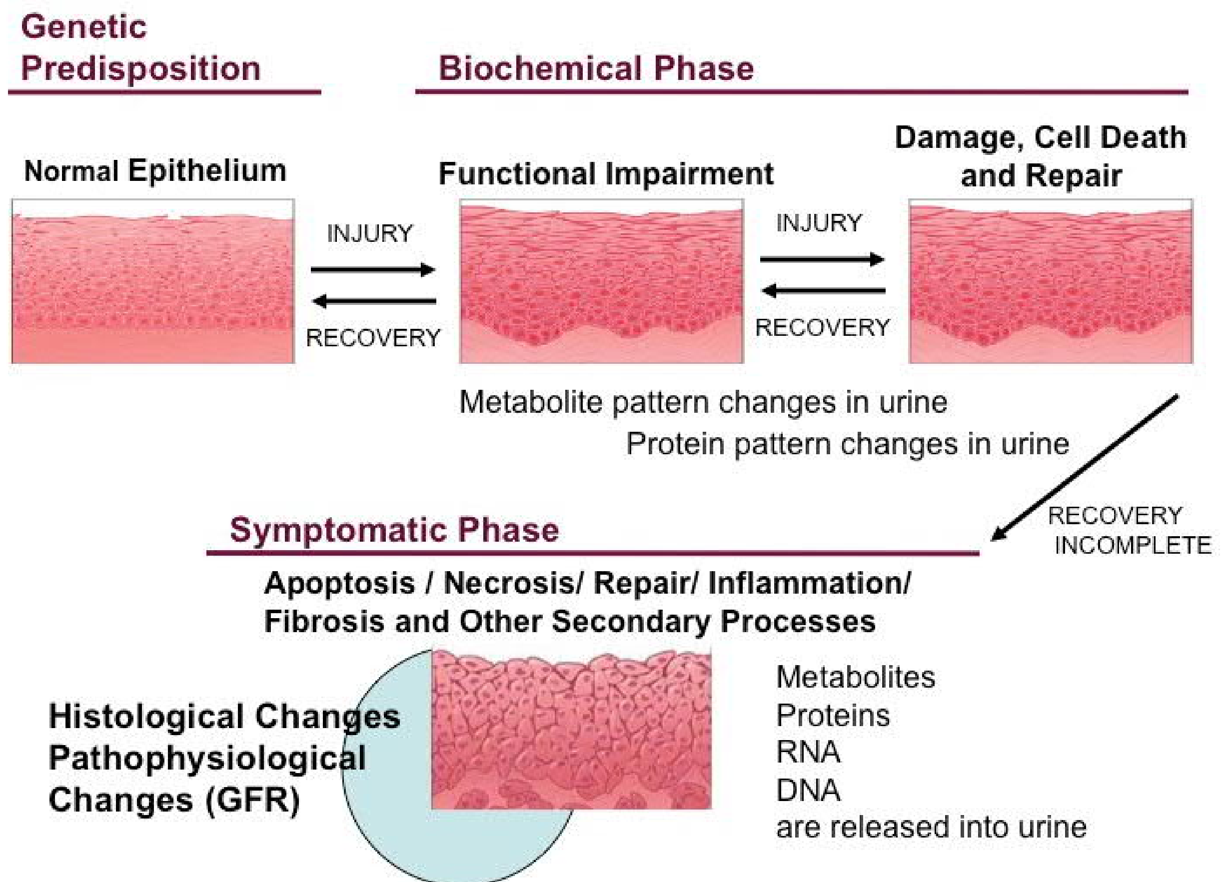


Figure 2. *"Time-dependency of kidney tubular epithelium injury and molecular markers in urine" (22).*

Thus, the key to reduce in damage to a transplant kidney caused by immunosuppressants is early detection. Modern screening technologies in the fields of genetics/ functional genomics, protein profiling (proteomics) and especially biochemical profiling (metabolomics) have created new opportunities for the development of more sensitive and specific diagnostic tools (4).

Metabolic profiling seems to be a good strategy since all organs are directly or indirectly in contact with biofluids. It is reasonable to expect that changes in cell biochemistry are ultimately reflected in the protein and metabolite patterns of (easily accessible) biofluids such as plasma

and urine. Such monitoring of cell metabolites in biofluids is an attractive concept since changes in cell biochemistry are ultimately responsible for histological and pathophysiological changes of the transplanted kidney (13, 17, 18). Furthermore, biochemical changes usually precede detectable histological and pathophysiological changes. Another advantage of monitoring metabolite or protein patterns in plasma or urine is that it is a less invasive procedure than procuring biopsy samples.

A biomarker should be defined as a characteristic that is objectively measured (Figure 2) and evaluated to indicate normal biological processes, pathogenic processes or pharmacological response to therapeutic intervention (23). A more realistic and targeted approach than a single biomarker, is the development of combinatorial biomarkers (22). Such biomarker patterns typically consist of five or more individual parameters. Combinatorial biomarker patterns provide significantly more information than a single measurement and thus can be expected to have better specificity and sensitivity. While, for example, creatinine concentrations in serum need to increase by 20% before being considered clinically significant , signals in a pattern demonstrating certain small changes may be sufficient for drawing reliable conclusions (4).

We are still in an exploratory phase for such concept, non-targeted analytical screening and discovery strategies such as ^{1}H-nuclear magnetic resonance spectroscopy (NMR) or high-performance liquid chromatography- mass spectrometry (HPLC/MS) to detect biochemical patterns. (24). These analytical technologies however, are not usually available in routine clinical laboratories. This is one reason why serum creatinine changes and immunosuppressive drug concentration monitoring are still the gold standard for monitoring kidney transplant patients. However, a series of studies in rats and healthy individuals evaluating the effects of immunosuppressants on urine metabolite pattern showed that immunosuppressant-induced changes of metabolite patterns in urine were associated with a combination of changes in glomerular filtration, changes in secretion/ absorption by tubule cells and changes in kidney cell metabolism (4). These studies suggested that because urine metabolites constitute valid surrogate markers of these kidney functions, they should be included in the combinatorial biomarker used for toxicodynamic therapeutic drug monitoring of immunosuppressants (4, 23, 25).

The development of a disease process can be divided into three stages: the genetic, the biochemical and the symptomatic stage (4).

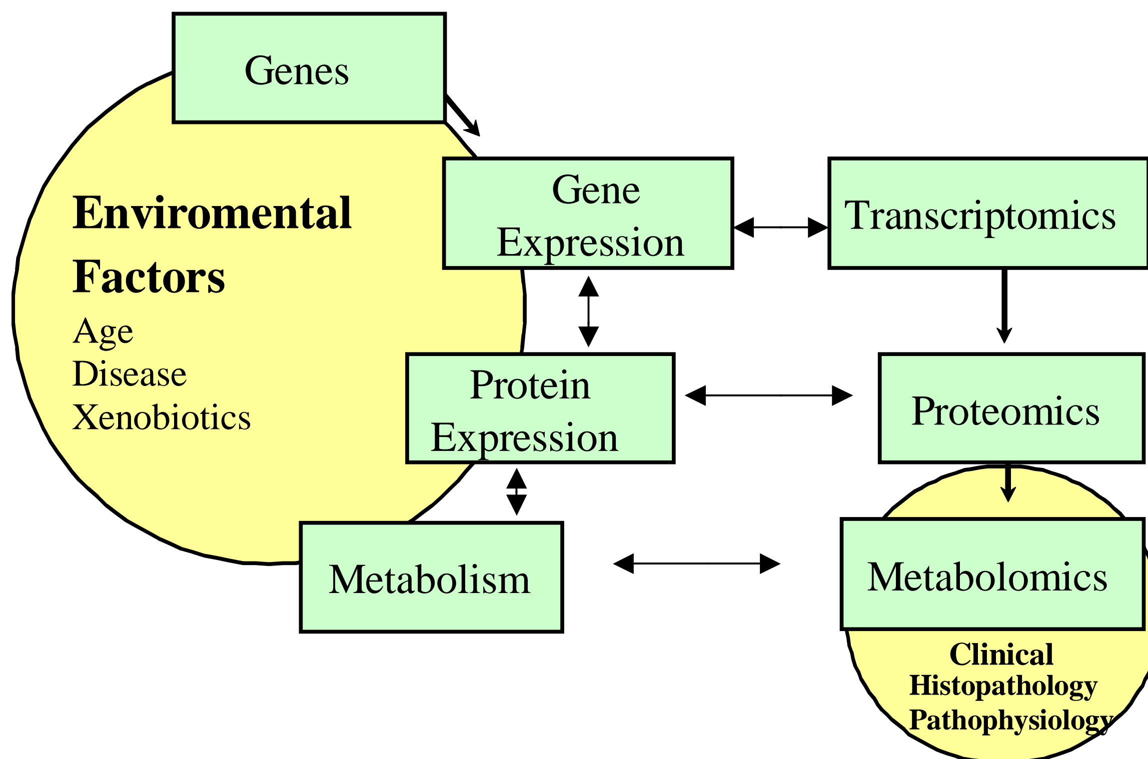


Figure 3. *Overview off different fields of so-called "omics" technologies (22).*

The genetic risk can be evaluated by assessing the presence of certain gene combinations, variants or polymorphisms that are known to be associated with the risk of developing a certain disease, or toxicodynamic response to drug exposure. Biochemical profiling or metabolomics seems to be a very attractive concept since ultimately, biochemical changes of the cells will cause the histopathological and pathophysiological changes that move a disease forward into the symptomatic stage.

However, the first phase, preceding everything, is the genetic stage. At this point, an individual bears a certain risk for developing a disease or experiencing a specific drug effect due to a genetic predisposition. However, in most cases the disease or drug reaction will require exposure to a disease agent, environmental factors or drug exposure. Once this has occurred, the disease process moves into the second, the biochemical stage. Changes in gene expression, protein expression and biochemical profiles occur during this phase, but the cells and organs are still able to compensate. In this stage, the disease process could be detectable if sufficiently sensitive

assays were available. During the biochemical phase, no notable histological damage has occurred yet and the disease process may also be fully reversible if an appropriate therapeutic intervention is available. Once the third, the symptomatic stage is reached, biochemical changes of a cellular, organ or on a systemic level can no longer be compensated and will lead to the pathophysiological and histological changes that define the clinical symptoms of a specific disease process (Figure 3) (4).

"Metabonomic approach is complementary to other profiling technologies such as proteomics and genomics, and can provide a chemical or biochemical profile of a specific body fluid, organ or tissue during a continuous time-course analysis . Overall, the determination of metabolic profiles and the mapping of interactions between metabolic pathways across complete systems." (26, 27) As mentioned above, clinical biomarkers currently used for the management of kidney transplant patients such as creatinine in serum, only assist in the diagnosis of a disease process once it has already reached the symptomatic stage.

Genomics, proteomics and biochemical profiling have the potential for the development of new diagnostic tools that allow for monitoring early changes in cell signal transduction, regulation and biochemistry with high sensitivity and specificity (28). This would allow for the detection of disease processes at a much earlier stage when the chances of a full reversal are still much better than is possible with the clinical biomarkers currently in use. An ideal biomarker for acute kidney injury may help transplant physicians and scientists during drug development not only to detect kidney dysfunction in its early stage with high sensitivity, but also to differentiate chronic immunosuppressant toxicity from chronic rejection of a kidney transplant, or other kidney diseases. Such a marker could be used to risk-stratify patients with acute kidney injury by predicting the need for renal replacement therapy, the duration of acute kidney injury, the length of hospital stay and may predict mortality.

Inflammatory, renal tubular proteins that are excreted into the urine after injury and surrogate markers of tubular injury (1, 5, 29). As mentioned above, the use of easily available body fluids such as plasma and urine for biomarker analysis can be considered ideal. It is reasonable to assume that, to a certain extent, biochemical and protein changes are reflected in body fluids. Cells either directly or indirectly communicate with body fluids. Also, cell metabolites, peptides and proteins will be released by the cells via normal excretion, trans-membrane diffusion or transport after cell death (30). It was shown in a rat model that exposure to calcineurin inhibitors for 28 days increases concentrations of lactate and alpha-glucose in urine and decreases urine concentrations of the Krebs cycle intermediates succinate, citrate, and 2-oxo-glutarate as well as concentrations of creatinine hippurate and urea (25, 31). Just like a bar code contains more

information than a single number, the metabolic profiling technologies allowing for the identification of patterns, provide significantly more information than the measurement of a single parameter (4).

2.1.1. Immunosuppressants

2.1.1.1. Tacrolimus

Tacrolimus (FK506, Figure 4) was first discovered in 1984 as a product of a fungus, named Streptomyces tsukubaensi. Its immunosuppressive activity in animal transplantation models and in vitro was first published in 1987 (32). Tacrolimus was originally developed and approved as immunosuppressant to be used after liver transplantation (33), followed by transplantation of other solid organs such as kidney, heart (34, 35), small bowel, pancreas, lung and trachea. In addition, it was used recently for the treatment of atopic dermatitis, collagen-induced arthritis and graft versus host disease (36, 37).

2.1.1.2. Cyclosporine

Cyclosporine, a lipophilic cyclic endecapeptide (Figure 5), was originally isolated from a filamentous fungus, named Tolypocladium inflatum. Its specific anti-T lymphocyte activity was already described in 1976 (38).

Administration of cyclosporine led to impressive results in animal models of transplantation and was followed by first studies in human kidneys and bone marrow.

It was approved by the United States Federal Drug Administration (FDA) in 1983 for treatment and prevention of transplant rejection. Currently, cyclosporine is used for prevention of graft rejection in liver, kidney, heart, lung, bone marrow and combined heart/lung transplantation (39-42). It is also approved for the prevention of graft-versus-host disease as well as in atopic dermatitis, psoriasis, rheumatoid arthritis and other autoimmune diseases (43-46).

2.1.1.3. Sirolimus

The macrolide sirolimus, also known as rapamycin, was first discovered as a product of the bacterium Streptomyces hygroscopicus in a soil sample from Easter Island (an island also known as "Rapa Nui", hence the name rapamycin) (47). Originally, sirolimus was developed as an anti-fungal agent but development was discontinued due to its immunosuppressive properties (Figure 6A).

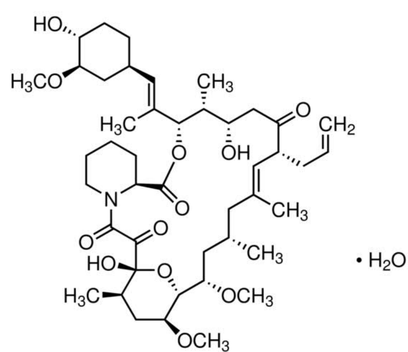
In the 1990s, sirolimus was rediscovered, at which time it was developed as an immunosuppressant used after solid organ transplantation, and was approved in 1999 under the brand name Rapamune as an anti-rejection drug in kidney transplants (48, 49). During its testing as a transplant rejection drug, anti-tumor effects were also observed (50). Rapamune is indicated for the prophylaxis of organ rejection in patients receiving renal transplants (51).Studies in experimental models show that sirolimus prolongs allograft kidney, heart, skin, small bowel, pancreatic-duodenal, and bone marrow survival in primates and other animals (52-55). Furthermore, sirolimus suppresses immune-mediated events associated with lupus erythematodes, collagen-induced arthritis, autoimmune type-1 diabetes, autoimmune myocarditis and graft-versus-host-disease (56-58).

2.1.1.4. Everolimus

Everolimus, a rapamycin derivative and mTOR inhibitor, was developed by introducing a hydroxyl group into rapamycin to improve its pharmacological properties (Figure 6B). Preclinical studies have shown that cyclosporine and everolimus exert synergistic immunosuppressive effects on the inhibition of lymphocyte proliferation and the prevention of allograft rejection (59, 60). In animals, rapamycin and its analog everolimus have been shown to prevent vascular smooth-muscle cell proliferation and intimal thickening, which occurs during the development of graft arterial sclerosis (61-63).

2.2.1.5. Mycophenolic acid

Mycophenolic acid (Figure 7) is derived from the fungus Penicillium stoloniferum (64). To improve oral bioavailability, it was initially marketed as the prodrug mycophenolate mofetil . Mycophenolic acid is commonly marketed under the trade names CellCept® and Myfortic®, available for oral administration. The incorporation of MMF into immunosuppressive drug regimens has been associated with decreased rates of acute rejection and chronic allograft loss (65-67).



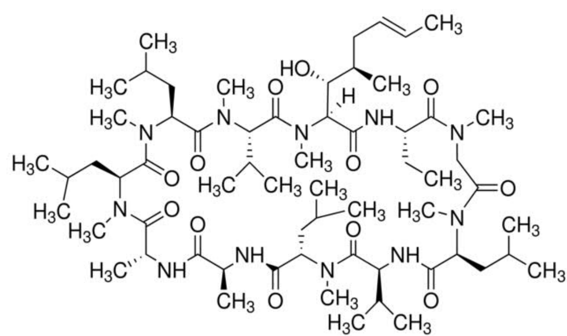
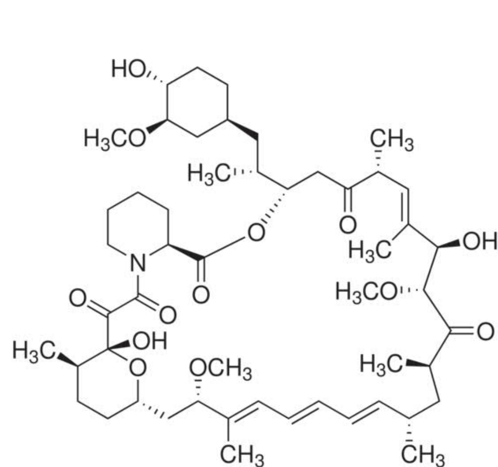
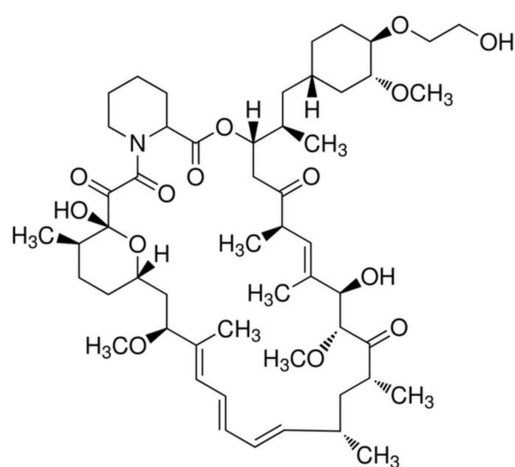


Figure 4. *Structure of tacrolimus (68).*

Figure 5. *Structure of cyclosporine (68).*



6A)



6B)

Figure 6. A. and B. *Structure of sirolimus (A) and everolimus (B) (68).*

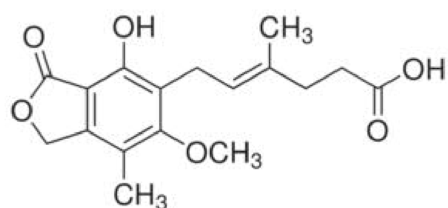


Figure 7. Mycophenolic acid (68).

2.1.2. Mechanisms of Immunosuppressants

The immunosuppressive mechanisms of tacrolimus and cyclosporine are similar except that tacrolimus binds to another family of immunophilins, the FK-binding proteins (FKBP), mainly FKBP-12, while in lymphocytes cyclosporine binds to cyclophilin, most importantly cyclophilin A. The cyclosporine/cyclophilin and the tacrolimus/FKBP complex inhibits calcineurin and the dephosphorylation of NFAT.

Sirolimus also binds to FKBP-12, and the 3-dimensional structure of the sirolimus-FKBP12 is in part similar to the tacrolimus-FKBP12 structure (69), however, the sirolimus-FKBP12 complex inhibits mTOR, and thus cytokine-driven T-cell proliferation from the G_1 to the S phase of the cell cycle. As shown in in-vitro and animal studies (70-72), sirolimus synergistically enhances cyclosporine as well as tacrolimus immunosuppressive activity. This was confirmed in various clinical studies (21, 73, 74). Everolimus is a rapamycin derivative and works similarly to rapamycin as an mTOR inhibitor (75). Everolimus and sirolimus do not prevent IL-2-mediated lymphocyte proliferation by blocking the synthesis of IL-2. Instead, everolimus and sirolimus block IL-2-receptor (CD25)-dependent signal transduction in activated lymphocytes, which results in an arrest of the cell cycle in the early G1 phase (76).

MPA is a potent reversible inhibitor of inosine monophosphate dehydrogenase, the enzyme that controls the de novo synthesis of guanosine nucleotides necessary for the proliferation of B and T lymphocytes and the synthesis of specific messenger RNA in the transition from G_0 to G_1 (64).

2.2. Kidney Dysfunction & Immunosuppressants

Renal failure is a rapid loss of renal function, which is characterized by oliguria or anuria, leading to accumulation of substances (77). The acute form wich can be caused by various factors, is reversible. Acute renal failure is classified as prerenal, intrarenal, and postrenal failure. Prerenal failure is caused by a hypovolaemic problem that manifests as decreased blood flow to the kidneys. This occurs with acute drop of blood pressure, myocardial infarction or high blood loss. Intra-renal failure results from injury to the glomeruli and tubules of the kidney. Common causes are glomerulonephritis, pyelonephritis, and tubular damage caused by drugs, heavy metals, and viral infection. Post renal failure is caused by obstruction in the urinary tract below the kidneys, which can occur as a result of urinary tract stones, tumors, and anatomic obstruction due to benign prostatic hypertrophy (77). Chronic renal failure which is a slow process that may follow repeated episodes of acute renal failure, is irreversible and may often result from acute glomerulonephritis or pyelonephritis. Other reasons for chronic renal failure are diabetes

mellitus, hypertension, kidney stones, atherosclerosis of the renal blood vessels, polycystic kidney disease and chronic exposure to nephrotoxic drugs (77).

2.3. Nephrotoxicity and Drug Development

Metabolomic approaches are useful to:

- identify the target organ or region of toxicity.
- identify the biochemical mechanism contributing to toxicity.
- identify molecular marker profiles of nephrotoxicity in plasma and urine.
- monitor the time course of nephrotoxicity, its dose-dependency and its recovery.

Following metabolite signatures in urine have been associated with injury to specific regions of the kidney:

- Proximal straight tubules: increase of lactate, phenylalanine, tryptophan, tyrosine and valin,.
- Proximal convolute tubules: increase of glucose; reduction of trimethylamine-N-oxide, xanthurenic acid and kynurenic acid.
- Cortical injury: increased glucose, alanine, valine, lactate and hippurate and decreased citrate, succinate and oxoglutarate,.
- Papilla and medulla: increase of glutaric acid, creatine, and adipic acid; reduction of citrate, succinate, oxoglutarate and trimethylamine-N-oxide. " (28)

3. Hypotheses and Aims of the Study

The aim of this dissertation was to win some results by the translational analysis from animal model to transplant patients for developing new specific biomarkers. The overall hypothesis was that kidney transplant function is reflected by metabolite changes in plasma and urine.

To test this hypothesis, I carried out a translational project to evaluate:

A) Effects induced by immunosuppressants in kidney metabolism in the rat as reflected by changes in urine metabolite patterns.
B) Changes induced in kidney metabolism by different immunosuppressants and their combinations (CyA/Tac in combination with Evrl or Srl).

Furthermore the following questions were asked:

1) Can changes in urine metabolite pattern predict changes in kidney function and kidney injury (immunosuppressant nephrotoxicity and/or rejection) with better sensitivity than the currently established clinical biomarkers typically used to monitor and manage kidney transplant patients such as GFR, serum creatinine concentrations and histological changes in biopsies?
2) Is it possible to predict the clinical outcome in a transplant patient population by assessing these novel biomarkers?

4. Materials and Methods

4.1. Materials and Equipment

- *Electric tissue homogenizer (Fisher Scientific, Pittsburgh, USA)*
- *NMR-tubes; Norell S 600 (Fisher Scientific, Pittsburgh, USA)*
- *HPLC-vials (Agilent Technologies, Santa Clara, USA)*
- *Ultrasonic bath (Aquasonic, VWR Scientific Products, Aurora, USA)*
- *Freeze dry system (Labconco Cooperation, Kansas City, USA)*
- *Centrifuge (Beckman Coulter, Palo Alto, USA)*
- *Speedvac (Labconco Corporation, Kansas City, USA)*
- *Vortexer (VWR International, West Chester, USA)*
- *Light microscope with cam (Leica, Wetzler, Germany)*
- *PH-electrode with 3-point calibration (Sigma Aldrich Chemie GmbH, Steinheim, Germany)*
- *API 5000 LC /MS/MS, (ABSciex, Foster City, USA)*
- *API 4000 LC /MS/MS, (ABSciex, Foster City, USA)*
- *HPLC (1100 series, Agilent Technologies, Palo Alto, USA)*
- *Binary pump (1100 Series, Agilent Technologies, Palo Alto, USA)*
- *Amix software package version 3.5.1 (Bruker, Germany)*
- *Topspin software package (Bruker BioSpin, version 1.3.b.17, Germany)*
- *Microsoft® Word 2002 (Microsoft Corp, Redding, USA)*
- *Microsoft® Excel 2002 (Microsoft Corp, Redding, USA)*
- *SPSS 16.0 (SPSS Inc., Chicago, USA)*
- *Analyst QS Software 1.4.2 (ABSciex, Foster City, USA)*

4.2. Chemicals and Substances

- *Chloroform; 99,8% (Sigma- Aldrich, Milwaukee, USA)*
- *Methanol (Fisher Scientific, Pittsburgh, USA)*
- *Internal Standard: 8-iso-prostaglandin-F2a (Toronto Research Chemicals Inc., North York, Canada)*
- *Cyclosporine D (250µg/L, Novartis Pharma AG, Basel, Switzerland)*
- *Cyclosporine A (250µg/L, Novartis Pharma AG, Basel, Switzerland)*
- *Neoral® (Novartis Pharma AG, Basel, Switzerland)*
- *ABT-578 (Toronto Research Chemicals Inc., North York, Canada)*
- *MMF internal standard (Toronto Research Chemicals Inc., North York, Canada)*
- *Mycophenolic acid (MPA, Toronto Research Chemicals Inc., North York, Canada)*
- *Sirolimus (Sigma-Aldrich, Milwaukee, USA)*
- *Tacrolimus (Sigma-Aldrich, Milwaukee, USA)*
- *Prograf® (Astellas, Deerfield, USA)*
- *Sirolimus (Rapamune, Wyeth-Ayerst, Princeton, USA)*
- *Everolimus (Certican, Novartis Pharma, Basel, Switzerland)*
- *Bidest Water (HPLC Grade, Fisher Scientific, Pittsburgh, USA)*
- *Deuterated water (Cambridge Isotope Laboratories Inc., Andover, USA)*

4.3. Animal Study

All animal protocols were approved by the University of Colorado Institutional Animal Care and Use Committee. Rats were housed and maintained in the University of Colorado Center for Laboratory Animal Care in accordance with the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals [60000406(07)1F, date of approval 7/14/2006].

A nephrotoxicity model for immunosuppressant toxicity in rats was established and qualified at the University of Colorado in Denver, Department of Anesthesiology (78). This model was successfully used to assess and compare the effects of cyclosporine, tacrolimus, everolimus and sirolimus as well as combinations of the calcineurin inhibitors with sirolimus or everolimus on kidney function. Animals were treated with different study drugs for 28 days (oral dosing, once daily) and the effect of the study drugs on cell metabolism in kidneys was monitored using a well established method of metabolic profiling in urine and blood (Appendix,Table A).

It is important to note that there is a lower oral bioavailability of these drugs in the rat than in humans and that the doses applied resulted in systemic exposure within the same range as that observed in transplant patients (78).

4.3.1. Animal Transplant Model for the Evaluation of Drug Nephrotoxicity

The study was based on healthy Lewis rats that were fed with a normal diet.

The model was established in the workgroup at the University of Colorado in Denver for the study of ischemia reperfusion injury, and acute/chronic rejection following allograft kidney transplantation (31). All animals were housed in cages in a temperature- and light-controlled environment with free access to tap water and food.

All animal experiments and dosing were performed following at least two weeks of acclimatization to local environment and Denver altitude.

4.3.2. Treatment and Treatment Groups

256 male Lewis rats were randomly assigned to treatment groups (n=4/group). All rats were treated with immunosuppressants administered by oral gavage once daily for 28 days.

If animals were treated with combinations of CIs (cyclosporine or tacrolimus) and mTOR inhibitors (sirolimus or everolimus), both drugs were administered simultaneously.

Commercial oral formulations of tacrolimus (Prograf®), cyclosporine, sirolimus (Rapamune®) and everolimus (Certican®) were administered in a constant volume by oral gavage after previous dilution in skim milk.

The study used a checkerboard-dosing scheme (Table 1), combining 0 (control) and 3 different doses of a calcineurin and mTOR inhibitor in every possible combination (n=4/ dose combination). The following doses were combined: cyclosporine: 3, 6, 10mg/kg/d; tacrolimus, sirolimus and everolimus: 0.5, 1.5, and 3mg/kg/d.

Animals were euthanized after 28 days and blood, urine and tissues were collected for analysis. In addition to kidney histology, glomerular filtration rates (FITC inulin method), measurement of blood, urine and kidney tissue concentrations of the immunosuppressants and their metabolites, blood and urine metabolite patterns were assessed using a combined ^{1}H-MRS/ GC-MS metabolic profiling strategy.

Cs [mg/kg] \ Sirolimus [mg/kg]	0	0.5	1.5	3
0	4	4	4	4
3	4	4	4	4
6	4	4	4	4
10	4	4	4	4

Cs [mg/kg] \ Everolimus [mg/kg]	0	0.5	1.5	3
0	4	4	4	4
3	4	4	4	4
6	4	4	4	4
10	4	4	4	4

Tac [mg/kg] \ Sirolimus [mg/kg]	0	0.5	1.5	3
0	4	4	4	4
0.5	4	4	4	4
1.5	4	4	4	4
3	4	4	4	4

Tac [mg/kg] \ Everolimus [mg/kg]	0	0.5	1.5	3
0	4	4	4	4
0.5	4	4	4	4
1.5	4	4	4	4
3	4	4	4	4

Table 1. *Dosing scheme for the rat study to compare the effects of immunosuppressants alone and in combination. [Tac: tacrolimus, Cs: cyclosporine; drug treatment in mg/kg body weight for 28 consecutive days, n:numbers of animals.].*

4.3.3. Histopathological Analysis

Harvesting of kidneys followed a standardized protocol also routinely established at the University of Colorado Denver, USA (31).

For hematoxylin/eosin (H.E.) and periodic-acid Schiff (PAS), kidney tissue samples were fixed in 10% buffered formaldehyde and embedded in paraffin. Histological sections were cut at 3 to 4 microns. Then, slides were incubated for 5 minutes in hematoxylin solution and for 60 seconds in eosin solution for H.E. staining. PAS stainings were incubated with periodic acid Schiff reagent.

“Sections were washed with plain water, differentiated in 1% hydrochloric acid (HCl) + 50% ethanol, and stain intensity was optimized in ammonia water. Finally, sections were rinsed in 70% ethyl alcohol and dehydrated in xylene solution”. (31)

For trichrome (TC) staining, kidney tissue samples were fixed in 10% buffered formaldehyde and embedded in paraffin and incubated 60 seconds with TC-staining solution.

Tissue sections were washed with plain water and differentiated in 0.5% acetic acid. Evaluation of kidney histology was carried out in a blinded manner using a modified semi-quantitative scoring system.

Histologies were graded in regard to their tubular epithelial aspects, glomerular and vascular alterations according to modified criteria after Banff classification (Table 2) (79, 80).

PAS and silver stains enhance the identification of glomerulitis and tubulitis. These stains also enhance the detection of chronic morphological changes such as arteriolar hyaline and increased mesangial matrix. The trichrome stain was also useful in defining interstitial fibrosis.

Histologic grading scores were expressed as median and range.

In 20 randomly selected non-overlapping fields per rat after H.E. and PAS stains, tubular injury (TI) was graded (0 to 3) based on the presence of tubular atrophy (=interstitional widening) and presence of isometric tubular vacuolization as followed: 0 = no changes present; grade 1: 1 to 25% ; grade 2: 26 % to 50 % and grade 3: > 50% TI involvement.

Interstitial fibrosis (IF) was scored as a sign of architectural destruction based on TC stains: 0: no changes present, grade 1: 1 to 25%; grade 2: 26 % to 50 % and grade 3: > 50% tubular injury involvement.

Glomerular injury (GI) was graded 0-3 for sclerosis (n= 5) and mesangial matrix expansion (n= 5) as a marker for glomerular ischemia and damage.

Renal arterioles were evaluated with respect to the presence of hyalinosis or sclerosis (n=6). Grade 0: no arteriolar changes; Mild-moderate (grade 1): 1 arteriole affected; moderate-severe (grade 2): 1-2 arterioles affected; severe (grade 3): more than 2 arterioles affected.

Digitalized images ranging from 10x5 to 10x100 (with oil immersion) magnification were acquired using a light microscope fitted with a video camera.

HISTOLOGICAL FEATURE	EXTENT OF CHANGE	SCORE
% Glomerulosclerosis	0%; 1-25%; 26-50%; >50%	0-3
Mesangial matrix expansion	0%; 1-25%; 26-50%; >50%	0-3
Isometric tubular vacuolization	0%; 1-25%; 26-50%; >50%	0-3
Tubular atrophy	0%; 1-25%; 26-50%; >50%	0-3
Interstitial fibrosis	0%; 1-25%; 26-50%; >50%	0-3
Arteriolar hyaline expression	0%; mild-moderate (1 arteriole); moderate-severe (1-2 arterioles) severe (many arterioles)	0-3
Total score		0-18

Table 2. *Histopathological score for nephrotoxicity after a modified Banff Classification (79, 80).*

4.4. Clinical Trial

4.4.1. Patient Criteria, Study Protocol and Immunosuppressive Treatment Regimens

In a prospective, longitudinal, one-arm, single-center clinical trial, conducted at the Charité Universitätsmedizin Berlin, Germany, 48 consecutive de novo kidney transplant patients from cadaveric donors were enrolled and included in the analysis.

Follow-up period was 12 months. After transplantation, patients were started on standard immunosuppressive drugs consisting of a calcineurin inhibitor, steroids, mycophenolate mofetil (MMF) and often an induction therapy (Basiliximab/Simulect®). Since patients participating in other clinical research studies on immunosuppressive drug treatment were also eligible for inclusion, several patients received alternative drug regimens as part of their study protocols (for individual immunosuppressive treatment regimens please refer to Table 3).

Calcineurin inhibitor dosing was guided by blood concentrations (trough levels) following clinical standard procedures at the clinic. Initially, these levels were aimed to be in the range of 10-15ng/ml for tacrolimus and 150-250ng/ml for cyclosporine. After 3 weeks, these levels were tapered down to 5-8 ng/ml (tacrolimus) and 100-150 ng/ml (cyclosporine). MMF was initially introduced at a dose

of 1000 mg daily and later increased to 2000 mg daily if tolerated, based on clinical criteria (white blood cell count, platelet count and hematocrit).

The study protocol was approved by the Institutional Review Board of the Charité. After informed written consent, 14 ml of blood (4 ml heparinized, 10 ml EDTA) and 10 ml urine samples were collected from each patient at fixed time-points before and on days 1, 3, 7 and months 1, 3, 6, 9 and 12 after transplantation. All of these samples were obtained during routine blood draws either during the hospital phase immediately after transplantation or during routine follow-up visits.

Also, as part of the routinely applied diagnostic workup in the case of graft dysfunction (proteinuria and/or an increase in creatinine), renal allograft biopsies were performed (Appendix, Table B).

Corresponding demographic and clinical data were obtained for each patient at the time-points given above by using an electronic data base program (T-Base) and corresponding patient files.

There were no recruitment restrictions regarding gender, age, or ethnic background. Inclusion/exclusion criteria were screened during exams and based on medical records. Subjects could withdraw at any time during the study.

Inclusion criteria:

All patients scheduled to and undergoing primary renal transplantation at Charité Universitätsmedizin Berlin, Campus Virchow, Germany with written patient consents.

Exclusion criteria:

- Retransplantations or concomitant transplantation of another organ such as liver or pancreas.
- Additional diseases requiring immunosuppressive medication (e.g. arthritis, psoriasis).
- HIV-positive patients.
- Mental inability to follow or understand simple instructions.
- Drop-out patients.

The following data were collected and entered into a database:

- Relevant routinely obtained laboratory parameters (e.g. white blood cells, C-reactive protein)
- Laboratory kidney function parameters (serum creatinine serum, blood urea nitrogen)
- Co-medications
- Relevant clinical diagnoses and events

- Blood concentrations of immunosuppressants

For comparison and as healthy controls, equal amounts of identical samples (blood, urine) were obtained once from 10 healthy volunteers.

4.4.2. Sample Handling

Samples were centrifuged at 3000·g for 10min. (4°C), aliquoted, kept on ice and snap frozen at -80°C within 1 hour of collection. All collected samples were transferred on dry ice via courier service from the clinic to the collaborators' laboratory in the Department of Anesthesiology, University of Colorado Denver, Aurora, Colorado, USA, where they were stored at -80°C until further analysis.

Metabolite profiles in urine and plasma were determined using non-targeted nuclear-magnetic-resonance spectroscopy (^{1}H-NMR) and high performance liquid chromatography-with mass spectrometry (HPLC-MS/MS) assays. 4ml of heparinized blood was used for metabolic profiling, 10ml of EDTA blood for quantification of immunosuppressive drugs and isoprostane concentrations, 10ml of mid-stream urine for ^{1}H-NMR/HPLC-MS/MS metabolic profiling and isoprostane concentrations.

Human histopathological evaluations based on routinely performed biopsies revealed a diagnosis of rejection in sixteen of 48 patients at one or more time-points; 18 patients had events of immunosuppressant toxicity; and 7 of the patients fitting in these categories were diagnosed with both immunosuppressant toxicity and rejection at different biopsy time-points.

Patient ID	IS 1	IS 2	IS 3	Other IS
N1	FK+MMF+Cortison	no switch	no switch	yes
N4	FK+MMF+Cortison	no switch	no switch	no
N9	FK+MMF+Cortison	no switch	no switch	yes
N10	FK+MMF+Cortison	no switch	no switch	yes
N12	FK+MMF+Cortison	no switch	no switch	yes
N13	FK+MMF+Cortison	no switch	no switch	yes
N14	FK+MMF+Cortison	no switch	no switch	yes
N17	FK+MMF+Cortison	no switch	no switch	yes
N19	FK+MMF+Cortison	no switch	no switch	no
N22	FK+MMF+Cortison	no switch	no switch	no
N23	FK+MMF+Cortison	no switch	no switch	no
N25	FK+MMF+Cortison	no switch	no switch	yes
N29	FK+MMF+Cortison	no switch	no switch	no
N30	FK+MMF+Cortison	no switch	no switch	yes
N32	FK+MMF+Cortison	no switch	no switch	yes
N35	FK+MMF+Cortison	no switch	no switch	no
N38	FK+MMF+Cortison	no switch	no switch	yes
N39	FK+MMF+Cortison	no switch	no switch	yes
N41	FK+MMF+Cortison	no switch	no switch	yes
N44	FK+MMF+Cortison	no switch	no switch	yes
N45	FK+MMF+Cortison	no switch	no switch	yes
N46	FK+MMF+Cortison	no switch	no switch	yes
N50	FK+MMF+Cortison	no switch	no switch	yes
N31	FK+Myfortic+Cortison	no switch	no switch	no
N47	FK+Cortison	no switch	no switch	yes
N5	FK+MMF+Cortison	CyA+MMF+Cortison	CyA+Imurek+Cortison	yes
N20	FK+MMF+Cortison	CyA+MMF+Cortison	no switch	no
N28	FK+MMF+Cortison	FK+Cortison	no switch	yes
N36	FK+MMF+Cortison	FK+Myfortic+Cortison	no switch	no
N37	FK+MMF+Cortison	CyA+MMF+Cortison	no switch	yes
N40	FK+MMF+Cortison	FK+Myfortic+Cortison	no switch	yes
N42	FK+MMF+Cortison	Myfortic+Cortison	FK+MMF+Cortison	yes
N2	CyA+MMF+Cortison	no switch	no switch	yes
N16	CyA+MMF+Cortison	no switch	no switch	yes
N33	CyA+MMF+Cortison	no switch	no switch	yes
N3	CyA+Myfortic+Cortison	no switch	no switch	yes
N48	CyA+Myfortic+Cortison	no switch	no switch	yes
N49	CyA+Myfortic+Cortison	no switch	no switch	yes
N7	CyA+MMF+Cortison	Sirolimus+MMF+Cortison	FK+MMF+Cortison	no
N24	CyA+MMF+Cortison	FK+MMF+Cortison	no switch	yes
N27	CyA+MMF+Cortison	CyA+Myfortic+Cortison	no switch	yes
N43	CyA+MMF+Cortison	Everolimus+Myfortic+ Cortison	no switch	yes
N51	CyA+MMF+Cortison	Sirolimus+MMF+Cortison	FK+MMF+Cortison	yes
N52	CyA+MMF+Cortison	FK+MMF+Cortison	no switch	yes
N11	Sirolimus+MMF+ Cortison	no switch	no switch	no
N34	Belatacept+MMF+ Cortison	no switch	no switch	yes
N15	Belatacept+MMF+ Cortison	Sirolimus+MMF+Cortison	Sirolimus+Myfortic+ Cortison	yes
N26	Belatacept+MMF+ Cortison	FK+Myfortic+Cortison	no switch	yes

Table 3. *Individual immunosuppressive treatment protocols of patients after kidney transplantation. Induction therapy with simulect ®. Switch referring to a protocol change within the observation period. [FK: tacrolimus, CyA: cyclosporine; MMF: mycophenolat mofetil].*

4.5. Metabolomic Analysis

4.5.1. Clinical Trial and Animal Study

A metabolic profiling strategy was used to analyze the samples from the animal and human studies.

Mass spectroscopy and ^{1}H-NMR are the most commonly used methods for detection and characterisation of small molecular substances. NMR spectroscopic techniques are advancing rapidly with increasing sensitivity of NMR pulse sequences, which enhance structural identification and quantification of small molecules in complex mixtures. It is likely that these technologies will significantly contribute to improving our understanding of renal processes and providing new diagnostic insights (81).

The advantage of ^{1}H-NMR is its non-destructive nature and simplicity of sample preparation.

HPLC with mass spectrometry (HPLC/MS(MS) constitutes a different, complementary method, which however requires extensive sample preparation including derivatization, but the number of metabolites that can be detected is higher than with NMR and the analytical sensitivity is better than that of ^{1}H-NMR spectroscopy.

In this study, a combination of non-targeted procedures and targeted approaches was used.

(A) Non-Targeted approach:

This method focused on changes in urine and blood metabolites and measure the components of combinatorial biomarker with ^{1}H-NMR spectroscopy:

1. Succinate, citrate, α-ketoglutarate, lactate (tubule cell metabolism),
2. Hippurate (active secretion),
3. Isoprostanes (oxidative stress).

(B) Targeted approach:

HPLC-MS/MS assays were used for immunosuppressant drug blood concentration measurements and the measurement of isoprostane concentrations in plasma and urine.

4.5.2. HPLC-MS/MS

The HPLC system consisted of 2 binary pumps, 2 vacuum degassers, a thermostated autosampler and a thermostated column compartment (all Agilent 1100 series) with an integrated 6-port column switching valve. API 4000 or API 5000 triple quadrupole mass spectrometers were used as detectors (Appendix, Table C) (82).

HPLC system and mass spectrometers were controlled by Analyst software. This setup allowed for online sample extraction and separation.

In the extraction position, the extracts were pumped onto the extraction column using pump I and cleaned ("online extraction"). After one minute, the switching valve was activated connecting analytical pump II to the cartridge column, which was now in line with the analytical column. Pump II back-flushed the analytes onto the analytical column that was connected to the mass selective detector (Figure 9 and Appendix, Figure D) (83).

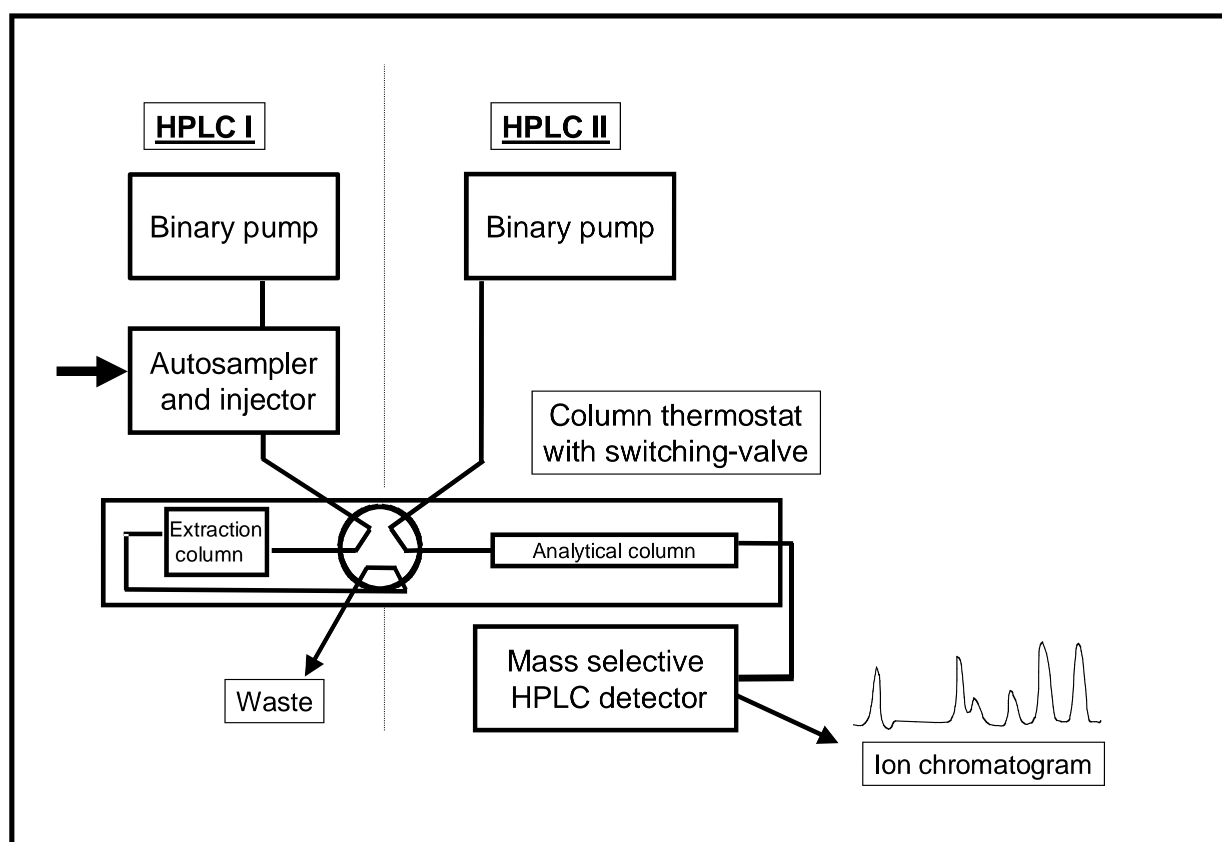


Figure 9. *Setup of HPLC system (77).*

4.5.3. Sample Preparation for the Quantification of Immunosuppressants using HPLC-MS/MS

All drug concentrations were determined 4h after the last dose. Whole blood samples (500µl) were collected in EDTA tubes. For protein precipitation, 800µl methanol and 0.2M $ZnSO_4$ (80/20, v/v) were added to 200µl of blood suspension (84).

Cyclosporin D (250µg/l, Novartis Pharma AG, Basel, Switzerland) was added as an internal standard for cyclosporine, ABT578 (Toronto Research Chemicals, North York, Canada) as an internal standard for sirolimus and everolimus and ascomycin (Toronto Research Chemicals, North York, Canada) as an internal standard for tacrolimus. For quantification of MPA, mycophenolate mofetil (MMF) was used as internal standard. After centrifugation (1300 *g*, 5min, 4°C), 100µl of the supernatant was injected into the HPLC system onto the extraction column.

Cyclosporine, tacrolimus, sirolimus and MPA were quantified using established and validated sensitive and specific HPLC-MS/MS assays. For more details, please see reference (82) (Appendix, Table E1-2 and Table F1-4).

4.5.4. Analysis of 15-F_{2t}-Isoprostanes

The assay is described in detail in reference (82). After a protein precipitation step using $ZnSO_4$ and methanol (3/7) and after centrifugation, 500µl of the supernatants was injected onto an extraction column (Eclipse XDB-C8 5-µm Agilent Technologies, 4.6 12.5mm) with a mobile phase of 30% methanol and 70% 0.1%-formic acid (flow rate 5ml/min.). After 1 min, the switching valve was activated and the analytes were back-flushed from the extraction column onto a column filled with polar endcapped-C18 material (Phenomenex Synergi Hydro-RP 80Å, 3.0, 250mm, 4mm.). Methanol and 0.1% formic acid at a flow rate of 0.6ml/min running the following gradient: 0–1 min 63% methanol; 1.1–10 min 63%–98%. The analytical column was kept at 98% for 1 min. and then reequilibrated to the starting conditions.

After 9min, the column-switching valve was switched into the extraction position and the extraction column was reequilibrated to the starting conditions. The total run time between injections was 13 min. Both columns were maintained at 60°C. The HPLC system was interfaced using a mass spectrometer with an atmospheric pressure chemical ionization (APCI) source. Nitrogen (purity: 99.999%) was used as collision-activated dissociation gas. The mass spectrometer was run in the negative multiple reaction monitoring (MRM) mode. The declustering potential was set to 70V, the entrance potential (EP) to 5V, the interface to 400°C, and the collision energy to 36eV. "The first quadrupole (Q1) was set to select the deprotonated molecular ions–of 15-F2t-IsoP (*m/z* 353) and 15-F2t- IsoP-d4 (IS, *m/z* 357), and the 3rd

quadrupole (Q3) to select the characteristic product ions of 15-F2t-IsoP (*m/z* 193) and 15-F2t-IsoP-d4 (IS, *m/z* 197). Peak area ratios obtained from MRM mode of the mass transitions for 15-F2t-IsoP (*m/z* 353 → 193) and 15-F2t-IsoP-d4 (IS, *m/z* 357 → 197) were used for quantification"(82) (Appendix, Table G1-4).

4.5.5. Sample Handling

4.5.5.1. General Sample Handling

All blood and urine samples of the animal and human study were collected under the same conditions, with same storage times at room temperature (rat urine over night in the metabolic cage) and 4°C (all blood samples and human urine for less than an hour). After short-term storage on ice, samples were aliquoted as appropriate for the measurements and stored at -80°C. This eliminated potential errors caused by differences in the number of freeze thaw cycles, if samples had to be remeasured. The blood extracts and urine samples for ^{1}H-NMR were prepared 24h prior to the measurement and extracts were stored at 4°C over night.

4.5.5.2. Creatinine Concentrations

Plasma was analyzed for creatinine concentrations in urine for the animal study by the University of Colorado Hospital Laboratory using established and validated clinical routine assays. Creatinine concentrations in blood for the human study were collected from patient files and electronic databases.

4.5.5.3. Urine Sample Preparation for ^{1}H-NMR Measurement

The collected urine samples were thawed, vortexed and 700 µl of each sample was transferred into a labeled Eppendorf tube.

The samples were centrifuged for 5min. at 20000g at 8ºC, and 550 µl of the supernatant was transferred into a labeled NMR tube. The urine was buffered with 73µl 1.5M potassium phosphate buffer in D_2O (K_2HPO_4/urine: 1/7.5). After 3-point calibration of the pH electrode, the pH was adjusted to 5.65-5.75 with NaOD and DCl.

4.5.5.4. Perchloric Acid Extraction for Drug Level Measurement in Tissue

See extraction details in (83). Rat kidney tissue was homogenized over liquid nitrogen using a mortar and pestle. The homogenates were pooled and used for all extraction procedures. Each of the following extraction procedures was carried out at 4°C. Five milligrams of powdered tissue was homogenized in 5ml of the particular extraction solution (12% perchloric acid-PCA) using

an electrical tissue homogenizer (10sec. at 4°C). The homogenate was centrifuged at 1400g/10min/4°C. After centrifugation, the supernatant was collected and the pellet was resuspended in 2mL of water. After centrifugation at 1400G/10min/4°C, the supernatant was combined with the supernatant of the first centrifugation and neutralized to pH 7 using $KHCO_3$ and KOH solutions. The resulting $KClO_4$ precipitate was then centrifuged and the pellet discarded. The remaining supernatant was lyophilized overnight.

4.5.5.6. Quantifications

The ratios between the analyte peaks (e.g. immunosuppressants) and the peaks of the internal standards were calculated.

Concentrations of other compounds of interest (e.g. isoprostanes) were calculated based on analogous external calibration curves.

Quality control samples were included in the analysis and a minimum of one calibration curve was used for every hundred samples.

4.5.6. NMR-Spectroscopy

Figure 10 A. *^{1}H-NMR spectrometer, 500 MHz, at the University of Colorado Denver, Anschutz Medical Campus.*

4.5.6.1. ^{1}H-NMR-Spectroscopy of Rat Urine

^{1}H-NMR urine analysis (84) was performed using a Varian Inova NMR 500 MHz spectrometer (Figure 10A). After preparation of urine (see 4.5.5.3), 550µl of urine and an external standard

compound (TMS/-trimethylsilyl propionic-2,2,3,3-d_4 acid solved in D_2O to 50 mM in a thin sealed glass capillary) were inserted into the NMR tube.

To suppress the water signal in urine, a standard Varian presaturation sequence was used. ^{1}H-NMR spectra were obtained at 500 MHz using a spectral width of 7200Hz, 32K data arrays, and 64 scans with 90° flip angle applied every 14.8 sec (Table 4). This left enough time for the relaxation of all proton signals integrated in this study.

Number of scans	64
Flip angle	90°
Repetitions time	14.8 seconds
Saturation delay	1.5 sec at 5dB
Spectral width	7200 Hz
Data size	32 K

Table 4. *Parameter setting for ^{1}H -NMR spectroscopy (77).*

4.5.6.2. ^{1}H-NMR-Spectroscopy of Animal and Human Heparin Plasma: Methanol/Chloroform Extraction

Blood samples were extracted using the following methanol/chloroform extraction technique prior to the ^{1}H-NMR-analysis.

1) 0.5 ml plasma was transferred into a glass tube.
2) 1 ml methanol/chloroform (in 1:1 ratio) was added and centrifuged (1400g/8°C/15min).
3) The upper phase was collected into a new glass tube. The middle pellet was then carefully punctured, the lower phase collected and combined with the top phase.
4) The pellet was resuspended in 1.0ml of ice-cold methanol/chloroform, vortexed and centrifuged (1400g/8°C/15min.), and the supernatant was added to that of step 3.
5) The pellet was resuspended in 0.5ml water, vortexed and centrifuged (3000g/8°C/5min), and the liquid phase was added to the united liquid solution of step 4.
6) 0.5ml water was added to the united solution and it was cooled down for 20min. at –20°C to allow for better phase-separation and then quickly centrifuged (2000g/4°C/2min.).
7) The top water layer was transferred to the pellet of step 5. The bottom residue was evaporated in the speedvac for 3h. The residue was stored at –80°C.
8) The pellet and the water phase of step 7 was vortexed and centrifuged at (1400g/8°C/15min).

9) The supernatant was collected into a new plastic tube and the pellet was resuspended in 1ml of water. After centrifugation (1400g/15min/4°C), the supernatant was combined with the supernatant of step 9, lyophilized overnight and stored at –80°C.

10) The protein pellet was kept and also stored at –80°C.

Before ^{1}H-NMR analysis, the lyophilized extracts were thawn to room temperature and 550 µl of D_2O was added into each tube.

After vortexing, the solution was treated with ultrasound for 2min. and centrifuged (2000·g/15min./4°C).

The supernatant was transferred into a labeled NMR-tube. ^{1}H-NMR blood analysis was performed with a set up similar to that used for spectroscopy of rat urine (see 4.5.6.1 above).

4.5.7. ^{1}H-NMR Data Processing and Analysis

Metabonomic profiling (28) (Figure 10 B) started with the acquisition of a set of spectra. The spectra were then reduced to histograms, which represented the area under the curve in a certain spectral region. This generated an ensemble of XY-tables (spectral region versus integral) also know as the Bucket tables. Next a statistical analysis, e.g. principal component analysis (PCA) was carried out. In the PCA, the principal components were constructed such as the first accounts for the most prominent variance in the ensemble, the second accounting for the second highest value etc. The clustering analysis of the scores plot, which showed PC_a versus the PC_b revealed the presence or absence of a difference among the treatment groups. This approach facilitated the discovery of hidden phenomena not revealed by the usual standard spectral dimension. The spectral regions that cause the separation were identified in the loading plots, which formed the link back to the spectral dimension. Through the use of 2D-NMR, the compounds under the signals in the first dimension were identified.

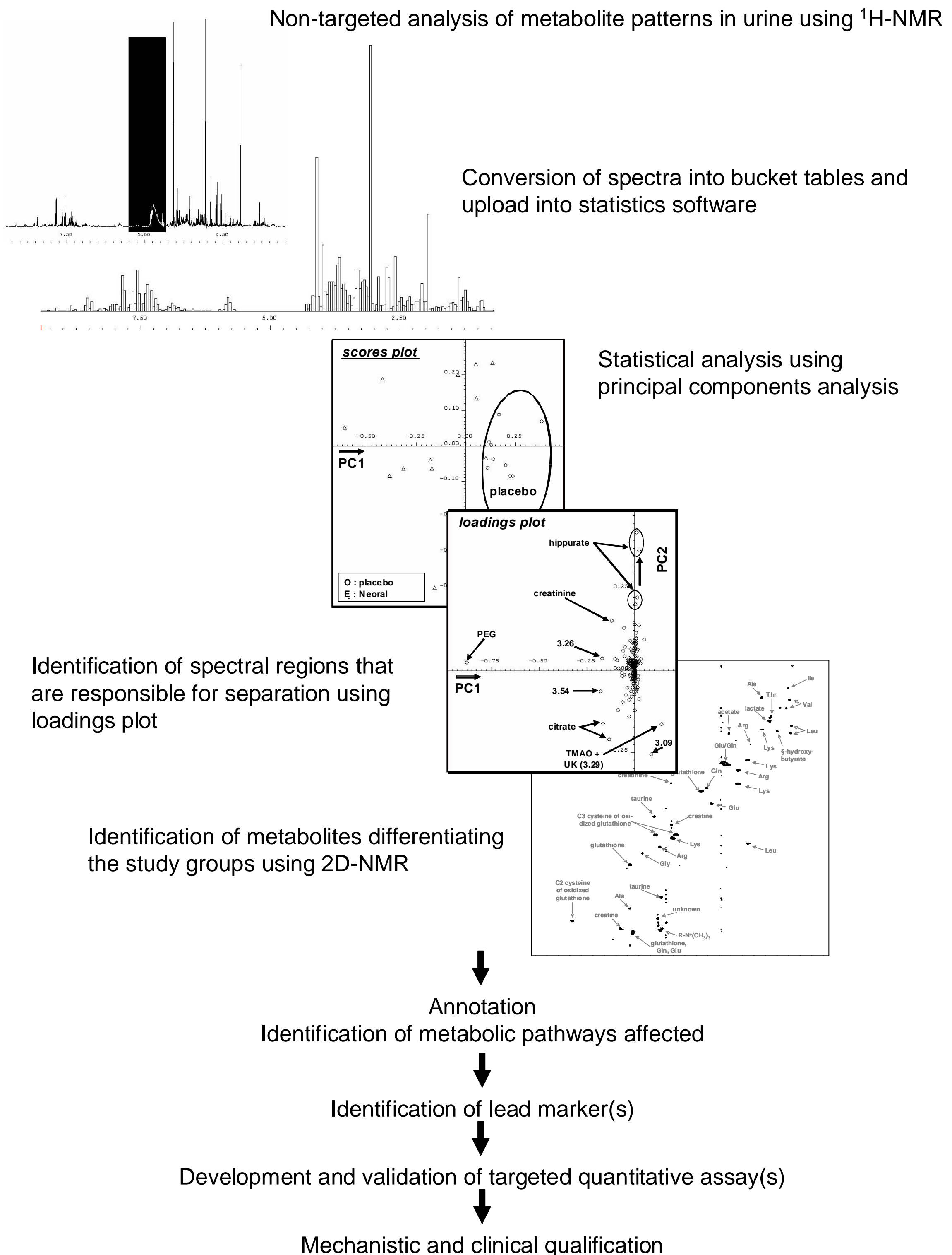


Figure 10 B. *Workflow of non-targeted metabonomics analysis (28).*

Prior to integration. all NMR spectra were manually corrected for phase and baseline distortions and calibrated to the corresponding chemical shift reference using the Topspin software package. The absolute concentrations of single metabolites were then referred to the integral and afterwards calculated.

Because of different overall urine concentrations, all urine spectra were normalized to the total integral.

For the non-targeted statistical approaches, including principal components analysis,

the transformation of the spectra into histograms was performed using the Amix software package. Spectra that were outside the model-space were excluded from the statistical analysis. This could be the case if for example urine was very highly diluted. The bucket width was usually 0.04 ppm and the spectra were scaled to the total intensity (if not described differently). One-way Anova test in combination with Tukey's post-hoc test or, as appropriate, an independent Student's T-test was performed to evaluate statistically significant changes in metabolites and histology scores between the study groups. P values < 0.05 were considered as statistically significant.

5. Results and Discussion of the Animal Study

5.1. Animal Study Results

5.1.1. GFR and Serum Creatinine

In the different treatment groups, the average glomerular filtration rates (GFR) ranged from 237± 41µl/min to 611± 492µl/min/100g body weight. However, no significant differences in kidney function among the different immunosuppressive drugs were detected when these were administered as monotherapy (Figure 11A).

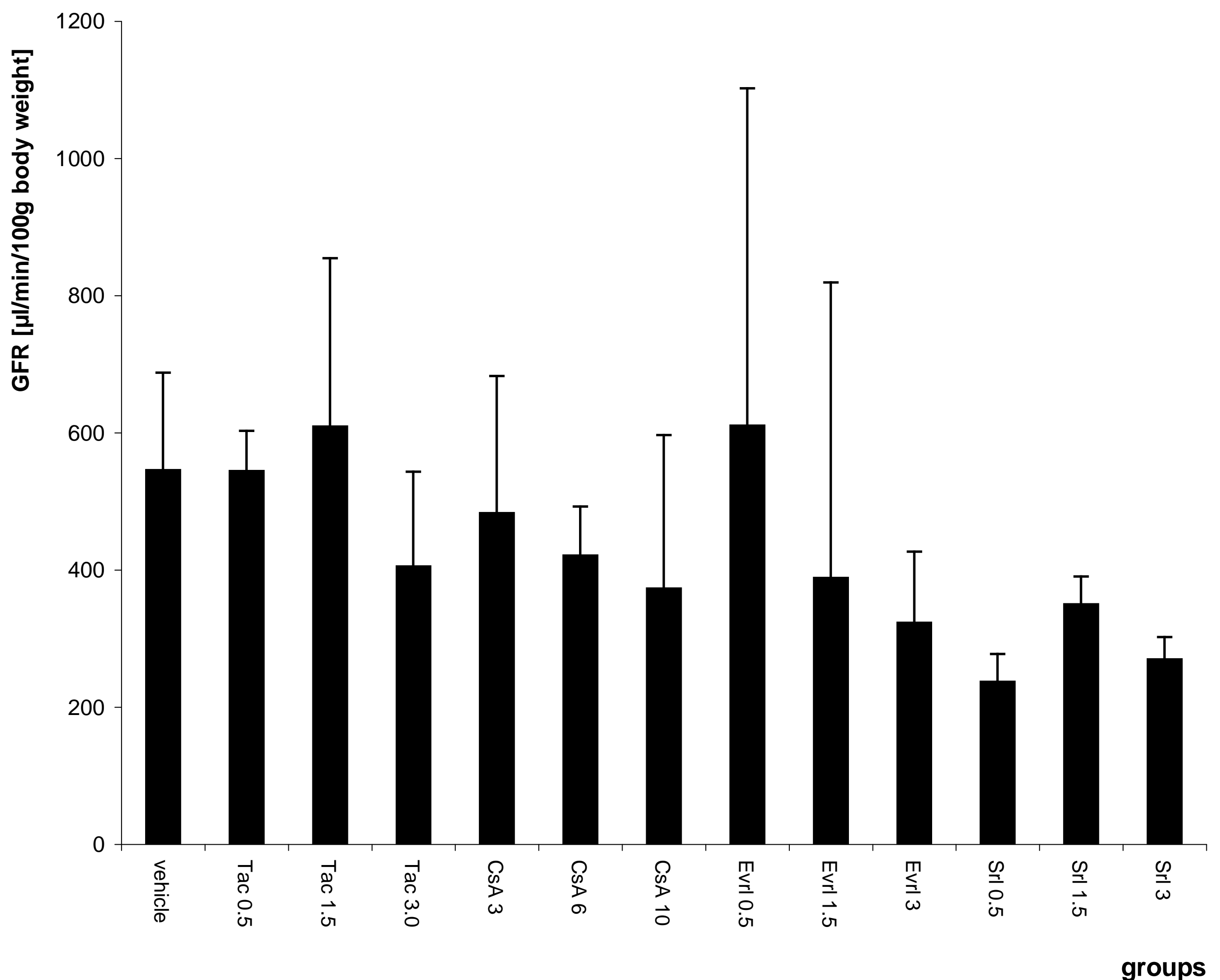


Figure 11A. *Glomerular filtration rates (GFR) in µl/min/100g body weight in groups exposed to different immunosuppressant drug regimens. [Although slightly lower in the high-dose sirolimus groups, none of the differences were significant. Vehicle: control, Tac: tacrolimus, CsA: cyclosporine, Evrl: everolimus, Srl: sirolimus. Numbers behind treatment groups: dose of specific immunosuppressant in mg/kg body weight, administered over 28 days.].*

In contrast, combination treatment of tacrolimus with either sirolimus or everolimus led to a significant decrease in glomerular filtration rates compared to the control group (545.9± 142.2µl/min/100g body weight). This effect was most pronounced in rats treated with the highest dose of tacrolimus (3mg/kg) in combination with sirolimus 1.5mg/kg (181± 119µl/min/100g body weight) and was further enhanced when sirolimus or everolimus were administered at a dose of 3 mg/kg (164+/-µl/min/100g body weight), (Appendix, Figure H.1. and H.2.).
Similarly, in the cyclosporine-treated groups, the greatest reductions in kidney function were observed in rats exposed to combinations of the highest cyclosporine and mTOR inhibitor doses (cyclosporine 10mg/kg plus sirolimus 3mg/kg (104± 18 µl/min/100g body weight) and cyclosporine 10mg/kg plus everolimus 3 mg/kg (100± 91µl/min/100g body weight) (Appendix, Figure H.3. and H.4.).

5.1.2. Immunosuppressive Drug Levels

5.1.2.1. Blood Drug Concentrations

When coadministered, neither sirolimus nor everolimus affected tacrolimus drug levels. When tacrolimus was administered at a dose of 3mg/kg body weight, blood trough levels remained below 4 ng/ml, irrespective of whether it is given alone or in combination with any mTor inhibitor (Figure 12.A.). This finding was similar in any tacrolimus treatment group where again no influence on tacrolimus blood levels by drugs given simultaneously could be observed.

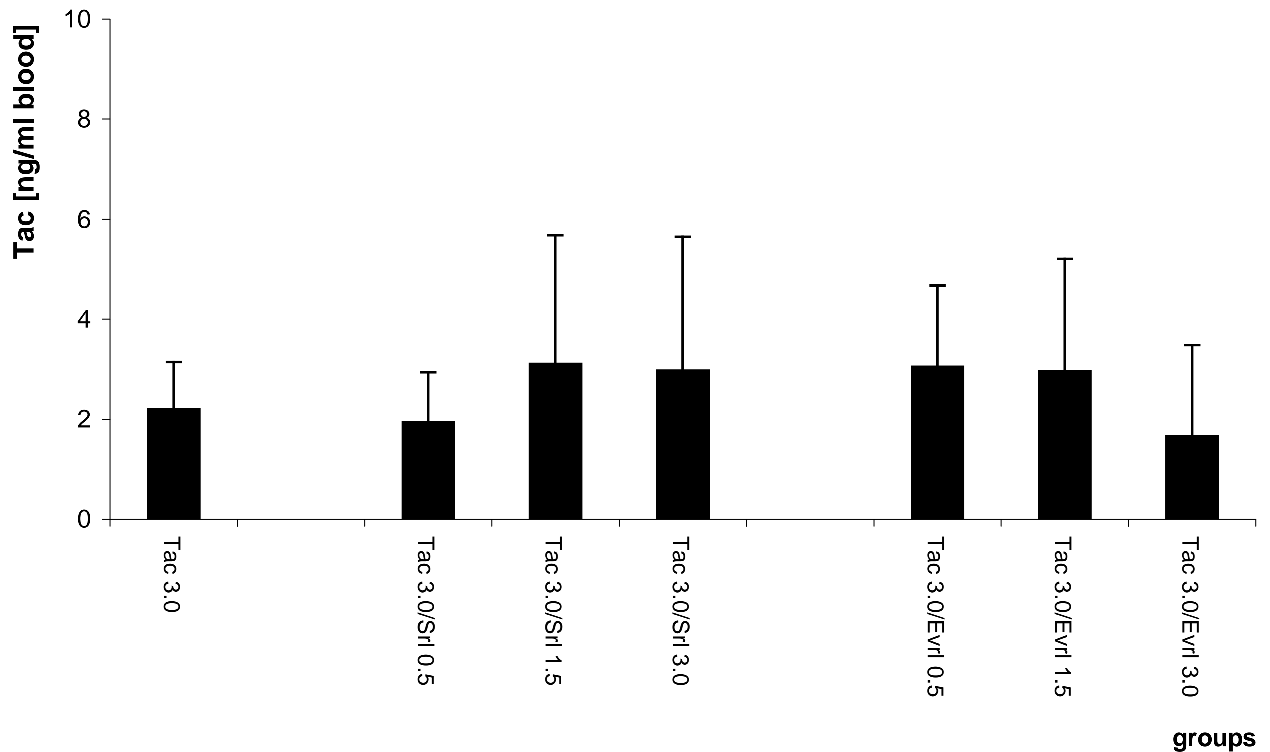


Figure 12A. *Tacrolimus blood concentrations in groups under tacrolimus treatment (3mg/kg) in combination with different doses of either sirolimus or everolimus. [No effect of simultaneous mTOR inhibitor treatment of tacrolimus blood levels was observed by One-way-Anova. Tac: tacrolimus, Srl: sirolimus, Evrl: everolimus].*

On the other hand, an increase of sirolimus blood concentrations was seen when administered in combination with tacrolimus (Appendix, Figure I.1.).

Also, everolimus blood concentrations displayed a trend to higher levels when co-administered with tacrolimus (Appendix, Figure I.2.).

A marked drug-drug interaction between mTOR inhibitors and cyclosporine was observed when tacrolimus was replaced by cyclosporine. This mainly affected the mTOR inhibitor blood concentrations and the cyclosporine concentration as well (Figure 13.A. and Appendix, Figures J.1. -3.).

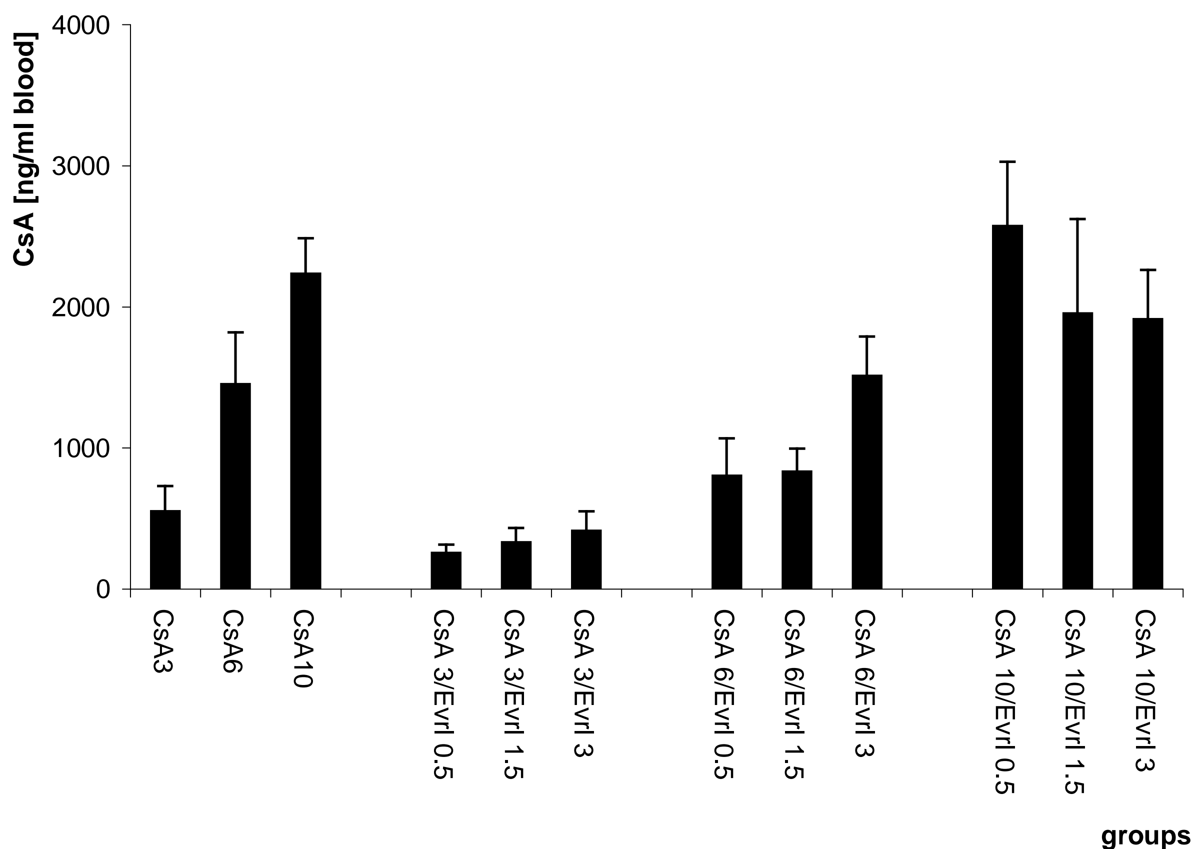


Figure 13A. *Cyclosporine concentrations in blood from rats treated with cyclosporine in combination with everolimus [*Significant differences by One-way-Anova between CsA10/Evrl0.5 vs CsA3/Evrl0.5; CsA10/Evrl0.5 vs CsA3/Evrl1.5; CsA10/Evrl0.5 vs CsA3/Evrl3; CsA10 vs CsA3/Evrl0.5; CsA10 vs CsA3/Evrl1.5; CsA10/Evrl3 vs CsA3/Evrl0.5. Significances are not shown in figure. CsA: cyclosporine, Evrl: everolimus.].*

5.1.2.2. Tissue Drug Concentrations

Although tacrolimus concentrations basically remained unaffected in blood when everolimus and sirolimus were coadministered, an analysis of corresponding kidney tissue revealed a significant increase of tacrolimus` main metabolite, 13-O-dimethyl-tacrolimus, which was most pronounced when tacrolimus and everolimus had been coadministered (Figure 14A).

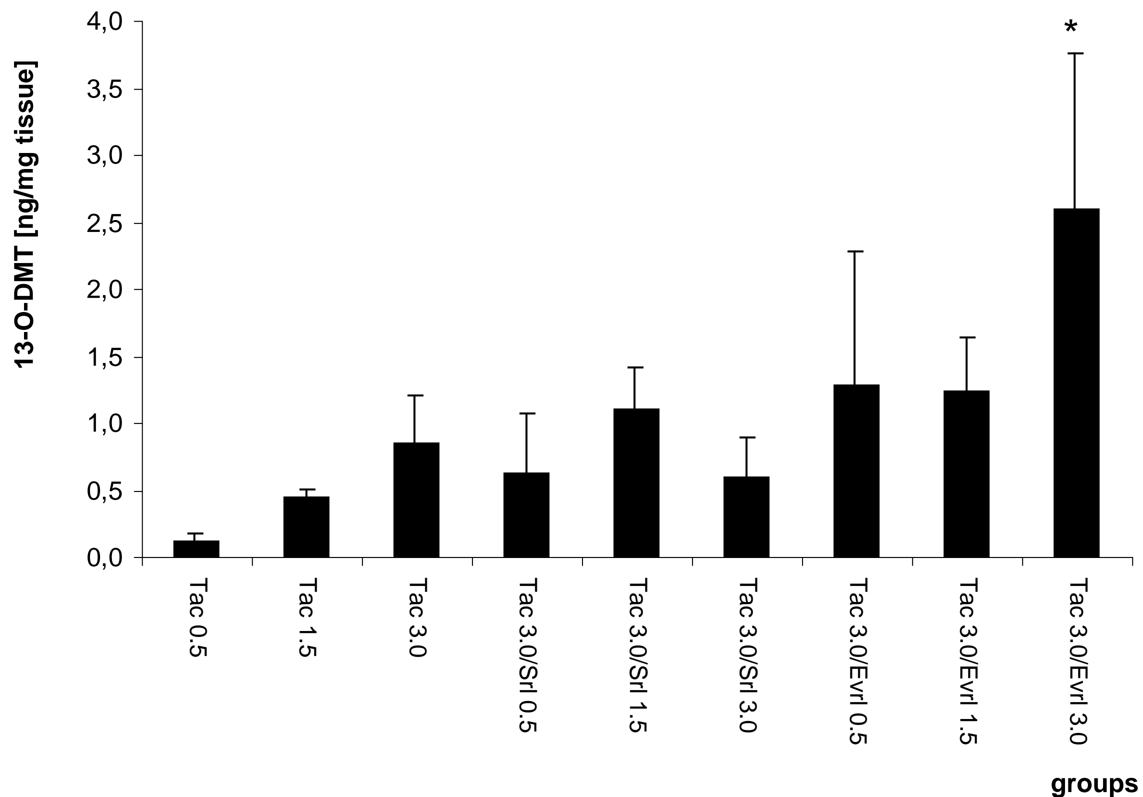


Figure 14A. *13-O-dimethyl-tacrolimus(13-O-DMT) tissue concentration in the kidney after 28 days of administration. [* Tac3.0/Evrl3 is significant different by One-way-Anova versus Tac 0.5, Tac 1,5, Tac 3, Tac 3/ Evrl 1.5, Tac 3/Srl 0.5, Tac 3/Srl 1.5 and Tac 3/Srl 3. Evrl:everolimus, Srl:sirolimus.].*

Tacrolimus tissue concentration ranged from 0.85+/-0.90 to 16.38+/-8.15ng/mg tissue.

Coadministration of everolimus significantly increased tacrolimus tissue concentrations after 28 days (Figure 14B).

Cyclosporine tissue concentration ranged from 3.4+/-2 to 20.9+/-3.9ng/mg tissue. The correlation of increasing tissue concentrations under increasing immunosuppressant doses is shown in Figure 14C. According to the results of cyclosporine blood levels, no influence of concomitant mTOR inhibitor administration on cyclosporine tissue concentrations was found.

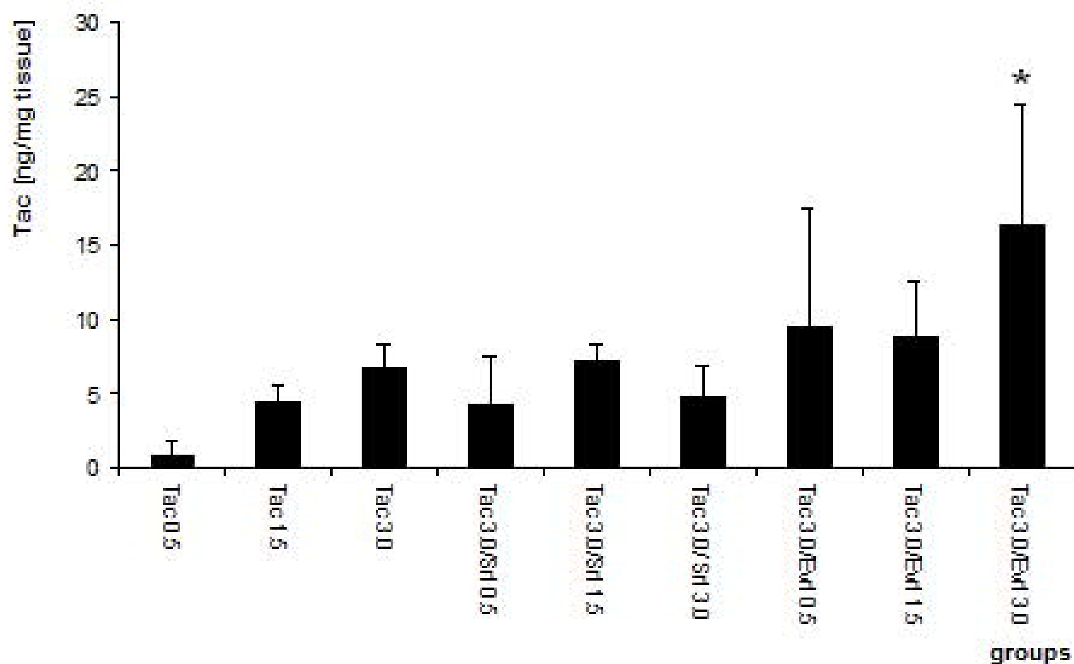


Figure 14B. *Tacrolimus tissue concentration in the kidney after 28 days of administration [*p value is significant by One-way-Anova versus Tac 0.5. Tac: tacrolimus, Srl: sirolimus, Evrl: everolimus.].*

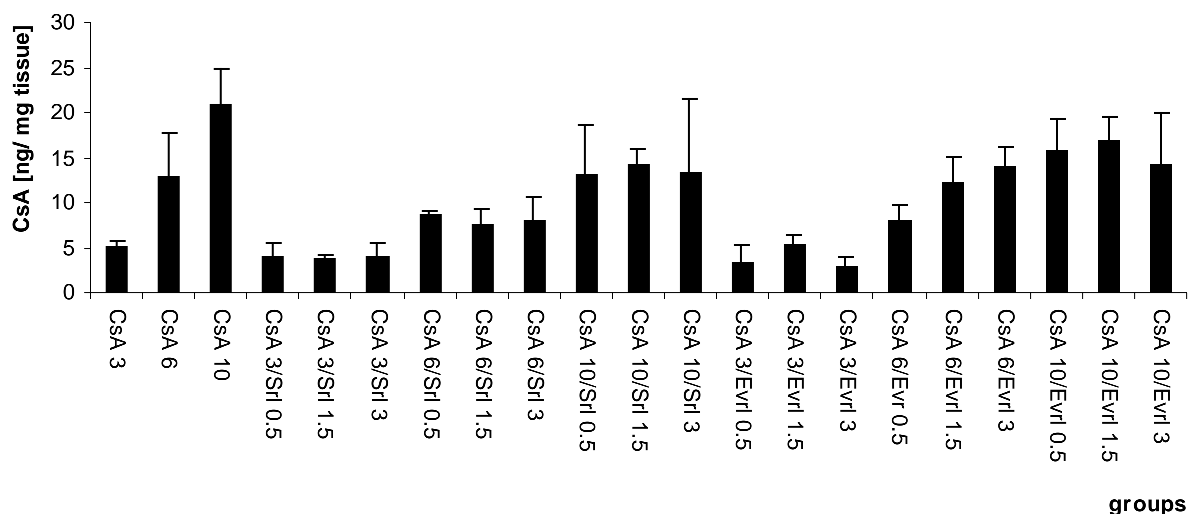


Figure 14C*. Cyclosporine tissue concentration in the kidney after 28 days of administration [*p value is significant by One-way-Anova for CsA10 versus CsA3/Evrl0.5, CsA3/Evrl3, CsA3/Srl0.5, CsA3/Srl1.5, CsA3/Srl3. Significances are not shown in figure. CsA: cyclosporine, Srl: sirolimus, Evrl: everolimus.].*

5.1.3. Analysis of 15-F2t-Isoprostanes

Urinary concentrations of 15-F_{2t}-isoprostane were measured as a stable marker for oxidative stress and an indicator for the generation of free radicals. 15-F_{2t}-isoprostane concentrations were similar in the urine of all treatment groups and there were no statistically significant differences (Figures 15 and Appendix, Figures K1-3) found.

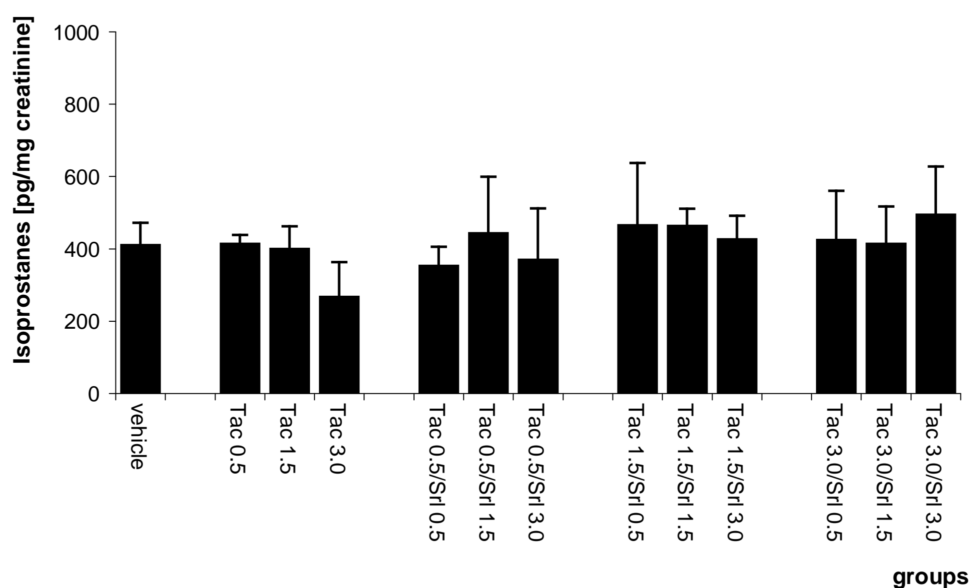


Figure 15. *15-F_{2t}-Isoprostane concentrations in urine after 1 week of treatment with tacrolimus and sirolimus. [No statistically significant differences between groups by One-way-Anova ($p>0.05$) were found. Vehicle: controls, Tac: tacrolimus, Srl: sirolimus.]*

5.1.4. Histopathological Analysis

Overall, analysis of histological slides based on modified Banff ´97 injury scores revealed only mild alterations in all groups. The most severe changes were found after drug exposure for 28 days. In terms of monotherapy, cyclosporine resulted in the highest cumulative histology injury scores in comparison to tacrolimus, everolimus and sirolimus (Figure 16). Surprisingly, treatment with everolimus alone appeared to cause more histopathological alterations than treatment with sirolimus. Appendix, L.1. and L.2. show exemplarily histopathological findings after treatment with tacrolimus and sirolimus. However, due to the low overall injury scores and the relatively small number of observations in each group (n=4), it was impossible to objectively assess whether this trend was of relevant impact. Under combined treatment, histological alterations were most pronounced in groups with high doses of cyclosporine in combination with sirolimus and everolimus, which presented itself as mild glomerulopathy and isometric tubular vacuolization (Figure 17). Such changes are considered typical for calcineurin inhibitor nephrotoxicity (Figure 18). These findings were consistent with a salt-depleted rat the study group I worked with conducted earlier, where the worst histopathological score was found in rats following a low salt diet and exposed to cyclosporine (85). The tubular cell morphology and architectural structure of the kidney cortex was affected. Tubular epithelial atrophy (exemplarily shown in Appendix, L.3.), vacuolization, glomerulopathy including glomerulosclerosis and mesangial matrix expansion were markedly found.

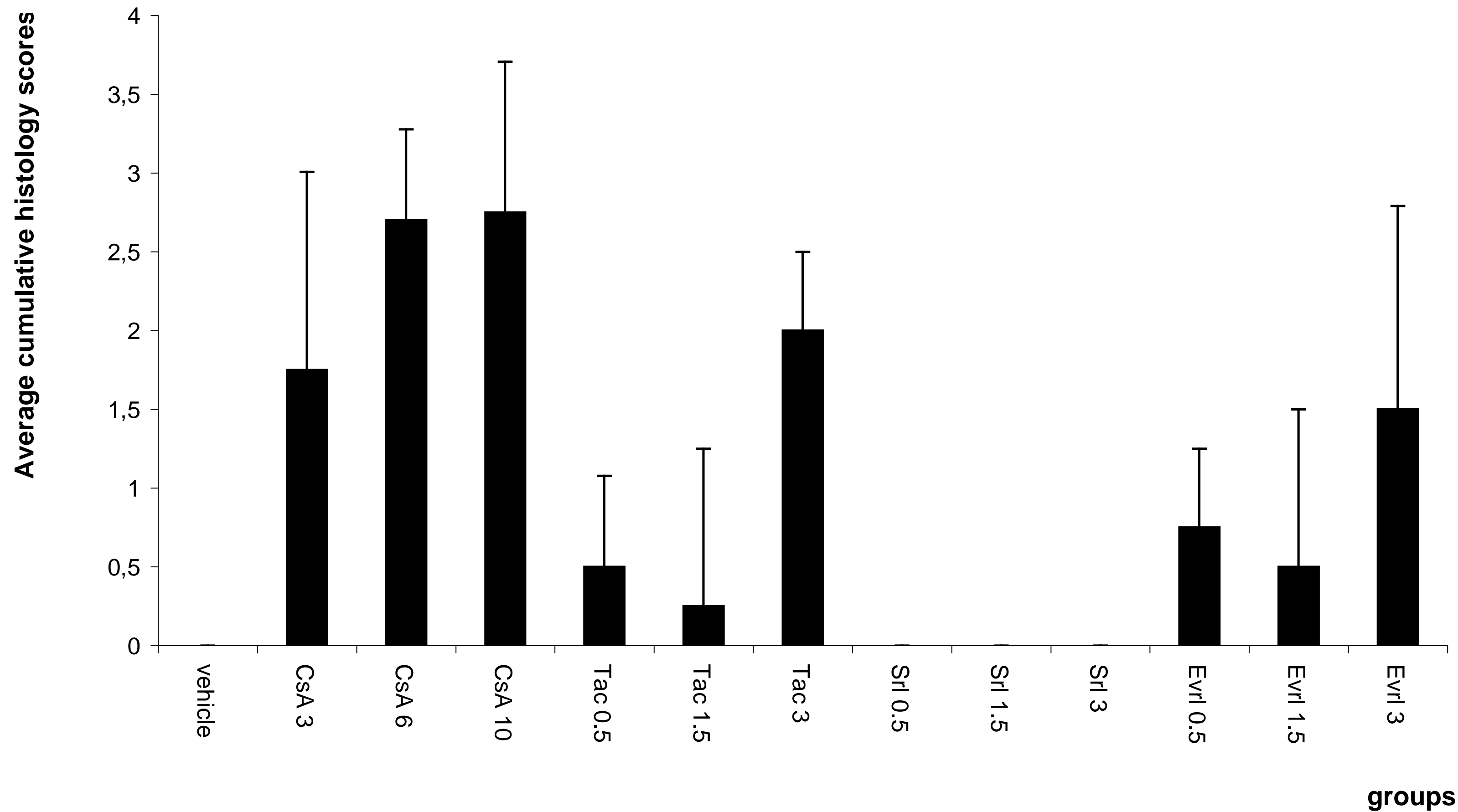


Figure 16. *Average of cumulative histology scores + standard deviation (n=4). [Comparison between cyclosporine, tacrolimus, sirolimus and everolimus given as monotherapy. Differences by One-way-Anova are not significant. Vehicle: control, Tac: tacrolimus, CsA: cyclosporine, Evrl: everolimus, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

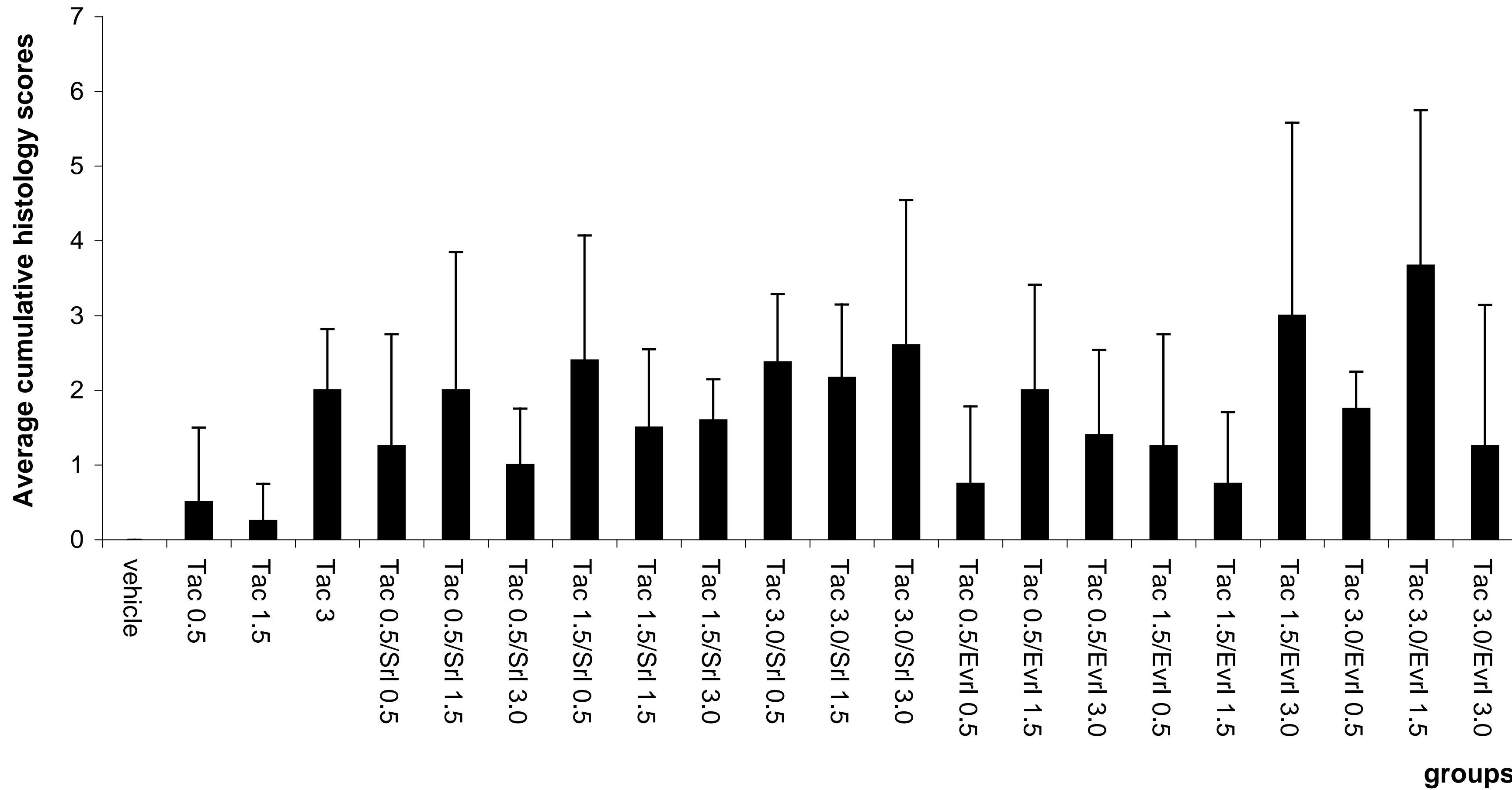


Figure 17. *Average of cumulative histology scores + standard deviation (n=4). [Comparison between tacrolimus and combination with sirolimus or everolimus. Differences by One-way-Anova are not significant. Vehicle: control, Tac: tacrolimus, CsA: cyclosporine, Evrl: everolimus, Srl: sirolimus. . Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

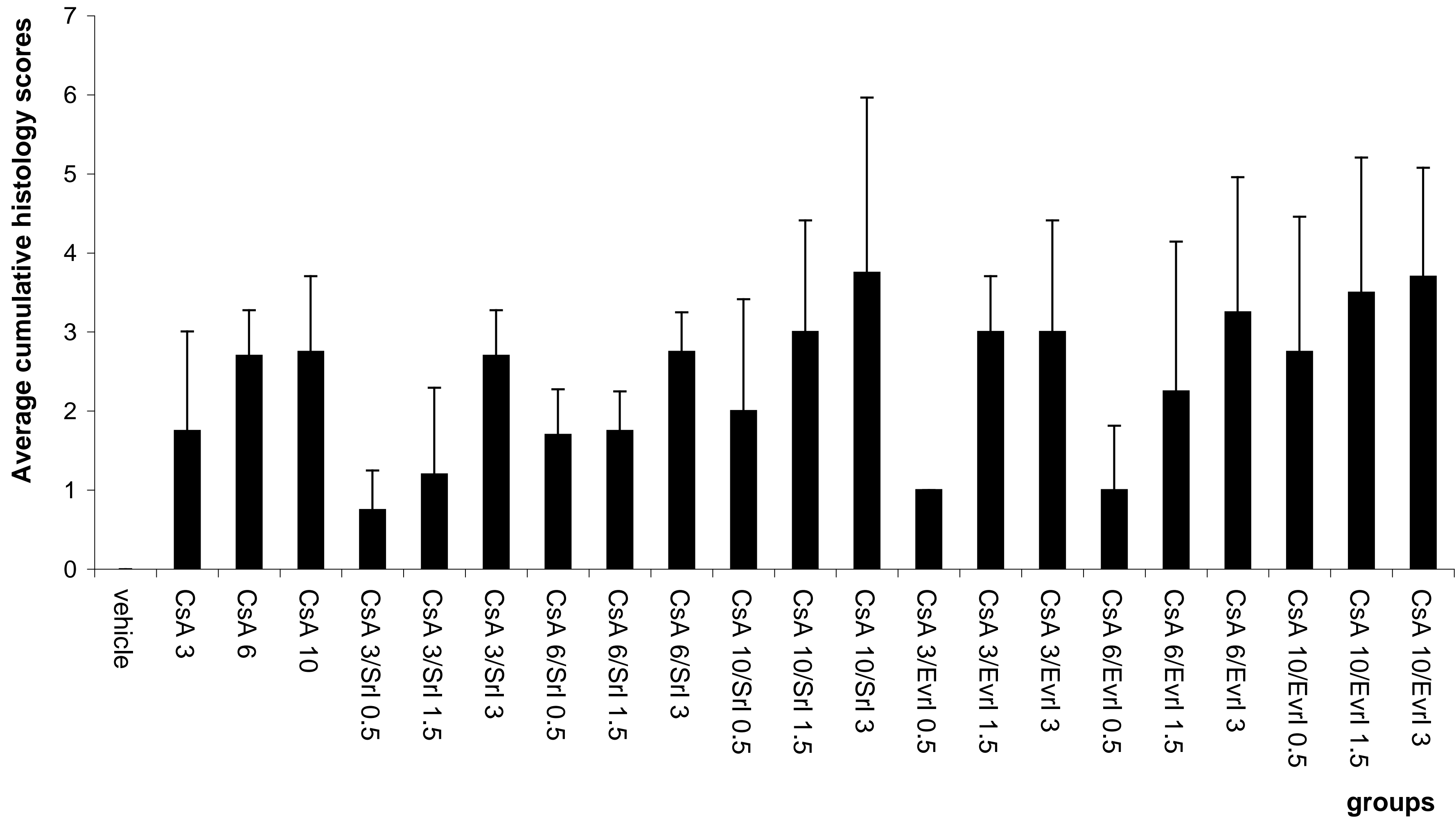


Figure 18. *Average of cumulative histology scores + standard deviation (n=4) [Comparison between cyclosporine and combination treatments with sirolimus or everolimus. Differences by One-way-Anova are not significant. Vehicle: control, Tac: tacrolimus, CsA: cyclosporine, Evrl: everolimus, Srl: sirolimus. . Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

5.1.5. ^{1}H-NMR Spectroscopy of Rat Urine

Metabolite patterns in urine were analyzed using ^{1}H NMR spectroscopy. A targeted analysis of key urine metabolites after manual integration of NMR spectra was carried out and the results for the different dose groups were compared using analysis of variance in combination with Tukey's post-hoc test. The urine concentrations of hippurate, creatinine, glucose, succinate, citrate, alpha ketoglutarate, urea and taurine after everolimus exposure in combination with cyclosporine were not statistically different compared to the control levels (Figure 19). However, there was a highly significant difference when cyclosporine was combined with either sirolimus or everolimus, indicating that sirolimus, but not everolimus may enhance the negative effects of cyclosporine on the kidney.

While Figure 19 shows selected metabolites that are known to be associated with proximal tubule function, the known major target of cyclosporine toxicity, we also employed a non-targeted unsupervised principal component analysis of the complete NMR spectra. The principal component analysis also revealed a distinct separation of the urine metabolite spectra of rats treated with sirolimus and everolimus alone (Appendix, Figure M.1.). By this means, urine metabolite patterns of rats, after exposure to combinations of cyclosporine and sirolimus and combinations of cyclosporine and everolimus, could clearly be differentiated. Interestingly, the combination of cyclosporine and everolimus was grouped in the same region as the controls. Overall, these results further confirmed the analysis shown in Figure 19.

As a first step, the concentrations of urine metabolites known to indicate proximal tubule function among the different treatment groups were compared. Metabolite patterns in urine were analyzed using ^{1}H-NMR spectroscopy, and ^{1}H-NMR spectra of urine after 28 days of treatment were manually integrated. As indicated by analysis of variance, the urine concentrations of hippurate, creatinine, glucose, succinate, citrate, alpha ketoglutarate, urea and taurine after exposure to a combination of tacrolimus and everolimus were not statistically different to those in urine samples from vehicle controls (Appendix, Figure M.2.). All drug concentrations were measured using a validated LC-MS/MS assay (see Materials and Methods). The effects of different doses and dose combinations of the immunosuppressants on trimethylamine oxide (TMAO) are shown in the Appendix, Figure M.3. Trimethylamine oxide is a metabolite that is found in relatively large concentrations in proximal tubule cells, and thus, an increase of its urine concentrations indicates proximal tubule damage (85) .

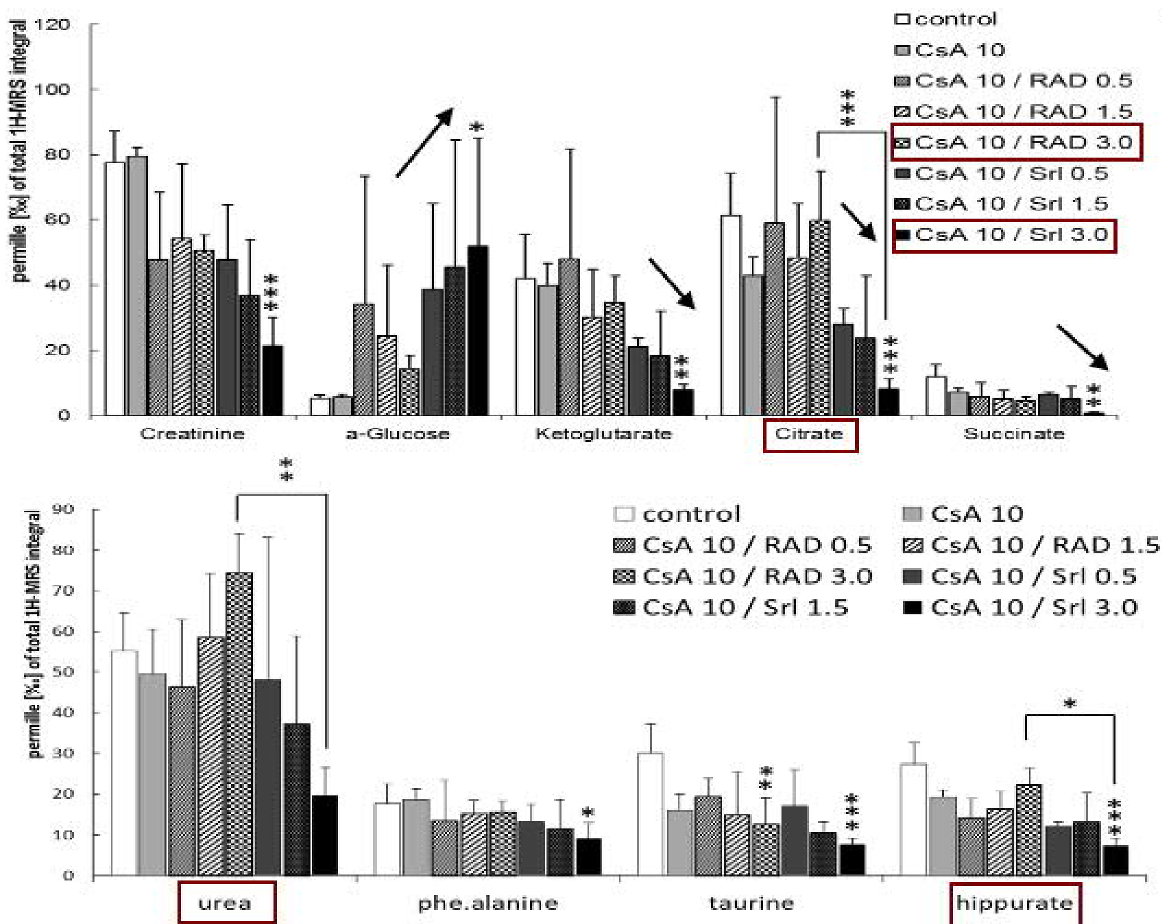


Figure 19. *Changes in urine metabolites as determined by ^{1}H-NMR spectroscopy after treatment of rats for 28 days. Columns refer to metabolite concentrations at different immunosuppressant doses (mg/ kg/ day) of respective drugs and combinations. [P-values were estimated using Anova, * = p < 0.05; ** = p < 0.001; *** = p < 0.001. CsA:cyclosporine.].*

Next step was to analyze the urine ^{1}H-NMR spectra after treatment with tacrolimus and its combination with sirolimus or everolimus for 28 days using an unsupervised principal component analysis. This analysis confirmed the trends already observed in the targeted analysis based on the relative concentrations of selected metabolites known to be associated with proximal tubule function. The analysis also separated urine ^{1}H-NMR spectra of rats treated with everolimus and tacrolimus alone or in their combinations with tacrolimus (Appendix, Figure M.4. and M.5.).

5.2. Discussion of the Animal Study

As expected, based on previous studies (3), at the doses administered in the present study, cyclosporine, sirolimus and everolimus blood concentrations were close to those reported in kidney graft patients.

Blood concentrations of tacrolimus were found at or below the concentration range of those typically targeted in transplant patients when administered as monotherapy, and were lower than in rats that were cotreated with tacrolimus and either sirolimus or everolimus.

Moreover, there was a marked drug-drug interaction when cyclosporine and sirolimus were co-administered. An increased relative bioavailability of sirolimus in the presence of cyclosporine (thus more negative effects) was observed.

Similarly, high tacrolimus doses led to higher blood concentrations of sirolimus. In this study, no such effect was detected for everolimus. The nephrotoxicity rat model used normally fed rats that were exposed to doses of tacrolimus and other immunosuppressants. As mentioned above, the so called salt-depleted rat model has become the standard in studying immunosuppressant nephrotoxicity since salt depletion accelerates the development of histological renal injury in the presence of immunosuppressants (86). However, as salt-depletion itself already has significant effects on the energy metabolism of the kidney, this was not an option for this study.

Isoprostanes are established in vivo markers for oxidative stress (87). After generation by free radical-catalyzed peroxidation of arachidonic acid, they are excreted into urine and can be detected since they are chemically stable.

Surprisingly and in contrast to previous results (78), no significant correlation between 15-F_{2t}-isoprostane concentrations in urine and immunosuppressant tissue concentrations after 28 days were found. Since it has been suggested that especially the enhancement of cyclosporine toxicity by sirolimus is mediated by an increase in reactive oxygen species and subsequently a decrease in mitochondrial energy metabolism (88), an increase in isoprostane concentrations would have been expected when calcineurin inhibitors and mTOR inhibitors were coadministered. It has to be noted that such previous data was generated only using short-term exposure to immunosuppressants in in vitro models (69). Thus, it is reasonable to expect that in the present, more physiological animal model using long-term exposure to the immunosuppressants, kidney cells adjusted to handle increased ROS levels, which explains the absence of detectable differences for oxidative stress among groups.

Overall, histology scores did not show statistically significant differences between Tac, CsA, Evrl and Srl and their combinations (Figures 16-18). In this context, it should be noted that the

primary outcome parameters of the rat toxicity model were the effects of the test drugs on kidney cell metabolism and urine metabolite patterns and not histological injury. Based on previous experience and the fact that no salt-depleted rat model was used, it was reasonable to expect histological changes after 28 days of treatment to be rather mild, and mostly insufficient to discriminate between treatment groups.

However, others have used a salt-depleted rat model to show that everolimus in combination with CsA indeed may lead to significantly less tubular injury than the coadministration of corresponding doses of sirolimus and CsA (89).

Besides measurement of creatinine and glomerular filtration rate, histology analysis of a kidney biopsy is still considered the current gold standard. However, this diagnostic strategy is not without problems. In many centers the procurement of a kidney biopsy, which bears the risk of complications such as bleeding, is guided by a rise in creatinine concentrations in serum. Thus, biopsies are usually taken at a relatively late time point when the disease process has already caused significant damage and is already driven by secondary disease processes such as inflammation and fibrosis. At such a late stage, it is difficult to determine the underlying cause that originally triggered the histopathological changes.

In many cases, the histological findings are inconclusive and do not allow for determination of the original disease mechanism or drug effects that triggered the processes leading to kidney injury and the observed histopathological changes.

As mentioned above, alterations such as interstitial fibrosis and tubular atrophy, as well as glomerular injury are non-specific responses to injury. Endothelial activation, immunosuppressive-induced toxicity, additional diseases, chronic inflammation, nonspecific immune mechanisms as well as diabetes mellitus have all been invoked as potential etiologies (28). Quality of histological sections should be critically evaluated. As most kidney injury processes are focal and not evenly distributed all over the kidney, additional slices may be needed to reduce the risk of false negatives (90). On the other hand, the evaluation of metabolite and protein changes in urine as a so-called “liquid biopsy” is an attractive concept (28). Urine as a proximal fluid is in constant contact and communication with the organ of interest. Metabolites and proteins are exchanged between urine and kidney cells via physiological processes that may be altered by diseases and drug effects. Under pathological conditions such as cell death, injury and repair, cells may also leak their contents into urine. Thus, the urine proteome and metabolome are dynamic and reflect the sum of processes occurring in the kidney at any given time (28).

Compared to untreated controls, sirolimus dose-dependently increased the negative effects of tacrolimus on urine metabolite patters. Although the results seem to match those with cyclosporine and its combination with sirolimus or everolimus, the differences are not pronounced. A potential explanation is that while cyclosporine trough blood concentrations were well within the concentration range targeted in transplant patients and slightly above, tacrolimus concentrations were at the lower limit and below, indicating that the exposure of tacrolimus was not comparable to that of cyclosporine.

In conclusion, this study showed that

- Long-term treatment of rats with immunosuppressant drug doses resulting in blood concentrations close to the target therapeutic range in renal transplant patients results in specific changes in urine metabolite patterns.
- The urinary metabolites mainly found changed after 28 days of exposure to immunosuppressants and their combinations of calcineurin and mTOR inhibitors were hippurate, creatinine, glucose, succinate, citrate, alpha ketoglutarate, and trimethyl aminooxide (TMAO).
- Isoprostanes, stable oxidative stress marker, were not found to be changed in comparision to baseline after treatment with immunosuppressants for 28 days.
- When combined with calcineurin inhibitors, sirolimus had different effects on urine metabolite patterns compared to the structurally related everolimus, which seemed to have a markedly less negative effect.

6. Results and Discussion of the Clinical Trial

6.1.Human Study Results

6.1.1. GFR and Serum Creatinine

Changes in glomerular filtration rate (GFR) were estimated using the MDRD (4 parameter) formula at different time points of certain patients in each study group. These were as follows: group I, patients with at least one event of immunosuppressant toxicity, group II: patients with at least one clinically relevant rejection episode, group III: patients with at least one event of immunosuppressant toxicity and clinically relevant rejection, group IV: patients with neither immunosuppressant toxicity nor rejection and group V: healthy individuals.

As expected, the average GFR (±standard deviations) improved during the first week after transplantation. (Figure 20) After 2 weeks, GFR of patients without events (group IV) started to improve more significantly than in those groups with events (toxicity, rejection or both, groups I-III). These findings in GFR also confirmed the assignment of patients to the no-event and adverse event groups. For more details, please see Figure 20.

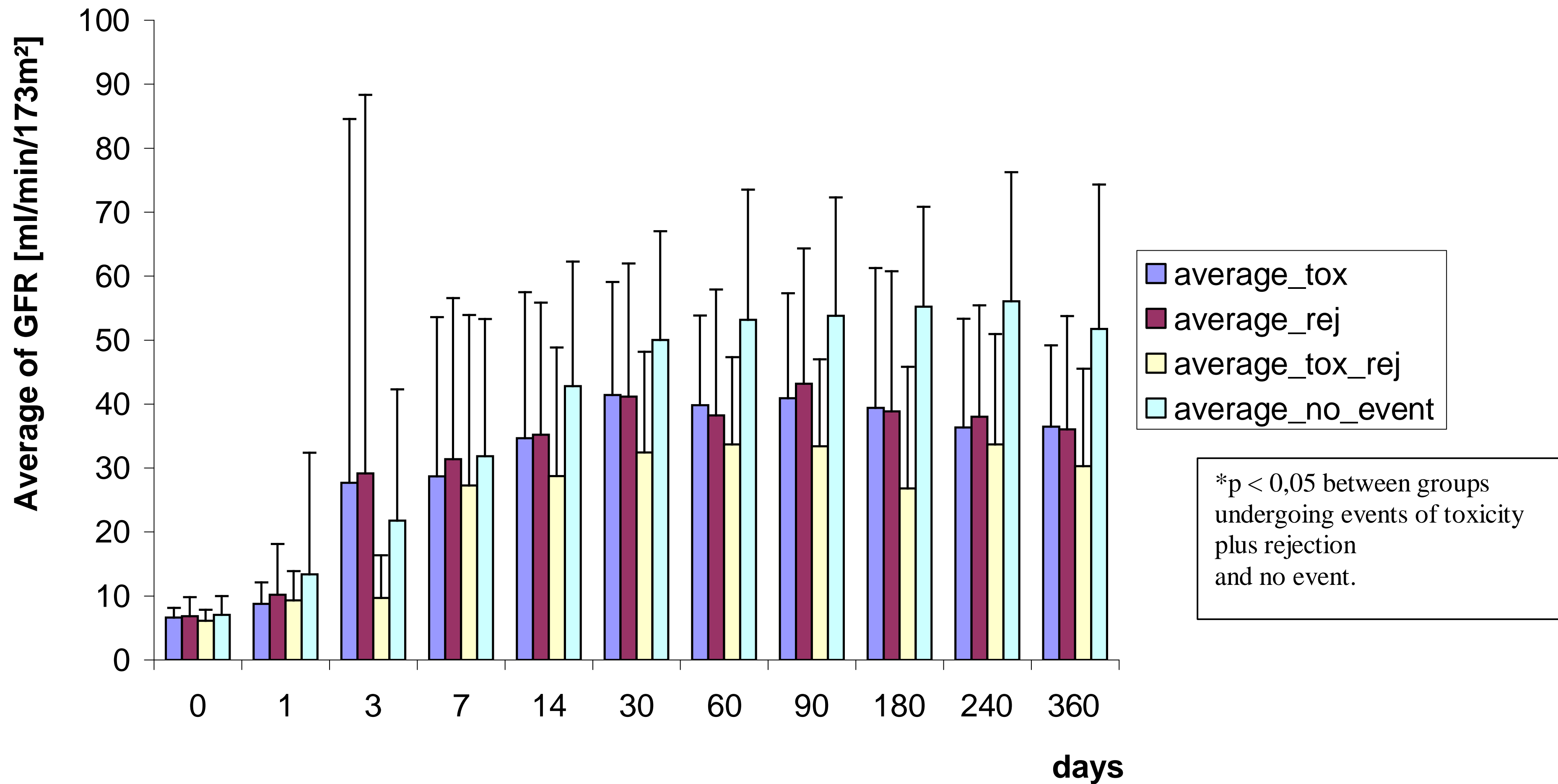


Figure 20. *Longitudinal changes of GFR after MDRD formula (average ± standard deviations) in the study groups over the observation period of 1 year after de novo kidney transplantation. Group I with at least one biopsy-proven event of immunosuppressant toxicity, group II with at least one biopsy-proven rejection episode, group III with both biopsy-proven immunosuppressant toxicity and rejection, group IV with neither*

immunosuppressant toxicity nor rejection events. [Statistical significance by One-way Anova were found between group III and IV. Rej: rejection, tox: toxicity.].

6.1.2. Immunosuppressant Blood Drug Concentrations

In the clinical study, tacrolimus blood concentration in patients with no event ranged from 1.7ng/ml to 22.3ng/ml (a representative example is shown in Figure 21 and in the Appendix, Figures N.1. and N.2.).

Tacrolimus blood concentrations in patients diagnosed with at least one event of biopsy-proven immunosuppressant toxicity ranged from 1.5 ng/ml to 15.7 ng/ml (a representative example is shown in Figure 22 and Appendix, Figures O.1. and O.2.).

Surprisingly, tacrolimus blood concentrations in patients diagnosed with biopsy-proven rejection events tended to be higher than in the other two groups and ranged from 3.2 ng/ml to 36 ng/ml (a representative example is shown in Figure 23 and Appendix Figures P.1. and P.2.).

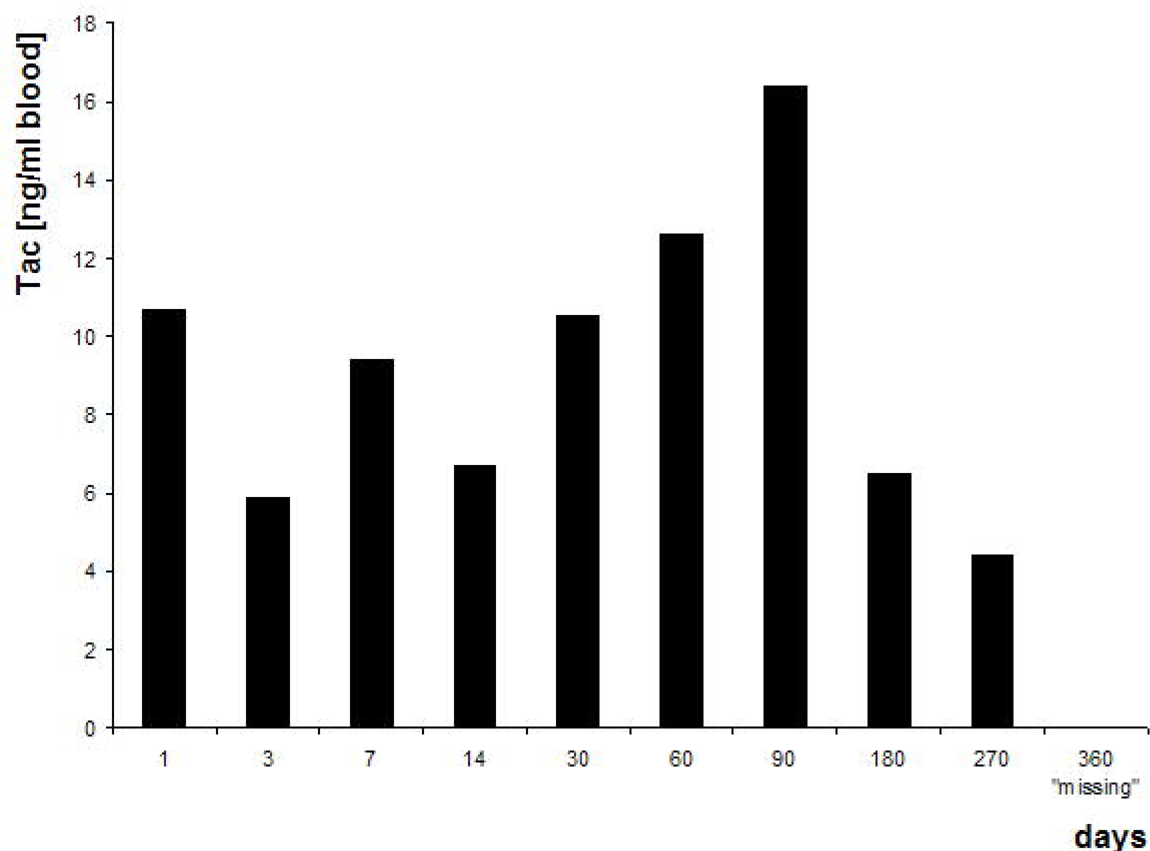


Figure 21 *Tac blood concentrations of patient # 4 as followed over the observation period of 360 days. No event of toxicity or rejection happened under treatment with cyclosporine [Tac: tacrolimus. Value day 360 is missing].*

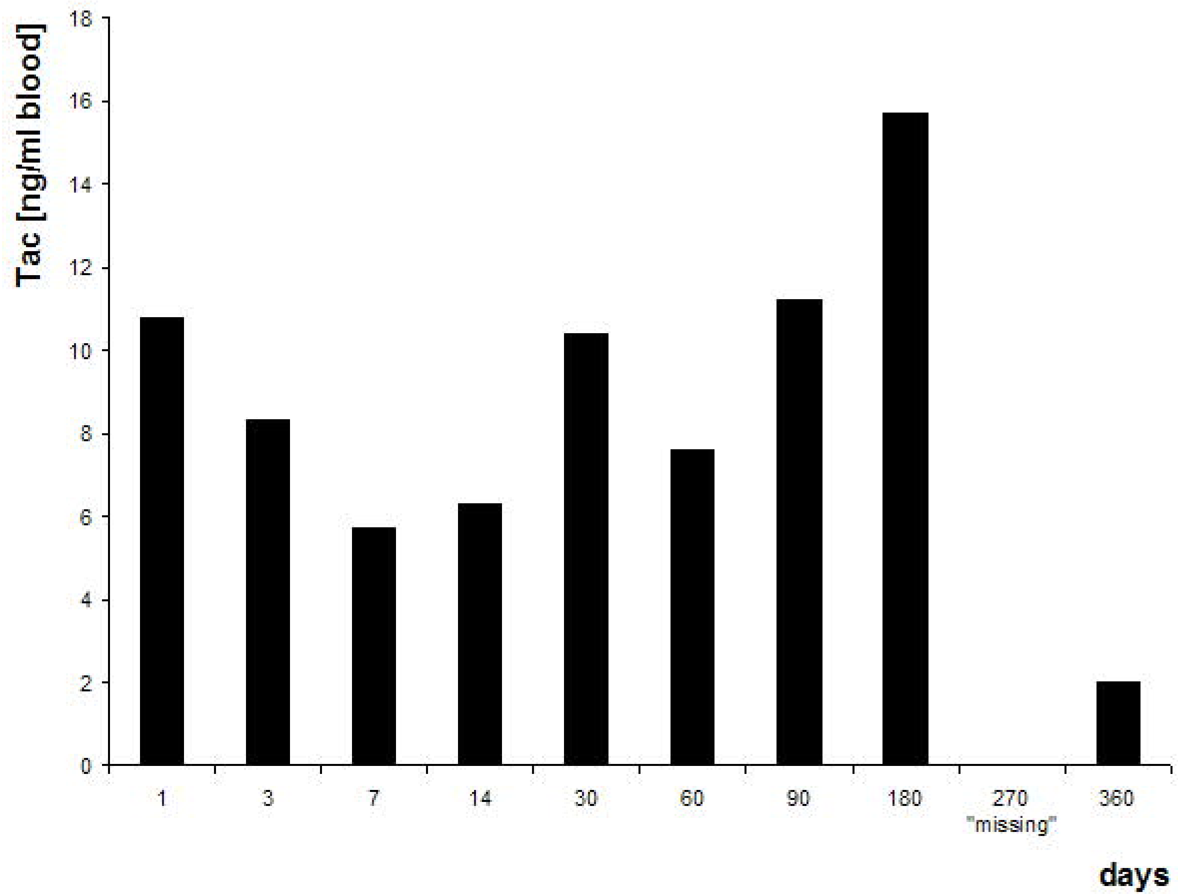


Figure 22 *Tac blood concentrations of patient # 1 as followed over the observation period of 360 days. Biopsy-proven toxicity was diagnosed on day 26 under treatment with tacrolimus. [Tac: tacrolimus. Value day 270 is missing.].*

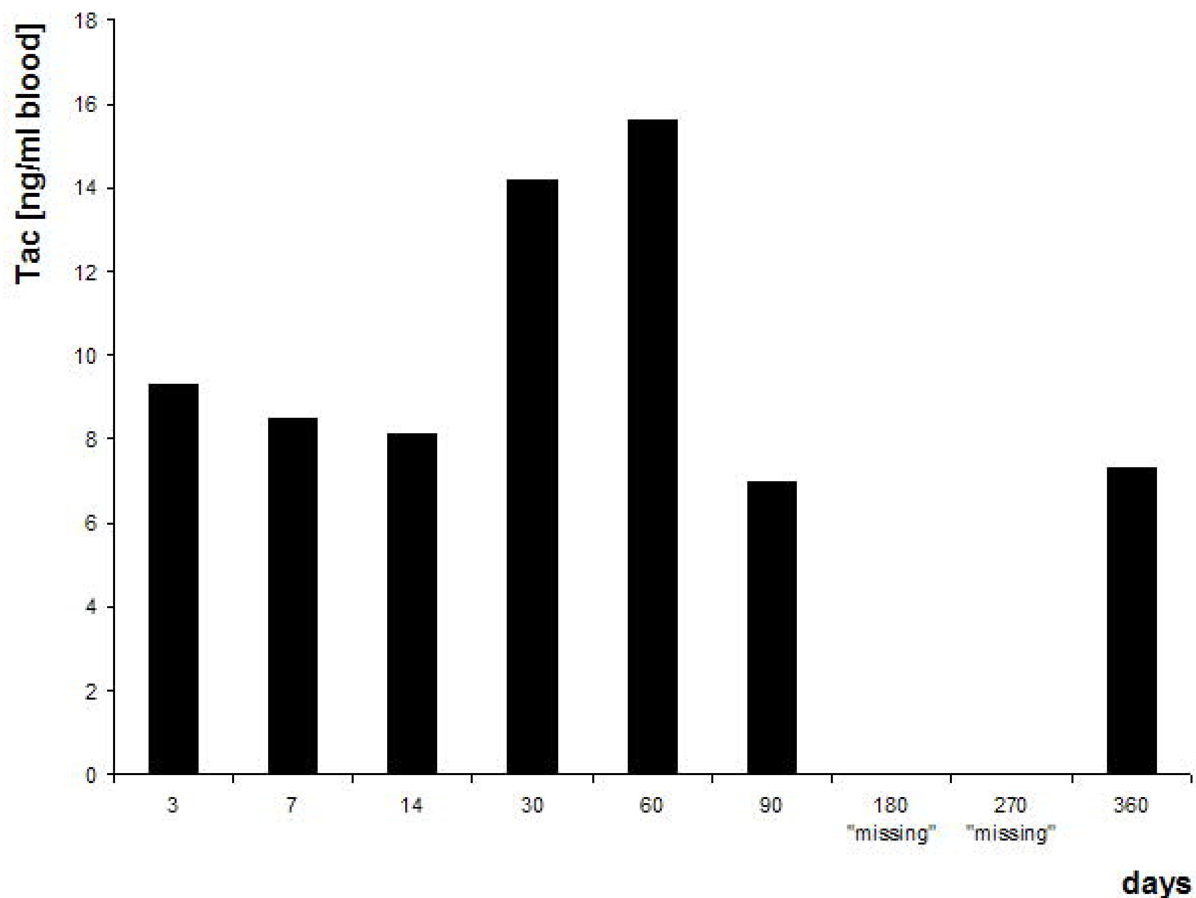


Figure 23. *Tac blood concentrations of patient # 12 as followed over the observation period of 360 days. Biopsy-proven rejection was diagnosed on day 6. [Tac: tacrolimus. Values days 180 and 270 are missing.].*

CyA blood concentration of patients with no event ranged from 73 to 305 ng/ml (a representative example is shown in Figure 24 and Appendix Figure Q.1.). CyA blood concentrations of patients with biopsy-proven rejection were similar and varied from 55-354 ng/ml (Figure 25).

As suggested by Figure Q.2, Appendix blood concentrations of cyclosporine seemed lower around the time of diagnosis of rejection. However, this observation was not backed up by statistically significances.

The representative sirolimus blood concentrations of stable kidney transplant patients without any diagnosed events of immunosuppressant toxicity or rejection over the observation period of 360 days are shown in the Appendix, Figure R.

Thus, in summary the data did not reveal any significant correlation between drug dosage, drug blood concentration and biopsy-proven immunosuppressant toxicity or allo-immune events occurring during the first year (360 days) after transplantation (Figures 26 and 27). These findings could be a result of the varying time periods between the scheduled sample draws and the diagnosis via biopsy. Also in the small study population, toxicity and rejection episodes were

rare events. Thus, small differences among groups may not have been detectable in the descriptive data analysis.

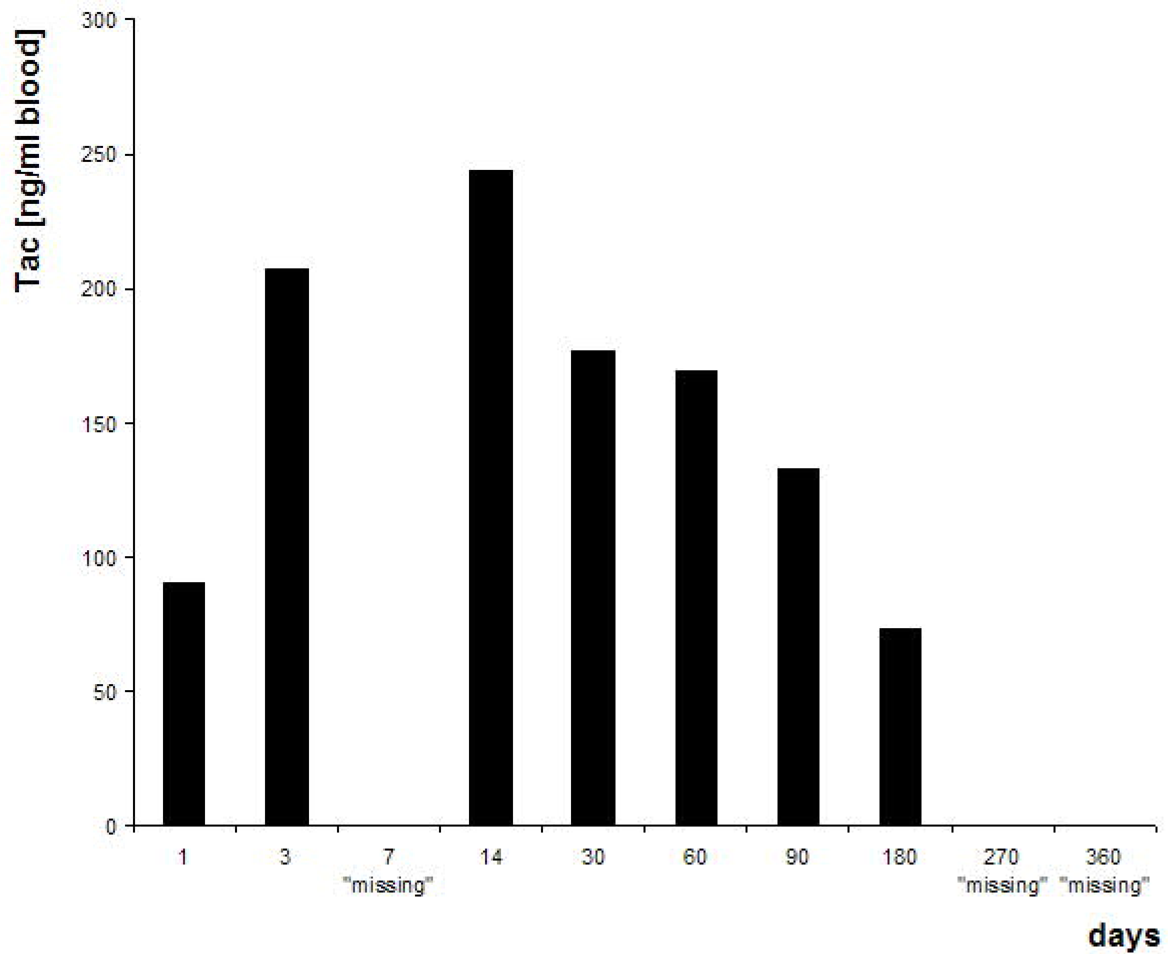


Figure 24. *Cyclosporine blood concentrations of patient # 48 as followed over the observation period of 360 days. No event such as alloimmune reaction against the transplant kidney or immunosuppressant toxicity was diagnosed. [CyA:cyclosporine. Values days 7, 270 and 360 are missing].*

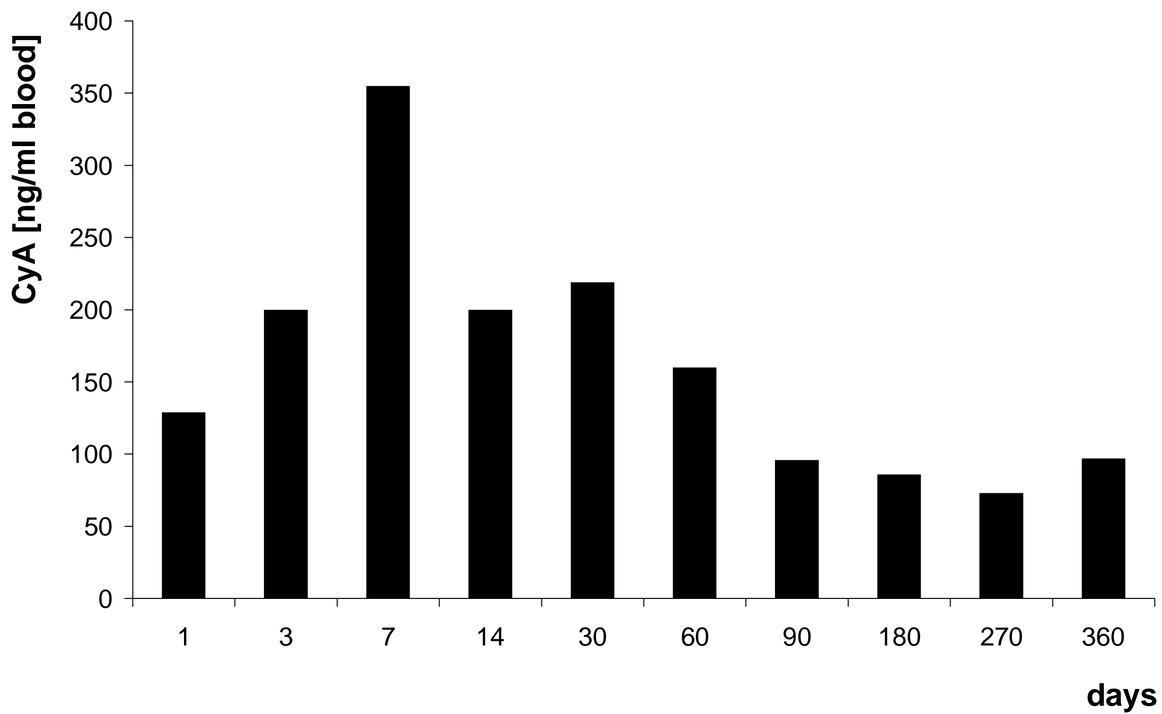


Figure 25. *CyA blood concentrations of patient # 2 as followed over the observation period of 360 days. Biopsy-proven rejection was diagnosed on day 11 under treatment with cyclosporine [CyA:cyclosporine.].*

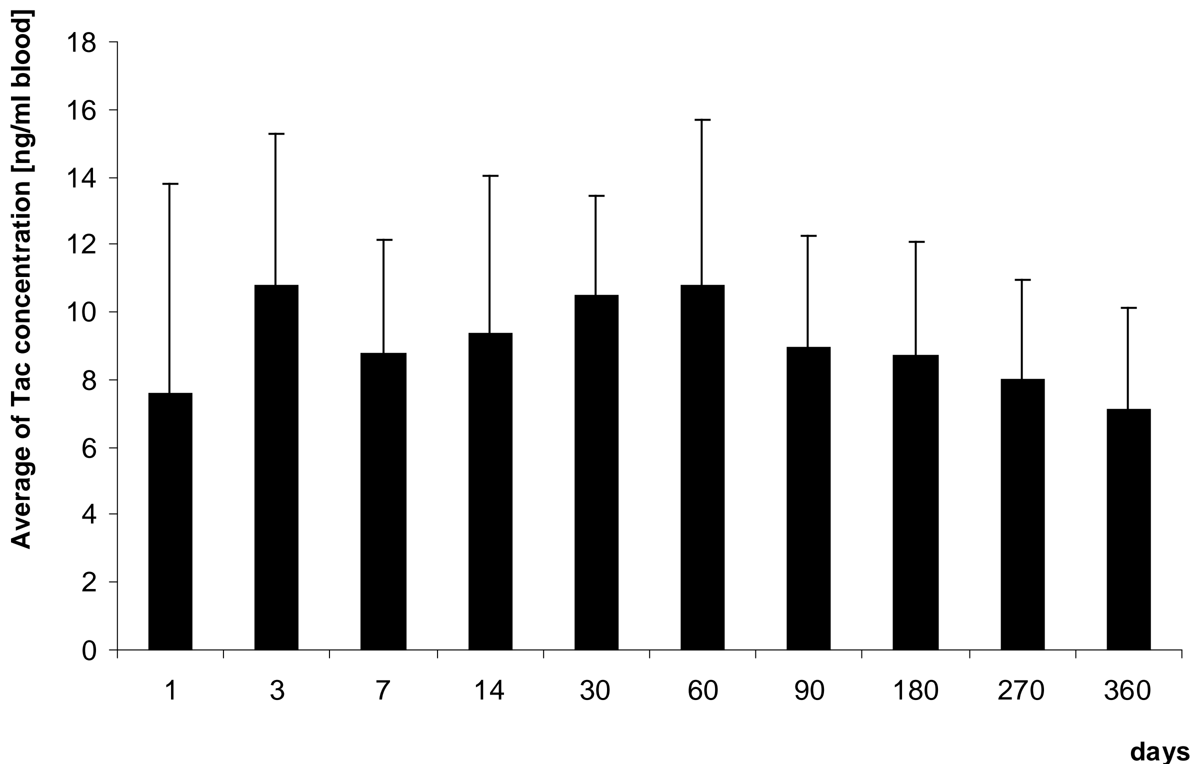


Figure 26. *Average of tacrolimus blood concentrations ± standard deviation over 360 days in patients who received only a tacrolimus-based immunosuppressive drug regimen (n=25) [Tac:tacrolimus.].*

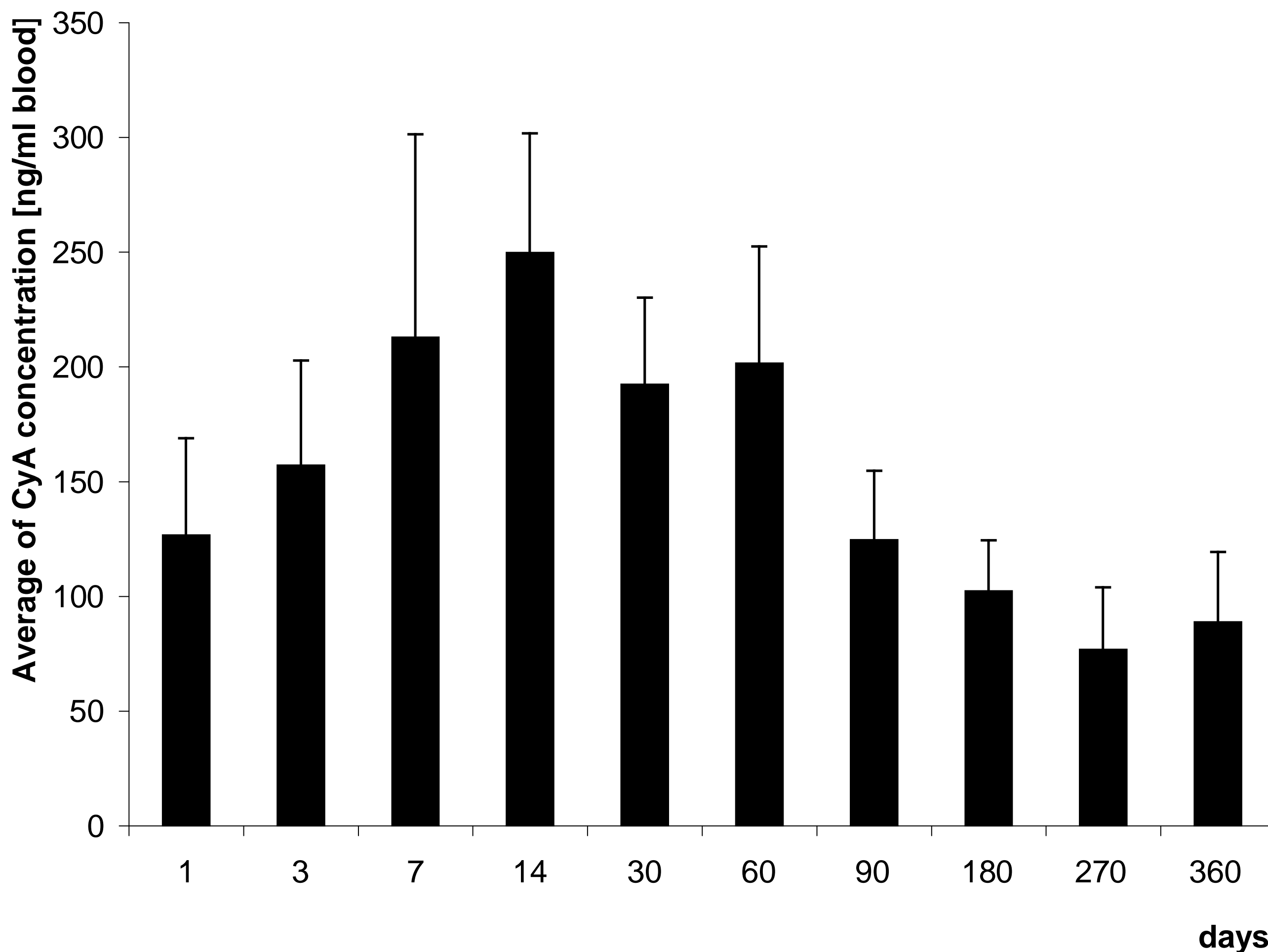


Figure 27. *Average of cyclosporine blood concentrations ± standard deviation over 360 days in patients who received only a CyA-based immunosuppressive drug regimen (n=6). [CyA:cyclosporine.].*

6.1.3. Analysis of 15-F2t-Isoprostanes in Human Urine and Plasma

Urinary isoprostane concentrations were normalized to urine creatinine concentrations. In healthy volunteers the isoprostane concentrations ranged between 278 and 716 pg/mg creatinine (Figure 28). In the patients with biopsy-proven events of immunosuppressant toxicity, isoprostane concentrations ranged between 74 and 851 pg/mg creatinine, while the patients with at least one documented rejection episode had concentrations ranging from 120 to 1066 pg/mg creatinine. Isoprostane concentrations of 21 transplant patients without the diagnosis of either biopsy-proven immunosuppressant toxicity or rejection (group IV) exhibited a range from 34 to 1737 pg/mg creatinine.

The analysis of urinary isoprostane concentrations of a total 16 patients diagnosed with an event of rejection as proven by biopsy (seven of them underwent an event of nephrotoxicity as well (group III), showed relatively high urinary isoprostane concentrations (n=10, 62.5%) (see representative examples in Figure 29 and Appendix, Figure S). In the other six out of 16 patients, the urinary

isoprostane concentrations were not different than in the other patient groups. Also, isoprostane analysis of urine from patients with no biopsy-proven events of immunosuppressant toxicity or rejection after kidney transplantation also showed substantial variability (Figures 30A and B). Moreover, analysis of isoprostanes in urine of 18 further patients which had been diagnosed with immunosuppressant nephrotoxicity as confirmed by biopsy (seven of them also had biopsy-proven rejection) showed no distinctive differences.

In these patients, significant increases of serum creatinine levels were found in 11 cases (representative patients are shown in Figure 31A and Appendix, Figure T.1.). It is important to note that the analyzed data of individual patients were followed longitudinally. When distribution statistics were calculated and the patient groups were compared, significant differences in neither the isoprostane in urine and blood nor in creatinine concentrations among patients with an event of immunosuppressant toxicity and/or rejection (groups I-III) or patients without any such event were found (group IV).

While in urine at least some patients seemed to show isoprostane concentration peaks preceding an occurrence of immunosuppressant and/or rejection events (Figure 30A and B), no such observations were made in plasma (Figure 32A-C and Appendix Figure U.1.).

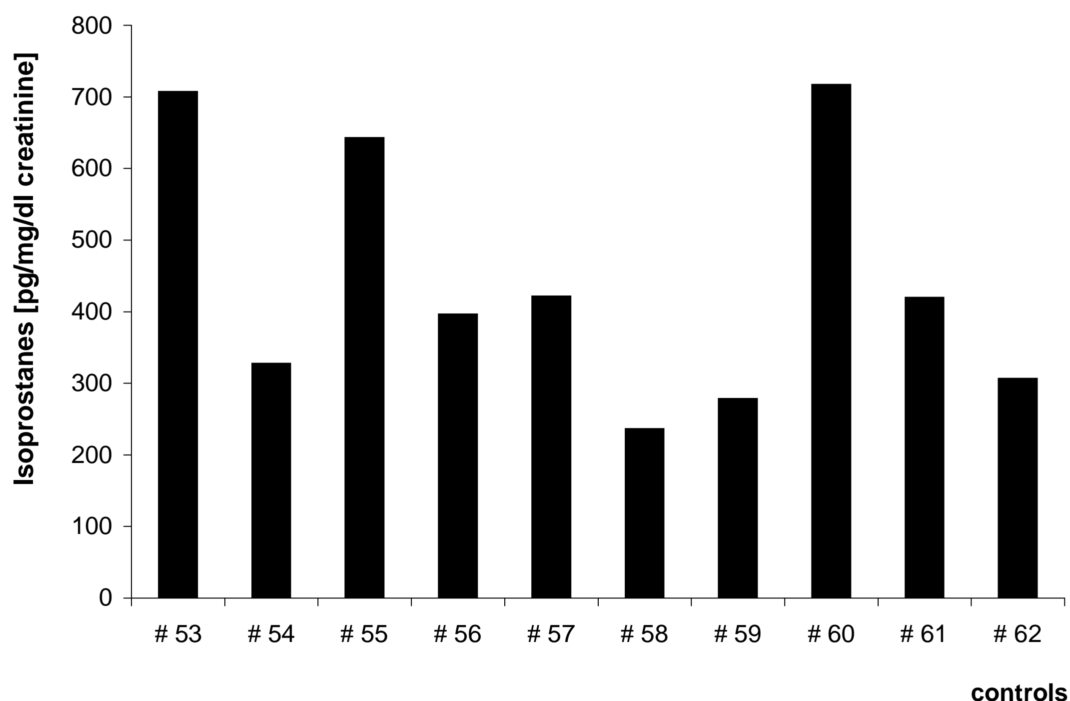


Figure 28. *Isoprostane concentrations in urine of ten healthy volunteers without any known disease. Range from 278 to 716 pg/mg creatinine.*

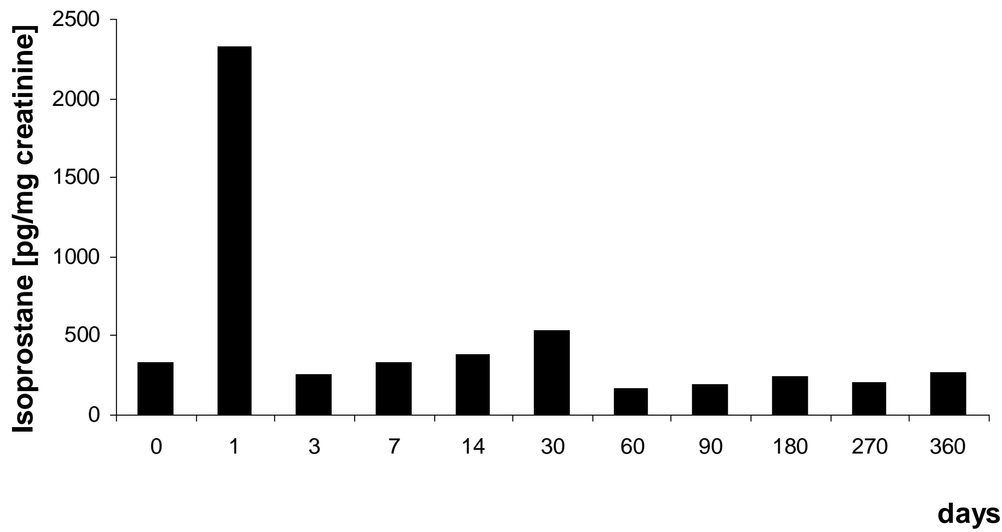


Figure 29. *Representative isoprostane concentrations in urine of patient #26. Biopsy-proven rejection episodes were diagnosed on days 59 and 77. A peak of isoprostane concentration before days of events could be observed [Crea: creatinine].*

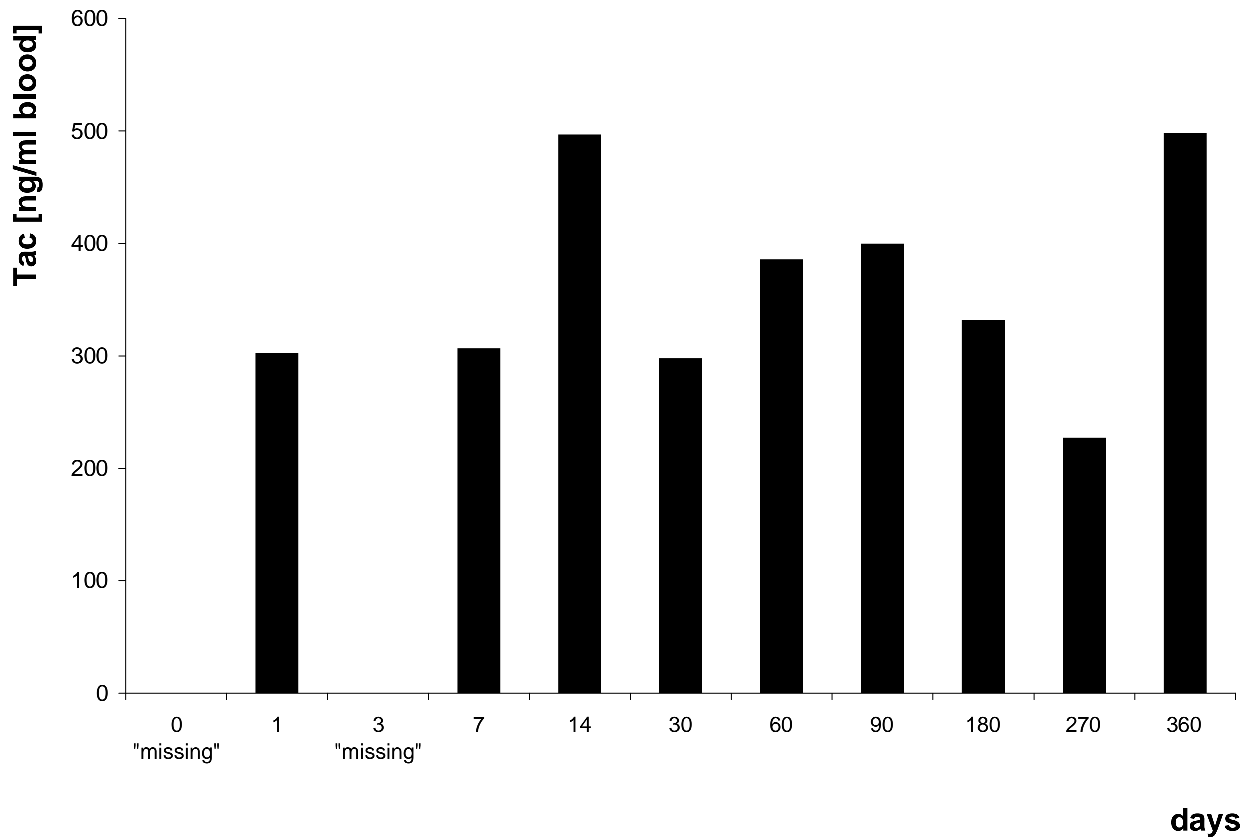


Figure 30A. *Representative isoprostane concentrations in urine of patient # 11. This patient was not diagnosed with nephrotoxicity and/ or rejection. [Crea: creatinine. Values days 0 and 3 are missing.].*

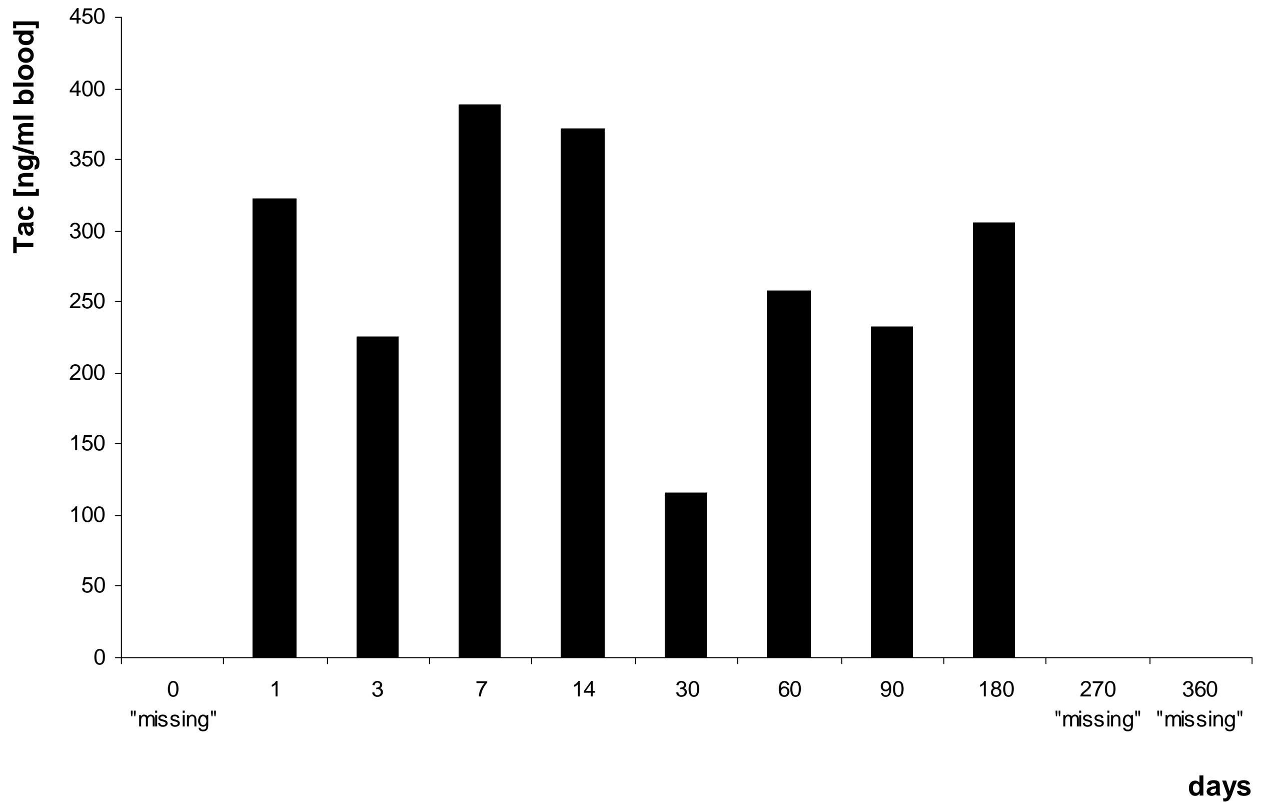


Figure 30B. *Representative isoprostane concentrations in urine in patient # 44 with biopsy-proven nephrotoxicity on days 2 and 5. No peak of isoprostane concentrations around these events could be observed. [Crea: creatinine. Values days 0, 270 and 360 are missing].*

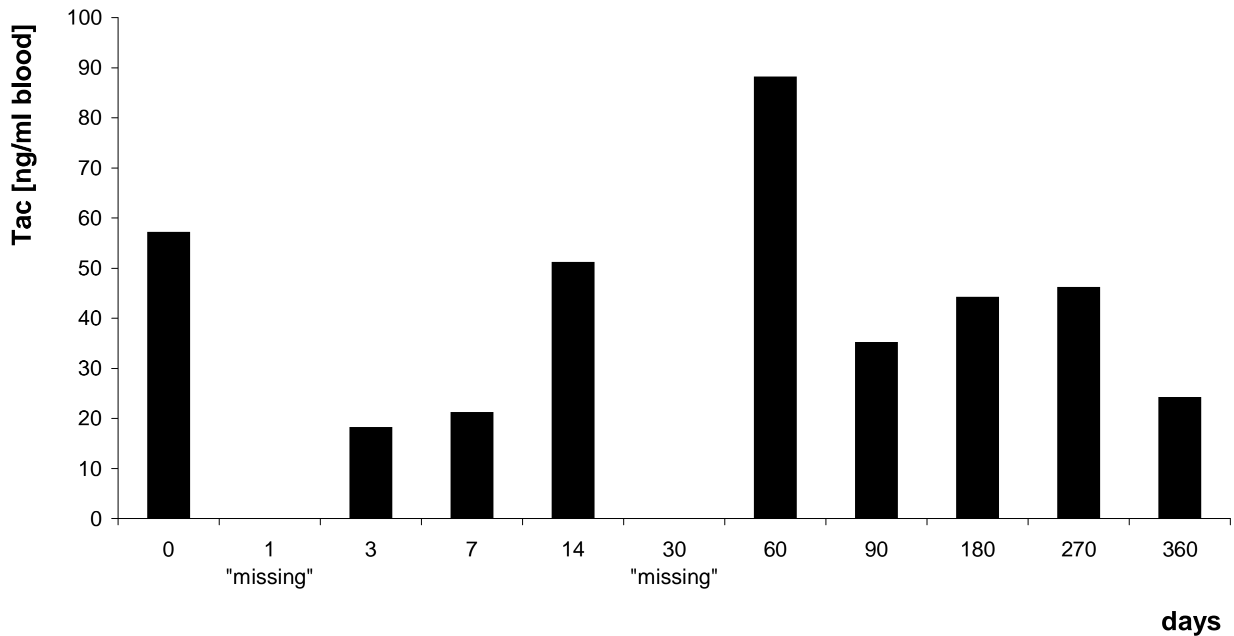


Figure 31A. *Creatinine concentrations in serum of patient # 24 who had a biopsy-proven event of immunosuppressant nephrotoxicity diagnosed on day 60. [Crea: creatinine. Values days 1 and 30 are missing.].*

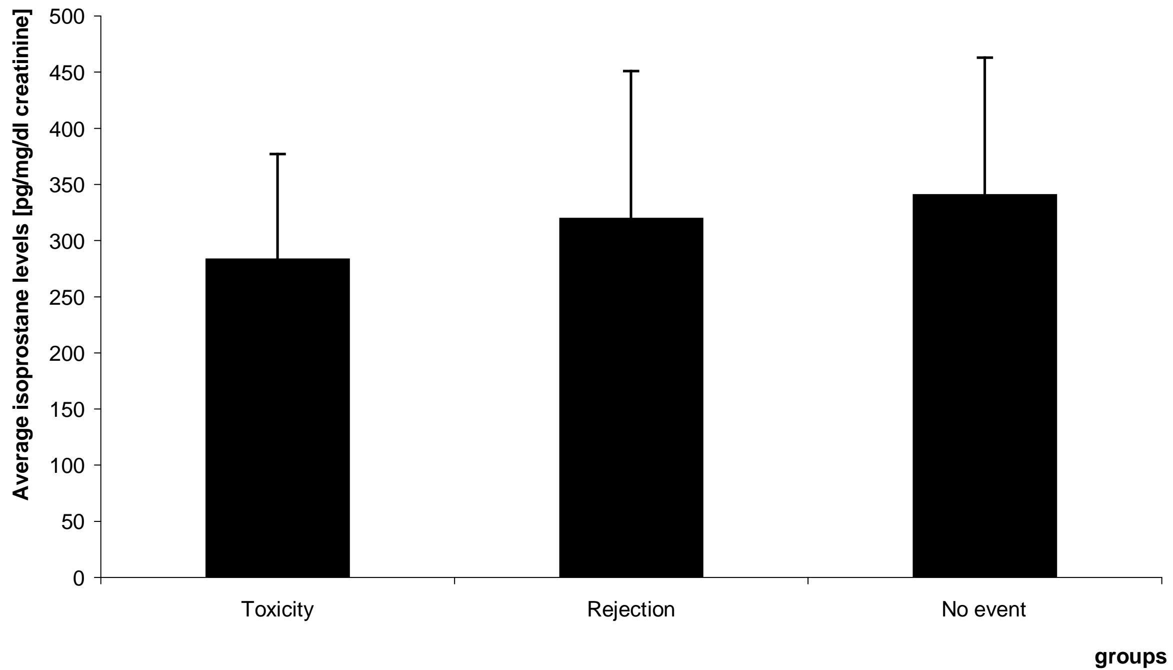


Figure 31B. Average (±standard deviations) of isoprostane concentrations normalized based on creatinine in urine in the study groups: patients with at least one event of biopsy-proven immunosuppressant toxicity (group I), at least one event of biopsy-proven rejection (group II) or no event (group IV). No differences were found.

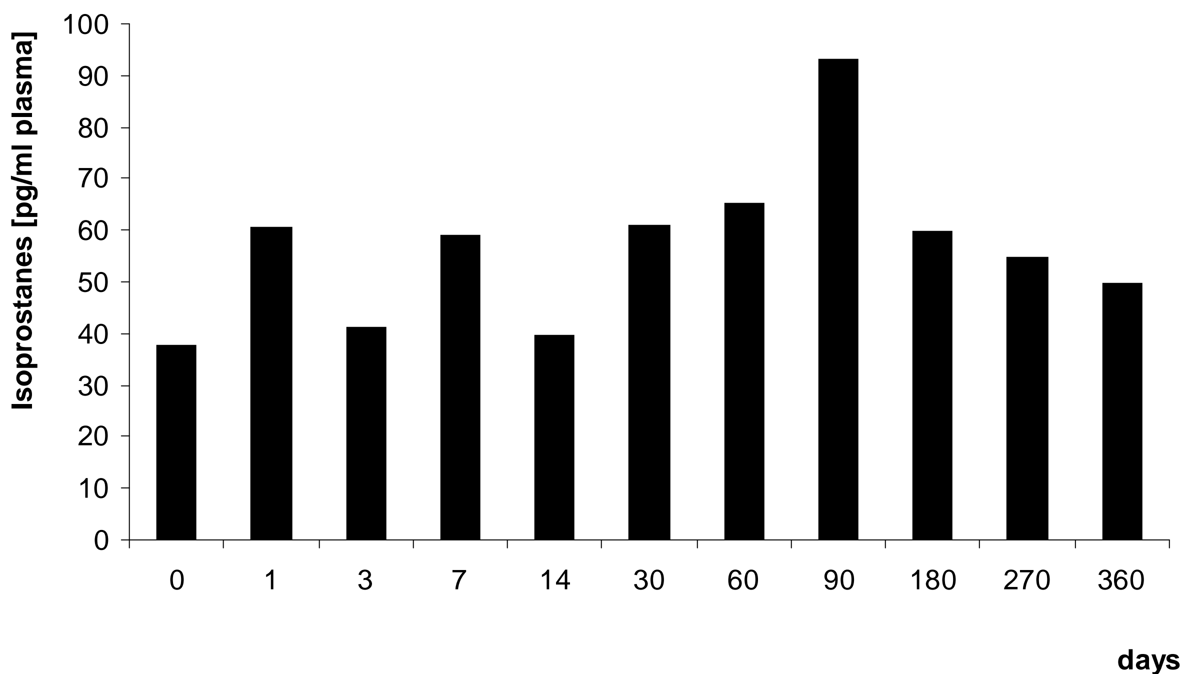


Figure 32A. Representative isoprostane concentrations in plasma of patient # 26 with a biopsy-proven alloimmune reaction on days 59 and 77. No peak of isoprostane concentration associated with the events could be observed.

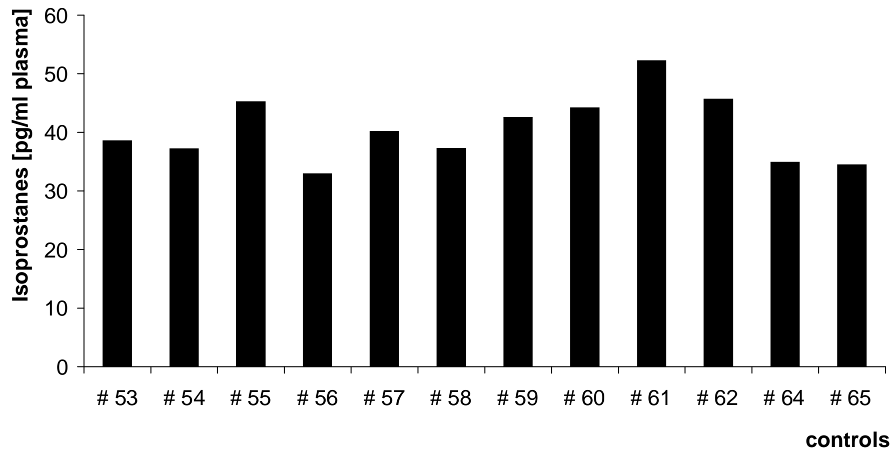


Figure 32B: *Isoprostane concentrations in plasma of patients in the control group. No statistical significance or trend could be observed.*

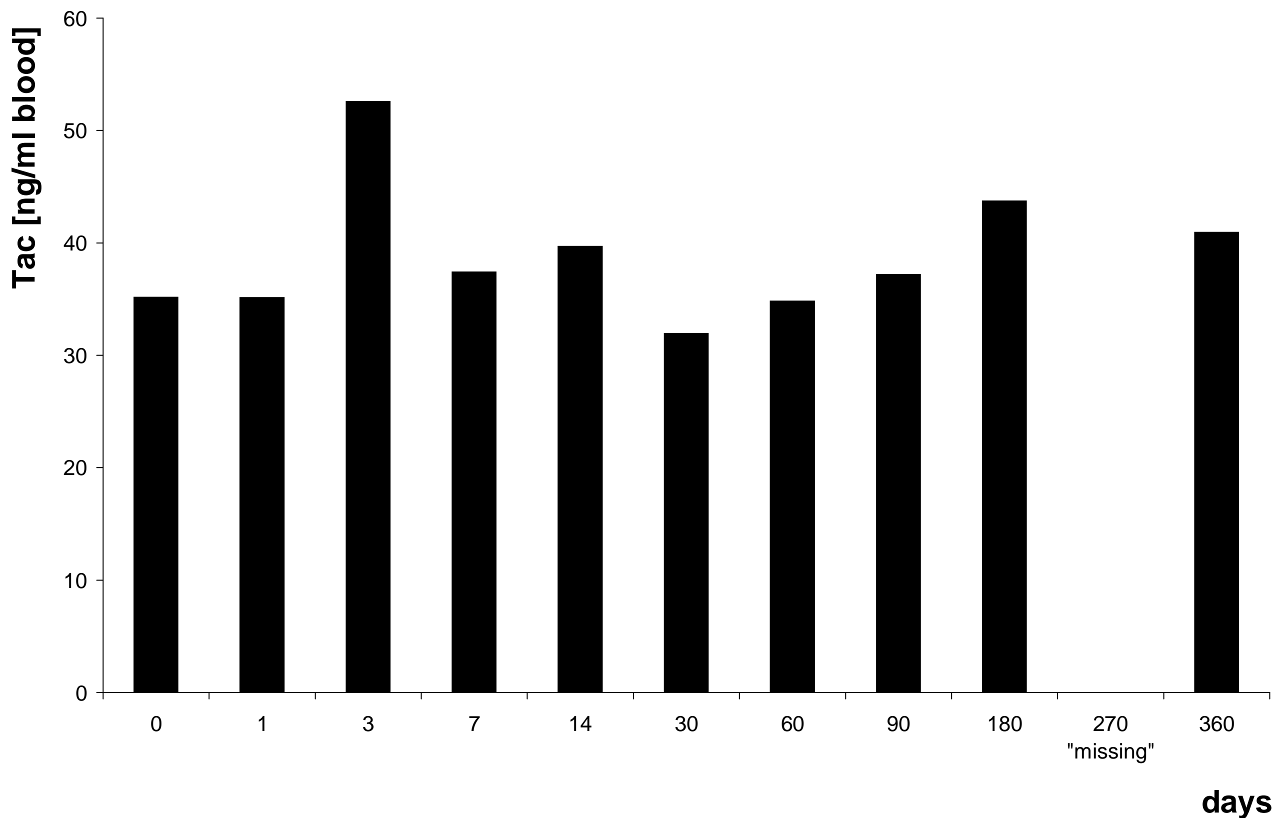


Figure 32C*. Representative isoprostane concentrations in plasma of patient #27 who had a biopsy-proven event of immunosuppressant nephrotoxicity diagnosed on day 147. No peak of isoprostane concentration around the event could be observed. [Value day 270 is missing.].*

6.2. Discussion of the Clinical Trial

Serum creatinine concentrations early after transplantation are an important predictive marker of graft survival (91). Monitoring changes in glomerular filtration rate (GFR) is the recommended gold standard method for assessing the progression of kidney disease (92).

Consequently, effective monitoring of early graft function and early detection of dysfunction will reduce the rate of late graft failure. However, as mentioned before, serum creatinine is neither a very sensitive nor a specific marker of early kidney graft function. Thus, guidelines have recommended estimating GFR using the MDRD (Modification of Diet in Renal Disease) formula, which takes into consideration further variables such as age, sex and race (93).

In addition to kidney graft biopsies and histological analysis following Banff criteria, GFR is considered one of the gold standard clinical outcomes parameters after kidney transplantation.

Drug	Starting oral dose	Target trough concentration range initial (first 90 days)	Maintenance
Cyclosporine	8 -17 mg/kg /day	250 to 350ng/ml	150 to 250ng/ml
Tacrolimus	0.3 mg/kg /day	10 to 20ng/ml	5 to 15ng/ml
Sirolimus	5-15 mg/day	10 to 15ng/ml	5 to 10ng/ml
Mycophenolate Mofetil	2-3 g/day	1,3-3,5mg/L	1,3-3,5mg/L

Table 8. *Recommended immunosuppressive drug doses and target trough blood concentration ranges for kidney transplant patients (94).*

Most immunosuppressants used for the prophylaxis of allograft rejection in transplant patients such as calcineurin inhibitors, mTOR inhibitors and possibly mycophenolic acid are considered narrow therapeutic index drugs. It is also known that, partially due to genetic polymorphisms of drug metabolizing enzymes such as CYP3A5 as well as drug transporters and nuclear receptors, the oral bioavailability and pharmacokinetics of these immunosuppressants exhibit marked inter-individual variability. Therefore most immunosuppressants used in transplantation require therapeutic drug monitoring and dose adjustments based on blood/plasma levels to maintain trough blood concentrations within the therapeutic target range (Table 8). However, especially with narrow therapeutic index drugs, of much greater concern than inter-individual variability is intra-individual variability. Variable exposure to immunosuppressants in an individual transplant patient can lead to episodes of drug concentrations that are considered too low, resulting in an increased risk of acute or chronic rejection. Thus, individual patients may require different doses

and different target trough blood concentrations. Where drug concentrations are too high, the patient is exposed to a higher risk of immunosuppressant toxicity. It is well established that patients with high intra-individual variability of their systemic immunosuppressant exposure, e.g. African American patients, have poorer long-term outcomes than patients with stable exposure. Intra-individual variability can be caused by a number of factors, including, but not limited to: non-compliance, gastrointestinal function, drug-drug interactions, disease-drug interactions, and food-drug interactions.

No significant trend could be observed between drug dosage, drug blood concentration and immunosuppressant toxicity or rejection events occurring during the 360 days period of our transplanted patients.

It needs to be taken into account that in the present study, kidney biopsies were not protocol biopsies but were procured “for cause”, i.e. only when there was a clinical indication. Thus, trough blood levels as collected do not have a clearly defined temporal relationship with the procurement of the biopsies and may not have been collected at the time when relevant fluctuations in blood trough concentrations occurred. Therefore, although the data comparing the study groups as well as analyzing individual patients did not show any association between immunosuppressant blood trough concentrations and the clinical events of interest (immunosuppressant toxicity and rejection), due to the study design such an association cannot be entirely excluded.

F_2-isoprostanes, stable isomers of prostaglandin $F_{2\alpha}$,, are considered a reliable index of in vivo oxidative stress (95). Of the 64 potential F_2-isoprostane isomers , the most commonly used marker for oxidative stress is 15-F_{2t}-isoprostane (8-iso PGF_{2a}) . Elevated plasma and urine concentrations of isoprostanes have been reported, among others, in cardiovascular and pulmonary disease , Alzheimer disease and type-2 diabetes (95) .

Previously, it was shown by the workgroup that a single dose of cyclosporine in healthy volunteers is associated with an increase of urinary 15-F_{2t}-isoprostane concentrations already 4 hours after administration, whereas the administration of placebo showed no significant changes (22). The conclusion was that a single dose of cyclosporine has the potential to increase the oxidative stress in healthy patients (22).

To investigate as to whether chronic rejection or toxicity events will impact isoprostane concentrations and therefore induction of oxidative stress in the clinical study, I chose to profile the urinary as well as plasma 15-F_{2t}-isoprostane concentrations in kidney transplant patients.

The wide range of urinary isoprostane concentrations observed in all patient groups a can most likely be explained by the multimorbidity of these patients. It is well known that concentrations of

isoprostanes are increased in a large number of human diseases, such as diabetes (96), arteriosclerosis (97) and heart diseases (98). Also, different daily activities and food diets as well as immunosuppressive treatment are known potential causes of changes in the isoprostane concentrations in plasma and urine. The data indicates that urinary isoprostane concentrations are most likely a molecular marker with low specificity for directly transplant related events such as immunosuppressant toxicity and allograft reactions.

It should be reconsidered that creatinine in serum, still the hallmark for monitoring kidney function, is not perfect either. Since isoprostanes were normalized based on creatinine concentrations in urine, this may have affected the data. Normalization based on creatinine in urine to compensate for differences in urine dilutions is only without problems as long as excretion of creatinine through the kidney is not affected. In addition, creatinine in serum is not a very sensitive marker and a substantial amount of nephrons need to be damaged before creatinine concentrations in serum show a noticeable increase (28).

The results of the present clinical trial confirm previous results from the animal study (see 5.1.3 Analysis of 15-F_{2t}-Isoprostanes) in which no increase of isoprostane concentrations after treatment with high doses of immunosuppressants was found.

Another possible explanation, as mentioned in association with the animal results, is that kidney cells can be expected to adjust to better handle increased oxygen radical concentrations during chronic exposure to immunosuppressive drugs. In the clinical study, many other factors may have influenced the production of oxidative stress such as the fact that all of these kidney transplant patients received high doses of corticosteroids, which have been suggested to have antioxidant properties both in vitro and in vivo (99). Therefore, even if my hypothesis that the nephrotoxicity is associated with oxidative stress and impaired mitochondrial function is correct (100, 101), the real effects of immunosuppressive drugs on oxygen radical production could have been masked or compensated for by other drugs and possible drug-drug interactions.

7. Conclusions and Perspectives

Based on the results of the clinical trial, I conclude:

- There was no association between drug blood concentrations and biopsy-proven alloimmune or immunosuppressant nephrotoxicity.
- As already indicated by the rat studies, isoprostanes in urine and plasma seem to be more of an acute marker rather than a marker for long-term monitoring of renal transplant patients.

In this regard, it will be interesting to see how sensitive urinary metabolite changes based on ^{1}H-NMR spectroscopy and chromatography analyses will be. The rat studies already showed promising results (84) and the workgroup had previously shown that there exist further interesting results in healthy individuals exposed to a single dose of 5 mg/kg cyclosporine (102). This work has been continued after I finished the experimental part of my thesis work. First results based on the samples from the clinical trial described that were published at the 60 th American Society of Mass Spectrometry Meeting in Vancouver in 2012 look already encouraging (103).

Individual patient samples collected during time points preceding biopsy-proven acute rejection and/or immunosuppressant nephrotoxicity events as well as time points devoid of any graft dysfunction were analyzed. Levels of hippurate, TMAO, and sorbitol displayed significant changes with p values $< 0,05$. Creatinine, was less sensitive than the novel urine metabolite patterns in differentiating between stable and transplant patients with transplant kidney dysfunction.

Further analysis of the samples from this study have revealed another interesting set of metabolite markers: the trans-methylation pathway intermediates S-adenosyl homocysteine and S-adenosyl methionine which were analyzed using a highly sensitive and specific LC-MS/MS assay in plasma (83). Although increased levels of the thiol metabolites S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) have been implicated as markers for renal and vascular dysfunction, until now there have been no studies investigating their association with clinical post-transplant events such organ rejection and immunosuppressant nephrotoxicity. This study showed that SAM and SAH concentrations were significantly elevated in renal transplant patients preceding documented acute rejection and nephrotoxicity events when compared to healthy controls (n=8) as well as transplant patients devoid of allograft dysfunction (n=8) (83).

Overall, this thesis took a translational approach to identify potential molecular marker panels that have potential to be used in the management of transplant patients. First clinical data based on a prospective longitudinal proof-of-concept and pilot study clinical trial suggested that these markers are more sensitive and predictive than creatinine in serum. These markers will now have to be qualified in a larger prospective clinical trials.

It is reasonable to expect that such management tools will allow for better individualization, and will provide the basis for more efficient clinical risk evaluation and management strategies, and thus that they will have a positive effect on long-term outcomes after renal transplantation.

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9. Abbreviations

API	Atmospheric pressure ionization
CRP	C-reactive protein
CyA	Cyclosporine A
D_2O	Deuterated water
EDTA	Ethylenediamine tetraacetic acid
ESI	Electrospray ionization
Evrl	Everolimus
FDA	U.S. Food and Drug Administration
FK-BP	FK-binding protein
GFR	Glomerular filtration rate
GI	Glomerular injury
H.E.	Hematoxylin/Eosin
HCl	Hydrochloric acid
HEP	Heparine
HPLC	High-performance liquid chromatography
IF	Interstitial fibrosis
IL	Interleukin
IS	Immunosuppression
KHCO3	Potassium bicarbonate
LC/MS	Liquid chromatography with massspectroscopy
mg	Milligram
min	Minute
ml	Milliliter
MMF	Mycophenolate mofetil
MPA	Mycophenolate acid
MS	Massenspectroscopy
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NMR	Nuclear magnetic resonance
PAS	Periodic-acid Shiff
RAD	Tacrolimus
RAPA	Rapamycin (Sirolimus)
SD	Standarddeviation
Srl	Sirolimus
t	Time
T	Temperature
Tac	Tacrolimus
TC	Trichrome
TI	Tubular injury

10. Appendix

Day of Treatmen t	24 h Urine Collection (Urine Metabolomic s and Isoprostanes)	Study Drugs by Oral Gavage	Heparin Blood Draw (Blood Chemistry and Biochemistry)	EDTA Blood Draw (Immuno-suppressants Blood Level)	Harvest - Freeze Clamp Left Kidney (Metabolomic Profiling) Collection of Right Kidney- for Tissue Concentrations of Immunosuppressants and Histology)
00	X				
01		X	X		
02		X			
03		X			
04		X			
05		X			
06		X			
07	X	X			
08		X		X	
09		X			
10		X			
11		X			
12		X			
13		X			
14	X	X			
15		X	X		
16		X			
17		X			
18		X			
19		X			
20		X			
21	X	X			
22		X		X	
23		X			
24		X			
25		X			
26		X			
27		X			
28	X	X			
29		X		X	X

Table A. *Animal study: Schedule of samples procedures.*

Patient numbers	Rejection	Timepoints of rejection (days)	Toxicity	Timepoint of toxicity (days)
N12	br	6	-	-
N2	1b	11	-	-
N7	1a, 1a	7, 119	yes	124
N13	1b	30	yes	36
N16	1a,br	16, 60	-	-
N20	br	249	yes	280
N23	1b	106	-	-
N24	1a, 1a, br, br	74, 97, 170, 180	yes	60
N26	2,2	59, 77	-	-
N52	3,2	8, 17	-	-
N3	br	134	-	-
N33	1a	222	-	-
N42	1a, 1a, 1a, 1b	270, 278, 298, 314	-	-
N1	-	-	yes	26
N5	-	-	yes	70
N9	-	-	yes	41, 53
N14	-	-	yes	2, 16, 67
N17	-	-	yes	12
N27	-	-	yes	147
N29	br	496	yes	1, 19
N30	-	-	yes	180
N34	2, br, br	53, 65, 72	yes	9
N37	br, 1b	8, 23	yes	74
N38	-	-	yes	10
N40	-	-	yes	11
N41	-	-	yes	10
N44	-	-	yes	2, 5

Table B. *Histopathological results proved by clinical biopsy. [Br: Borderline Rejection, 1a: rejection grade 1a, 1b: rejection grade 1b, 2: rejection grade 2.]*

G1367A	autosampler
G1330A	thermostat
G1312A	binary pump
G1322	degasser
G1316A	column-thermostat

G1322	degasser
G1312A	binary pump
G1946A or API4000	mass-selective detector

Table C. The LC-MS/MS-system consisted of the above 1100 HPLC parts

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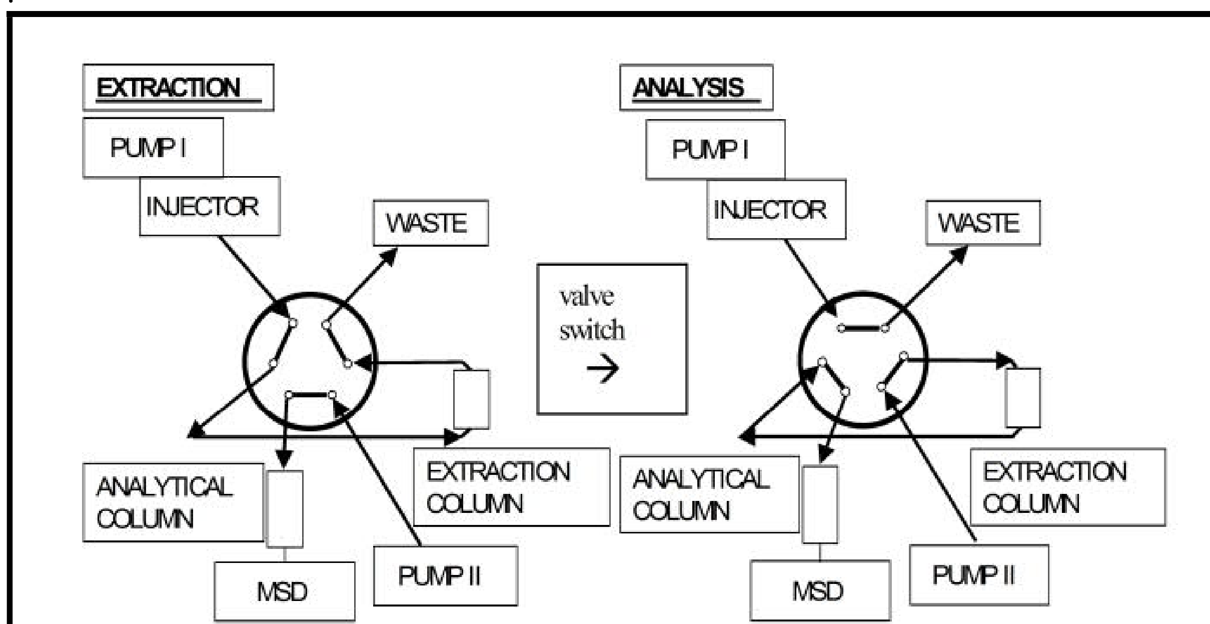


Figure D. *The HPLC system consisted of two columns and two pumps connected via a column switching valve (77).*

Stock #	From Stock #	Take amount [µl from Stock]	Add Amount [µl]	Total Concentration
1		/	/	1 mg/ml
2	1	100 Tac & 100 Srl	800 MeOH	100µg/ml
2b	2	10	990 Plasma	1 ng/ml
3	2a	100	900 Plasma	100ng/ml
4	3	500	500 Plasma	50 ng/ml
5	4	500	500 Plasma	25 ng/ml
6	5	400	600 Plasma	10 ng/ml
7	6	500	500 Plasma	5 ng/ml
8	7	500	500 Plasma	2.5 ng/ml
9	8	400	600 Plasma	1.0 ng/ml
10	9	500	500 Plasma	0.5 ng/ml
11	10	500	500 Plasma	0.25 ng/ml
12	11	400	600 Plasma	0.1 ng/ml
13	12	500	500 Plasma	0.05 ng/ml
14	13	500	500 Plasma	0.025 ng/ml
15	14	400	600 Plasma	0.01 ng/ml
16	15	500	500 Plasma	0.005 ng/ml
QC 1	3	100	400 Plasma	20 ng/ml
QC 2	QC 1	100	400 Plasma	4 ng/ml
QC 3	QC 2	40	360 Plasma	0.4 ng/ml
QC 4	QC 3	80	360 Plasma	0.08 ng/ml
QC 5	QC 3	100	100 Plasma	0.04 ng/ml

Table E.1. *Calibration curves and absolute recovery rate of tacrolimus drug levels.*

Stock #	From Stock #	Take amount [µl from Stock]	Add Amount [µl]	Total Concentration
1		/	/	1 mg/ml
2	1	100	900 MeOH	100 µg/ml
3	2	100	900 Plasma	10 µg/ml
4	3	150	850 Plasma	1500 ng/ml
5	3	125	875 Plasma	1250 ng/ml
6	3	100	900 Plasma	1000 ng/ml
7	4	500	500 Plasma	750 ng/ml
8	6	500	500 Plasma	500 ng/ml
9	8	500	500 Plasma	250 ng/ml
10	9	400	600 Plasma	100 ng/ml
11	10	500	500 Plasma	50 ng/ml
12	11	500	500 Plasma	25 ng/ml
13	12	400	600 Plasma	10 ng/ml
QC 1	3	90	910 Plasma	900 ng/ml
QC 2	4	100	900 Plasma	150 ng/ml
QC 3	QC 2	200	800 Plasma	30 ng/ml

Table E.2. *Calibration curves and absolute recovery rate of cyclosporine drug levels.*

Stock #	From Stock #	Take amount [µl from Stock]	Add Amount [µl]	Total Concentration
1a		/	/	1 mg/ml
1b	1	200	1800 MeOH	100µg/ml
2	1b	500	500 MeOH	50 ng/ml
3	2	500	500 MeOH	25 ng/ml
4	3	400	600 MeOH	10 ng/ml
5	4	500	500 MeOH	5 ng/ml
6	5	400	600 MeOH	2.5 ng/ml
7	6	500	500 MeOH	1.0 ng/ml
8	7	500	500 MeOH	0.5 ng/ml
9	8	500	500 MeOH	0.25 ng/ml
10	9	400	600 MeOH	0.1 ng/ml
11	10	500	500 MeOH	0.05 ng/ml
12	11	500	500 MeOH	0.025 ng/ml
13	12	400	600 MeOH	0.01 ng/ml
QC 1	3	400	200 MeOH	66.7 ng/ml
QC 2	QC 1	200	200 MeOH	33.3 ng/ml
QC 3	QC 2	100	900 MeOH	3.3 ng/ml
For total concentration take 1 µl (from stock) 1-13 and 1 ml plasma				

Table F.1. *Protocol of calibration curves and absolute recovery rate of MPA drug levels*

Total time [min]	A (%)	B (%)	Flow rate [µl/min]
0	0	100	5000
1,0	0	100	5000
1,1	100	0	100
4.80	100	0	100
4.81	100	0	5000
5.10	0	100	5000
6.50	0	100	5000

Table F.2. *Meassurement of MPA, gradientpump 1 (extraction): Flowrate and mobile phases/time. Mobile phase A: methanol, mobile phase B: 0,01% formic acid in water.*

Total time [min]	A (%)	B (%)	Flow rate [µl/min]
0	70.0	30.0	1000
01,0	70.0	30.0	1000
4.00	95.0	5.0	1000
4.50	100	0.0	1000
5.00	70.0	30.0	1000
6.50	70.0	30.0	1000

Table F.3. *Measurement of MPA, gradient pump 2 (separation and analysis). Flowrate and mobile phases/time. Mobile phase A: methanol, mobile phase B: 0,01% formic acid in water.*

Temperature, left	40.0°C
Temperature, right	40.0°C
Start aquisition tolerate	+/-0.5°C

Table F.4.*Agilent 1100 column thermostat: Temperature settings for MPA concentrations measurement.*

Stock No.	From Stock No.	Take Amount[from Stock]	Add Amount [ml urine/plasma]	Total Concentration
1		/	/	1 mg/ml
2	1	100 µl	9.9	10 ug/ml
3	2	100 µl	9.9	100 ng/ml
4	3	4 ml	6	40 ng/ml
5	4	5 ml	5	20 ng/ml
6	5	5 ml	5	10 ng/ml
7	6	5 ml	5	5 ng/ml
8	7	5 ml	5	2.5 ng/ml
9	8	4 ml	6	1 ng/ml
10	9	5 ml	5	0.5 ng/ml
11	10	5 ml	5	0.25 ng/ml
12	11	4 ml	6	0.1 ng/ml
13	12	5 ml	5	0.05 ng/ml
14	13	5 ml	5	0.025 ng/ml
15	14	5 ml	5	0.0125 ng/ml
QC0	3	1 ml	4	20 ng/ml
QC1	4	0.5 ml	4.5	4 ng/ml
QC2	QC1	1 ml	9	0.4 ng/ml
QC3	QC2	2 ml	8	0.08 ng/ml
QC4	QC3	3 ml	3	0.04 ng/ml

Table G.1. *Protocol of calibration curves and absolute recovery rate of isoprostanes.*

Total time [min]	A (%)	B (%)	Flow rate [μl/min]
0	70.0	30.0	5000
1,00	70.0	30.0	5000
1.10	5.0	95.0	200
8.00	2.0	98.0	200
8.50	5.0	95.0	2000
8.70	70.0	30.0	2000
9.80	70.0	30.0	5000

Table G.2. *Measurement of isoprostanes, gradientpump 1 (extraction): Flowrate and mobile phases/time. Mobile phase A: methanol, mobile phase B: 0,01% formic acid in water.*

Total time [min]	A (%)	B (%)	Flow rate [μl/min]
0	37.0	63.0	600
1.00	37.0	63.0	600
7.00	22.3	77.7	600
7.80	2.0	98.0	600
8.80	2.0	98.0	600
8.90	37.0	63.0	600
10.00	37.0	63.0	600

Table G.3. *Measurement of isoprostanes, gradient pump 2 (separation and analysis). Flowrate and mobile phases/time. Mobile phase A: methanol, mobile phase B: 0,01% formic acid in water.*

Temperature, left	60.0°C
Temperature, right	60.0°C
Start aquisition tolerance	+/- 0.5°C

Table G.4. *Agilent 1100 column thermostat: Temperature settings for meassurement of isoprostanes concentrations.*

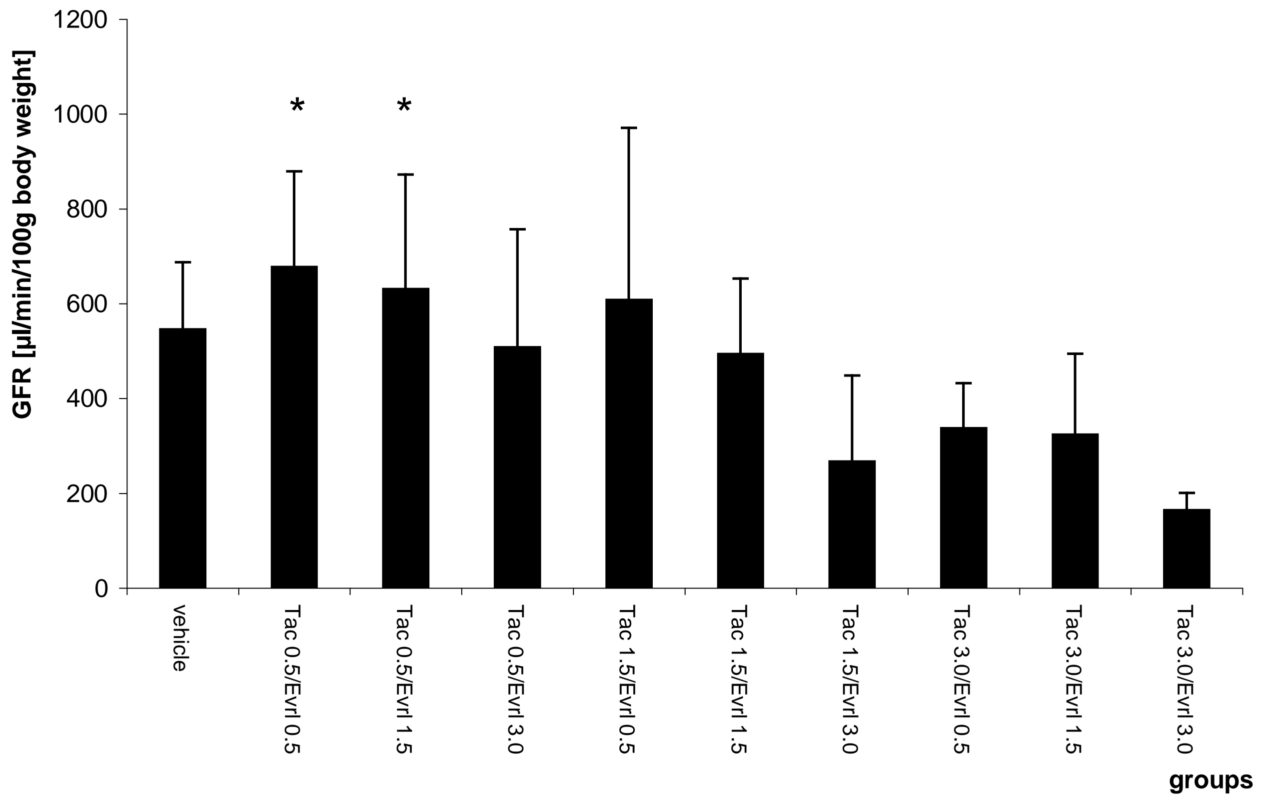


Figure H.1. *Glomerular filtration rate (GFR) in µl/min/100g body weight in groups with combinated treatment of tacrolimus and everolimus in different doses. [*p is significant versus control by One-way-Anova. Vehicle: control, Tac: tacrolimus, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight applied over 28 days.].*

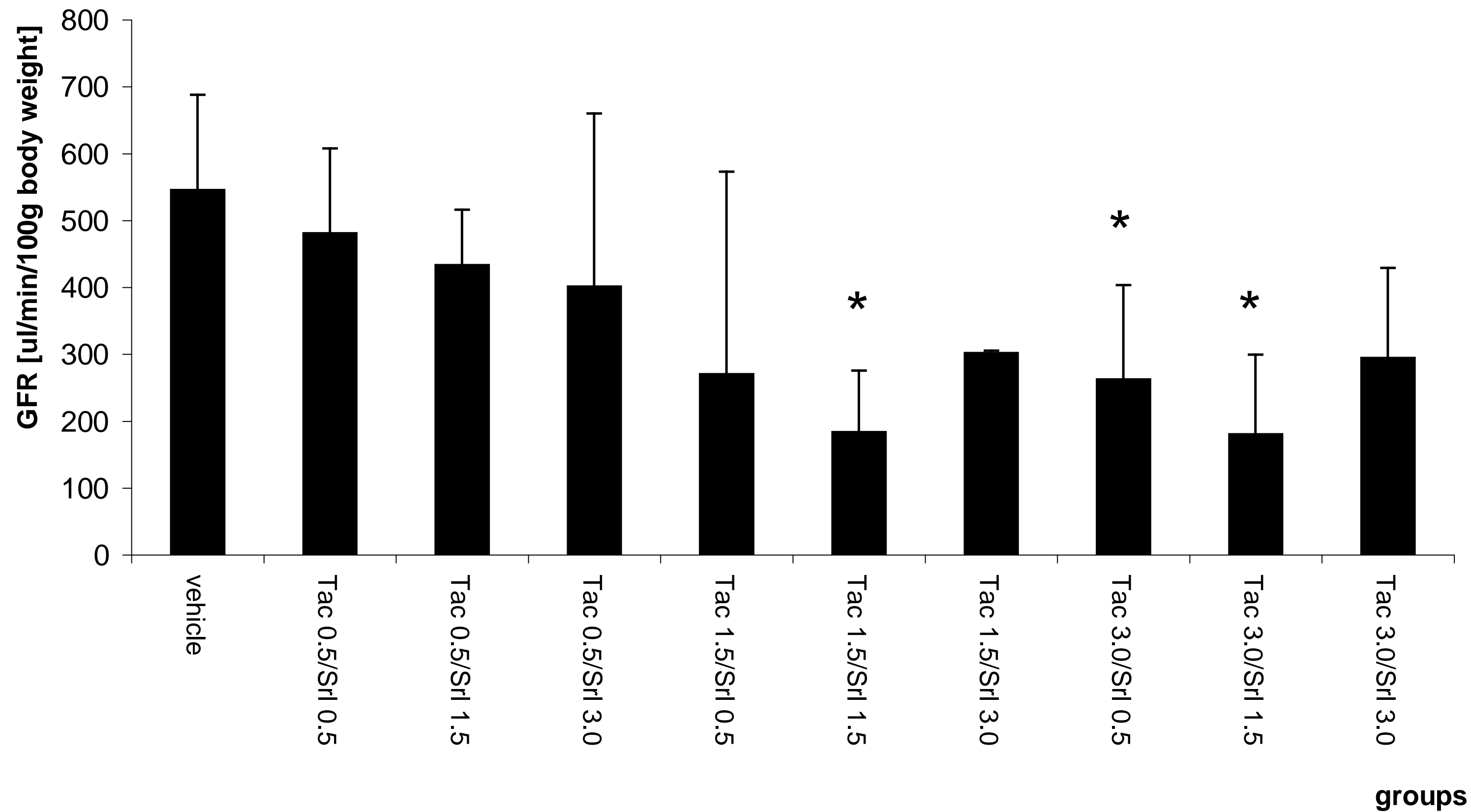


Figure H.2. *Glomerular filtration rate (GFR) in µl/min/100g body weight in groups with combinated treatment of tacrolimus and sirolimus in different doses. [*p is significant versus control by One-way-Anova. Vehicle: control, Tac: tacrolimus, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

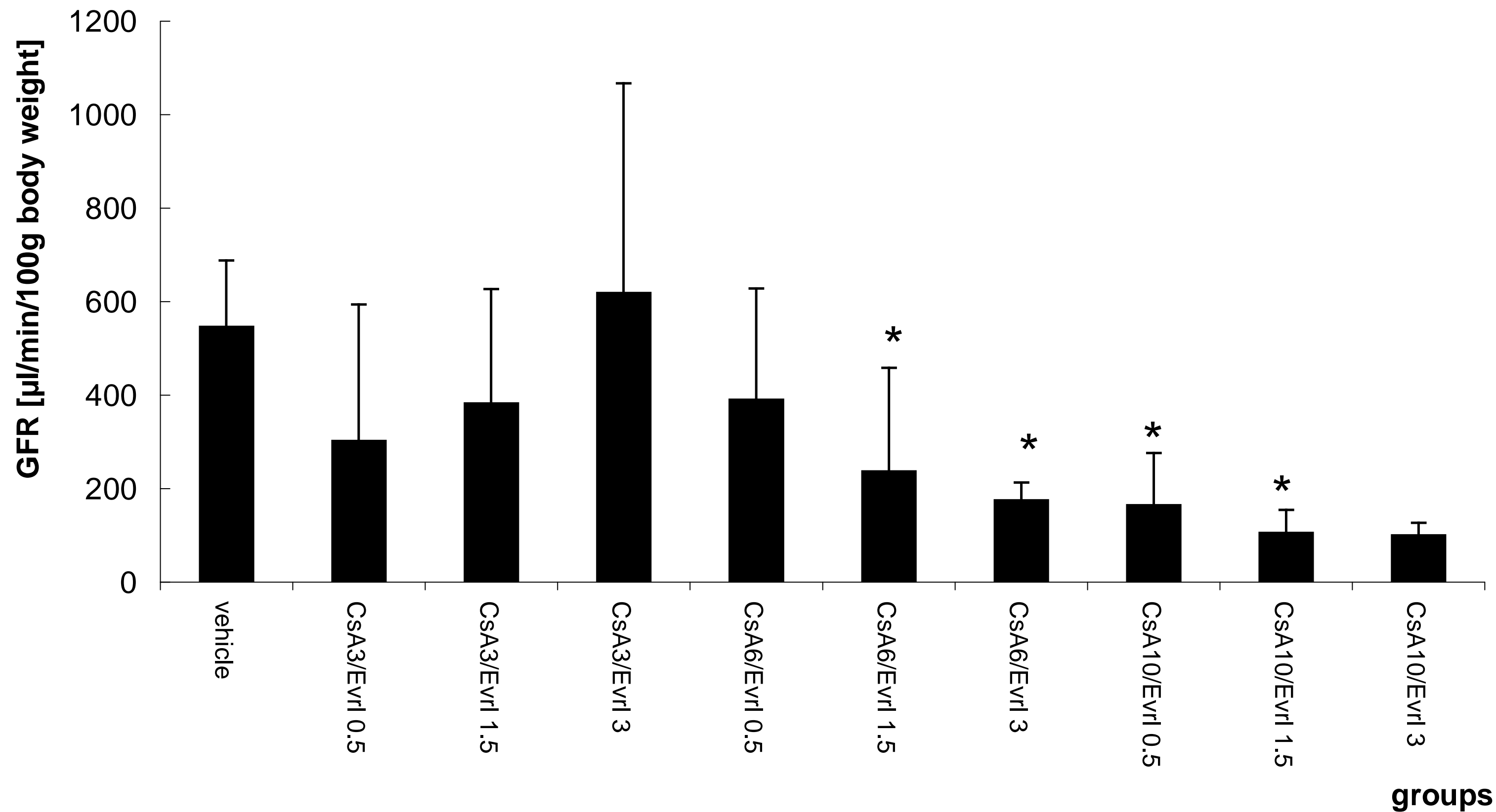


Figure H.3. *Glomerular filtration rate (GFR) in µl/min/100g body weight in groups with combinated treatment of cyclosporine and everolimus in different doses. [*p is significant versus control by One-way-Anova. Vehicle: control, CsA: cyclosporine, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

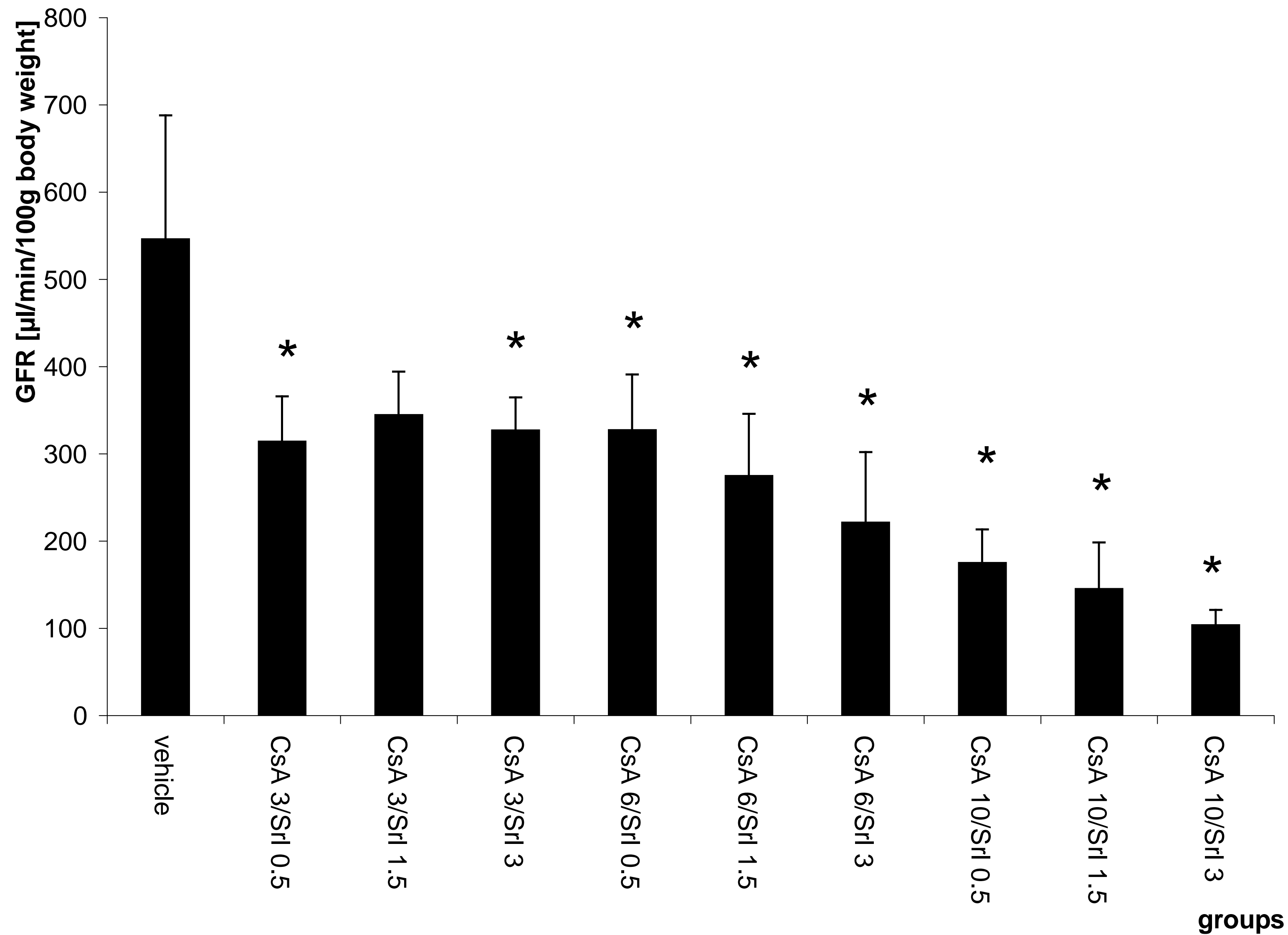


Figure H.4. *Glomerular filtration rate (GFR) in µl/min/100g body weight in groups with combination treatment of cyclosporine and sirolimus in different doses. [All groups excepting CsA3/Srl 1.5 are statistical significant; *p-value versus control by One-way-Anova. Vehicle: controls; CsA: cyclosporine, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

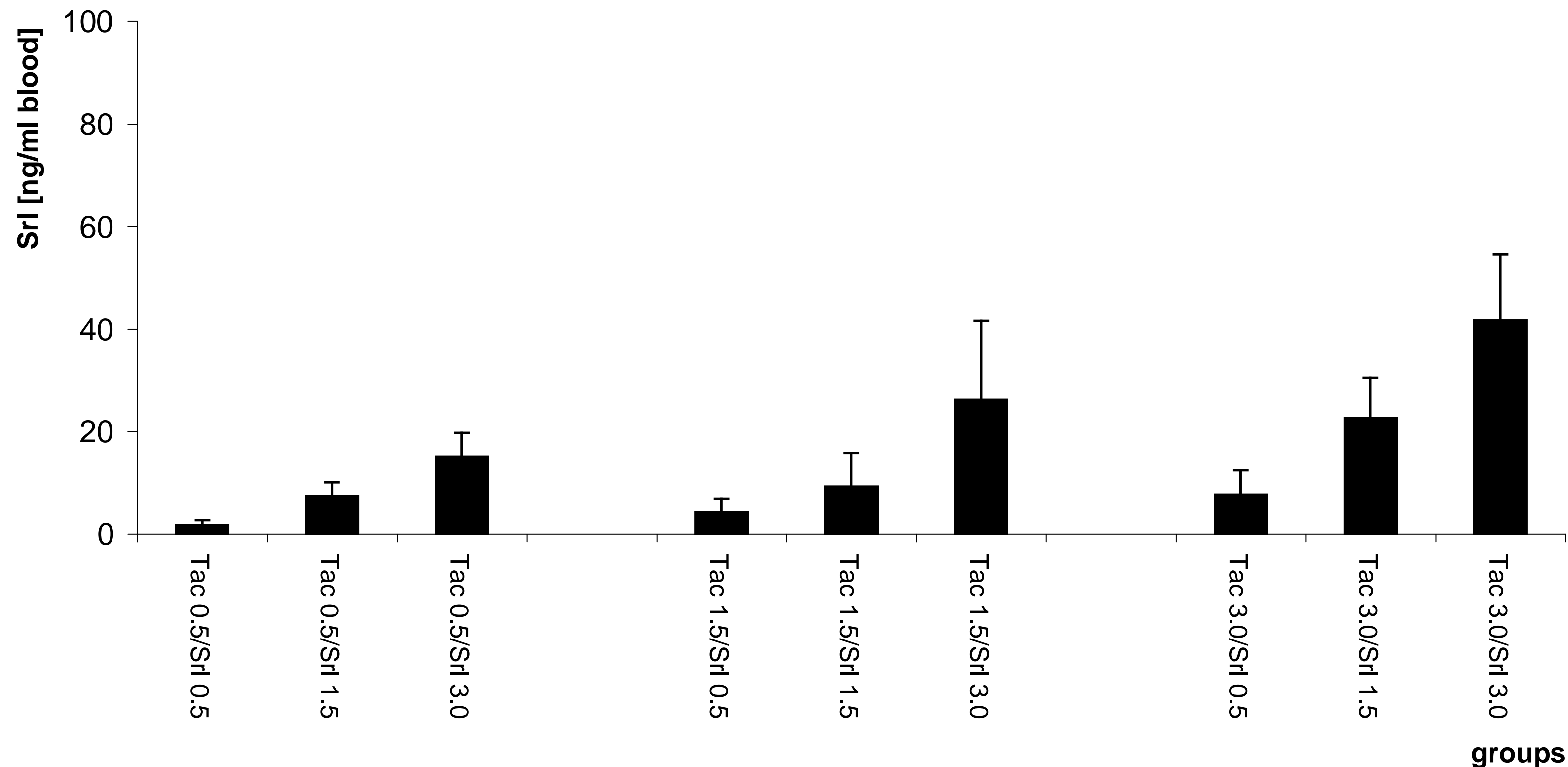


Figure I.1. *Sirolimus blood concentrations in groups with combined treatment of tacrolimus and sirolimus. Increasing drug doses of tacrolimus in groups with constant doses of sirolimus induce an increase of sirolimus blood levels. [Statistical significance by One-way-Anova were found in following groups: Tac3/Srl3 vs Tac0.5/Srl0.5; Tac3/Srl3 vs Tac1.5/Srl0.5; Tac3/Srl1.5 vs Tac0.5/Srl0.5; Tac3/Srl1.5 vs Tac1.5/Srl0.5; Tac1.5/Srl3 vs Tac0.5/Srl0.5; Tac1.5/Srl3 vs Tac1.5/Srl0.5; Tac0.5/Srl3 vs Tac0.5/Srl0.5. Results are not shown in figure. Tac: tacrolimus, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

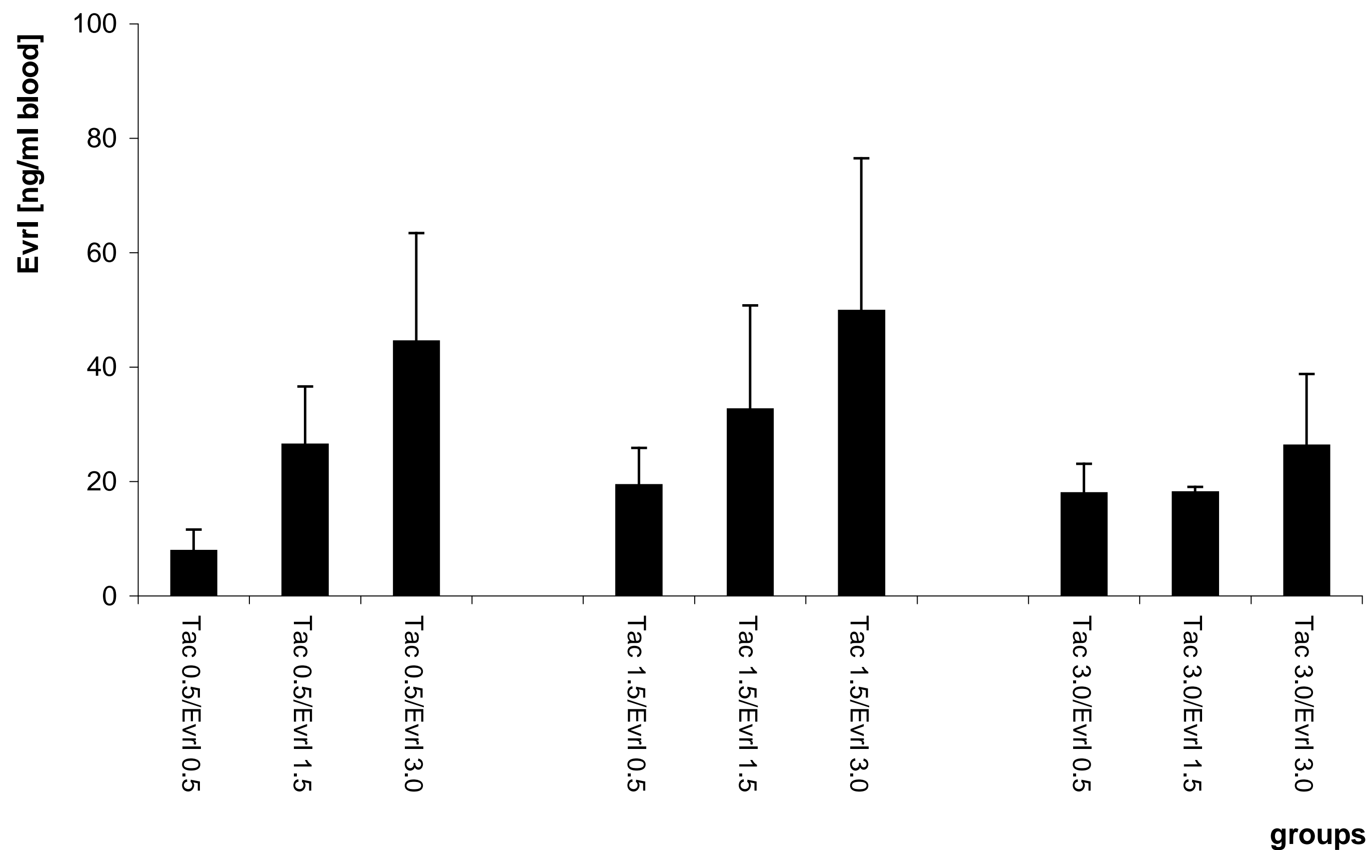


Figure I.2. *Everolimus concentrations in groups with combined treatment of tacrolimus. [Differences were not significant by One-way-Anova. Tac: tacrolimus, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

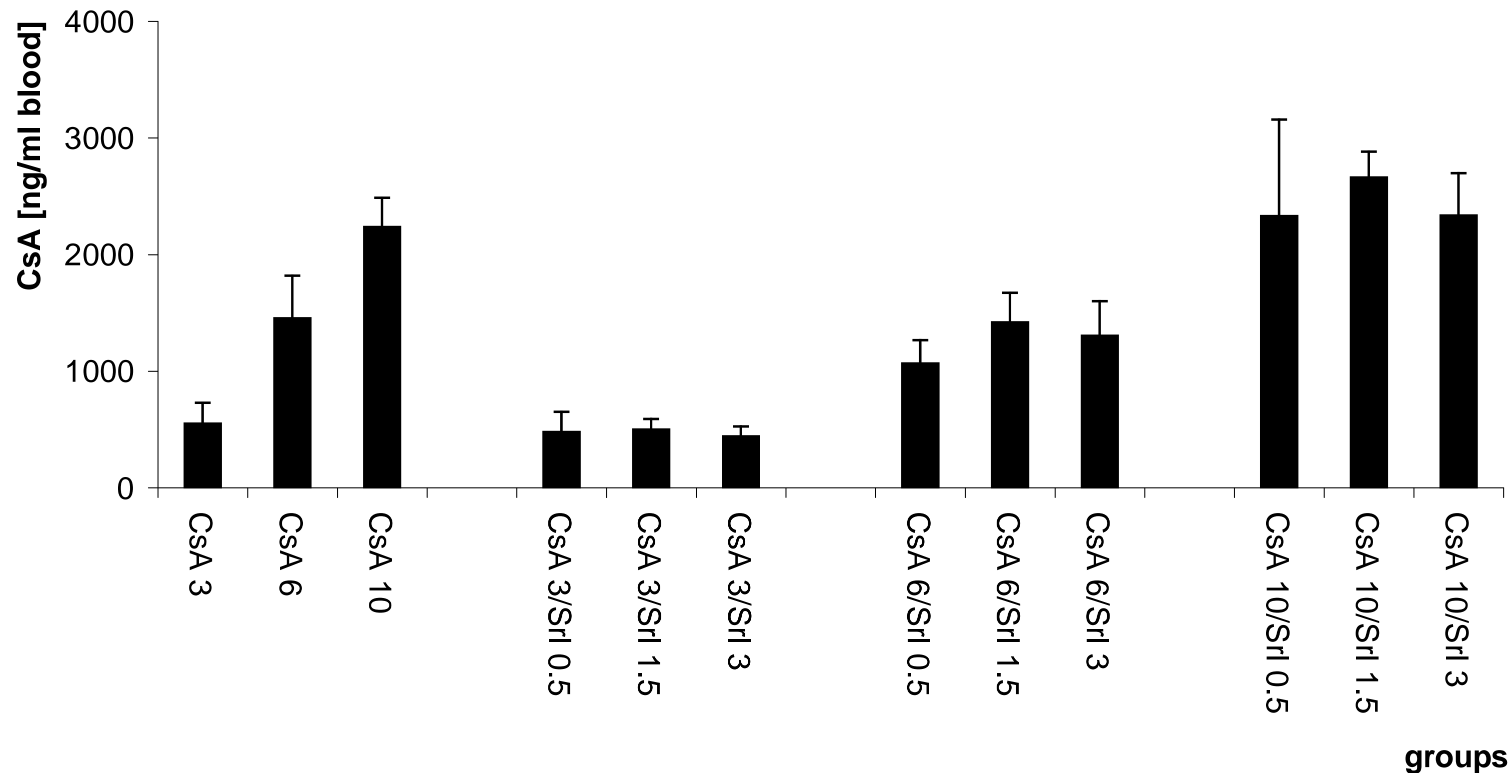


Figure J.1. *Cyclosporine drug blood levels of groups with treatment of cyclosporine in combination with sirolimus. [*p is significant difference by One-way-Anova: CsA10/Srl1.5 vs CsA3/Srl3; CsA10/Srl1.5 vs CsA3/Srl0.5; CsA10/Srl1.5 vs CsA3/Srl0.5; CsA10/Srl1.5 vs CsA3/Srl1.5; CsA10/Srl1.5 vs CsA3/Srl1.5; CsA10/Srl1.5 vs CsA3; CsA10/Srl3 vs CsA3/Srl3; CsA10/Srl0.5 vs CsA3/Srl3; CsA10 vs CsA3/Srl3. Significances are not shown in figure. CsA: cyclosporine, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

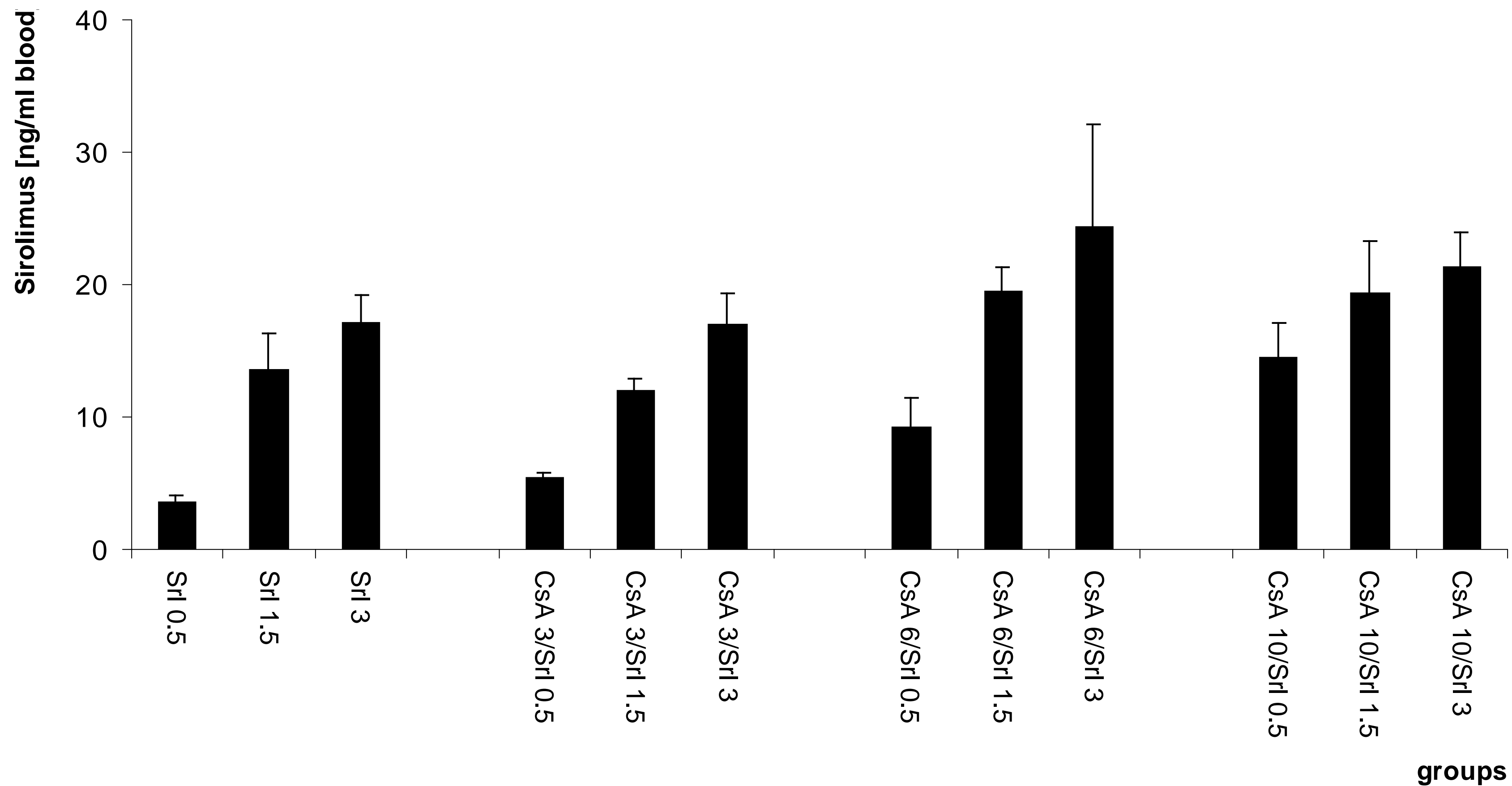


Figure J.2. *Sirolimus blood concentration of groups with treatment of cyclosporine in combination with sirolimus [* p is significant different by One-way-Anova: CsA6 vs Srl0.5; CsA6 vs Srl1.5; CsA6 vs Srl0.5/CsA3; CsA3 vs Srl0.5; CsA3 vs Srl1.5; CsA3 vs Srl0.5; CsA3 vs Srl1.5; Srl0.5/CsA6 and Srl0.5/CsA10. Significanes are not shown in figure. CsA: cyclosporine, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

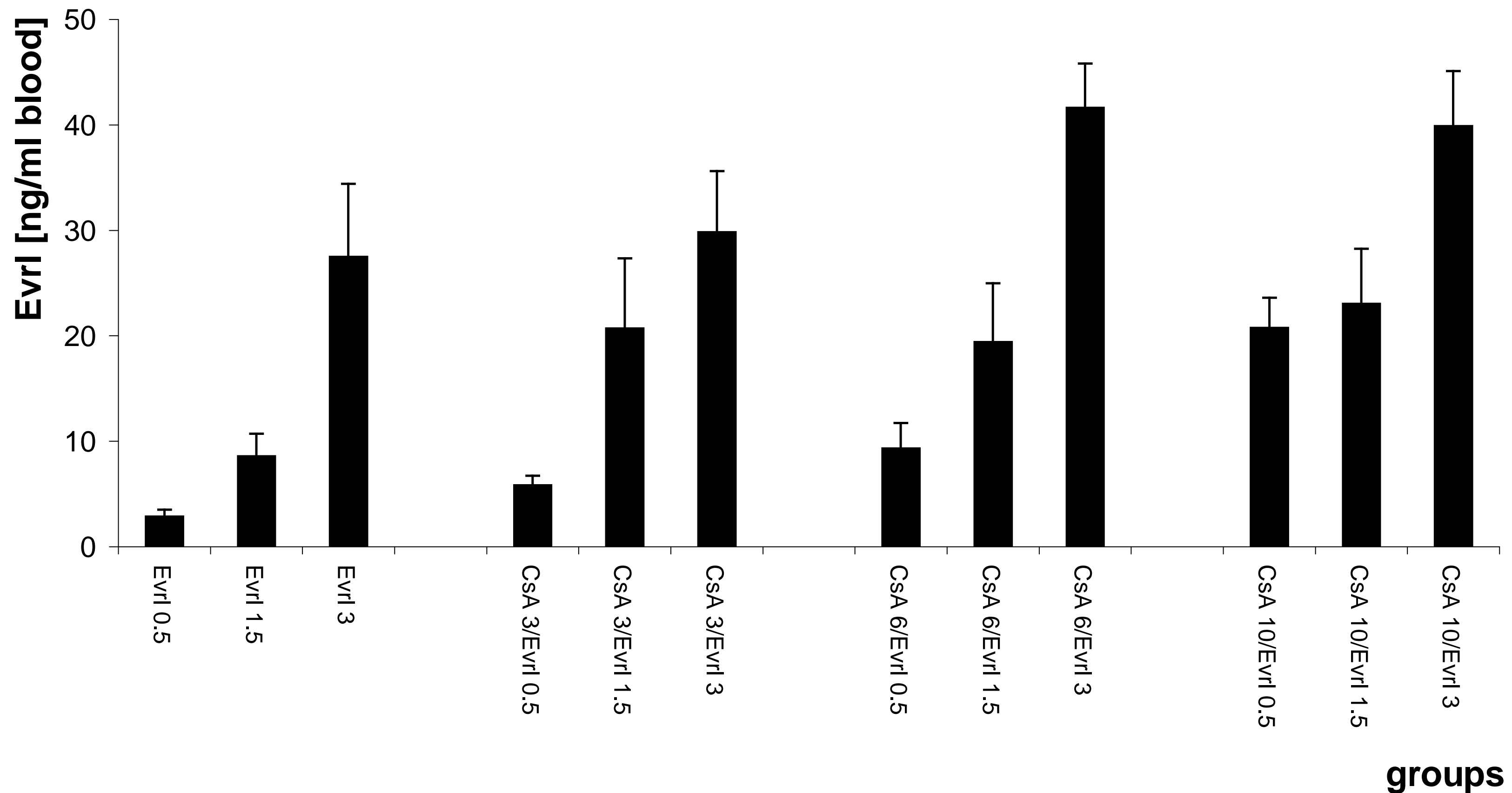


Figure J.3. *Everolimus concentration in groups with treatment of cyclosporine in combination with everolimus [*p is significant different by One-way-Anova: Evrl3/CsA6 vs Evrl0.5; Evrl3/CsA6 vs Evrl0.5/CsA3; Evrl3/CsA6 vs Evrl1.5; Evrl3/CsA6 vs Evrl0.5/CsA6; Evrl3/CsA10 vs Evrl0.5; Evrl3/CsA10 vs Evrl0.5/CsA3; Evrl3/CsA10 vs Evrl1.5; Evrl3/CsA10 vs Evrl0.5/CsA6; Evrl3/CsA3 vs Evrl0.5; Evrl3/CsA3 vs Evrl0.5/CsA3; Evrl3 vs Evrl0.5; Evrl3 vs Evrl0.5/CsA3. Significances are not shown in figure. CsA: cyclosporine, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

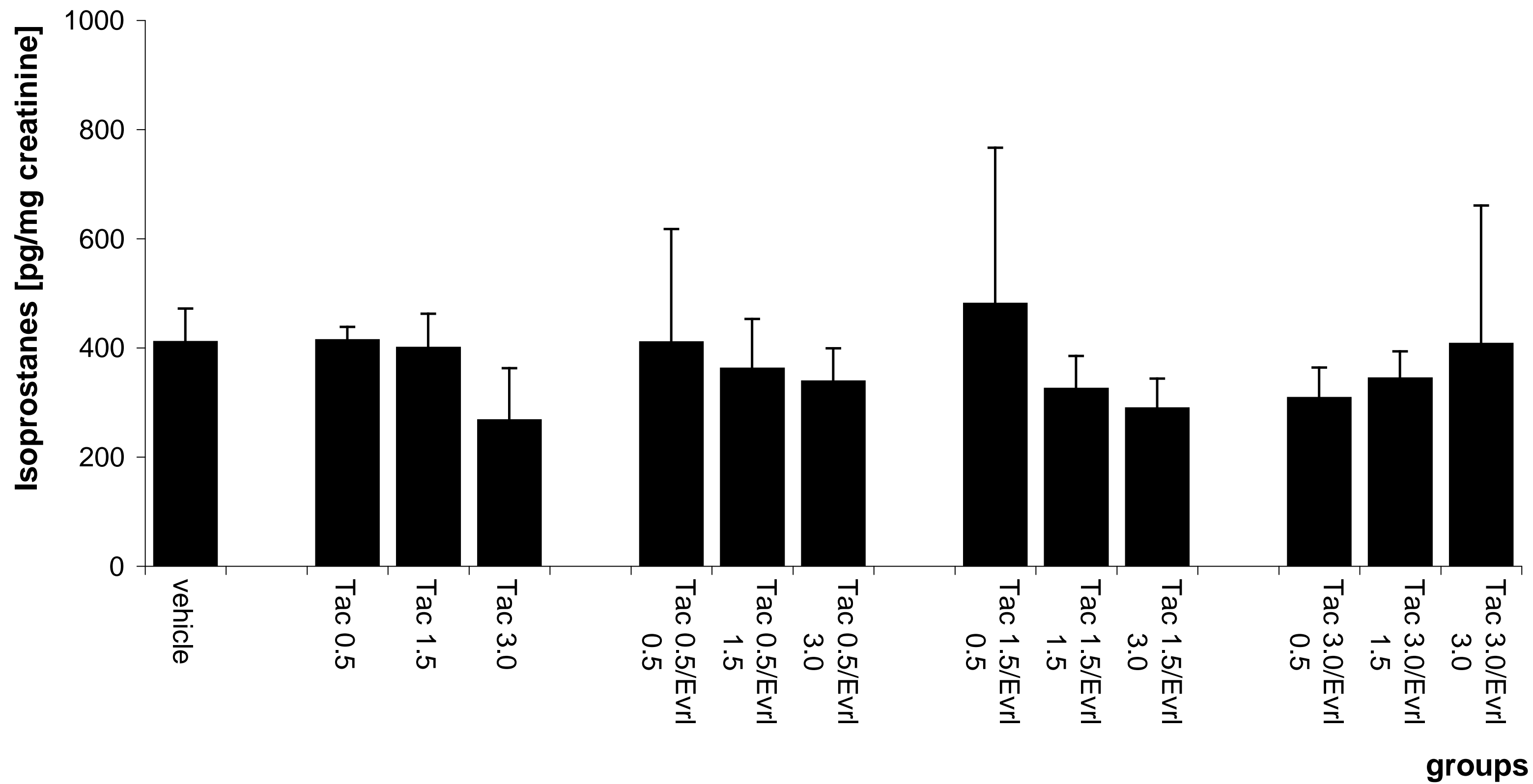


Figure K.1. *15-F_{2t}-Isoprostane concentrations in urine after 1 week of treatment with tacrolimus and everolimus. [No statistically significant differences between groups by One-way-Anova ($p>0.05$) were found. Vehicle: control Tac: tacrolimus, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

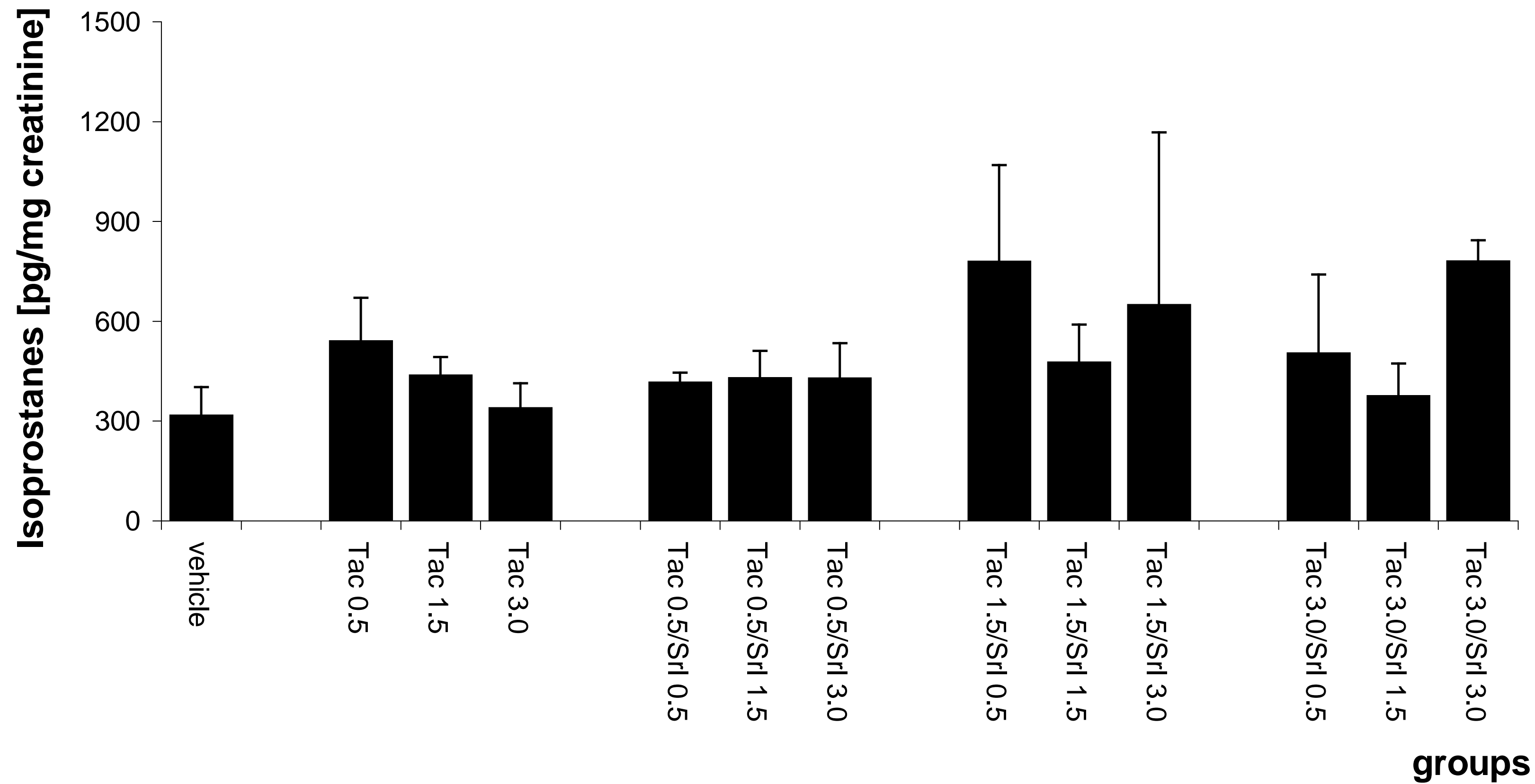


Figure K.2. *15-F_{2t}-Isoprostane concentrations in urine after 28 days of treatment with tacrolimus and sirolimus. [One-way-Anova showed no statistically significant differences between groups. Vehicle: control, Tac: tacrolimus, Srl: sirolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.].*

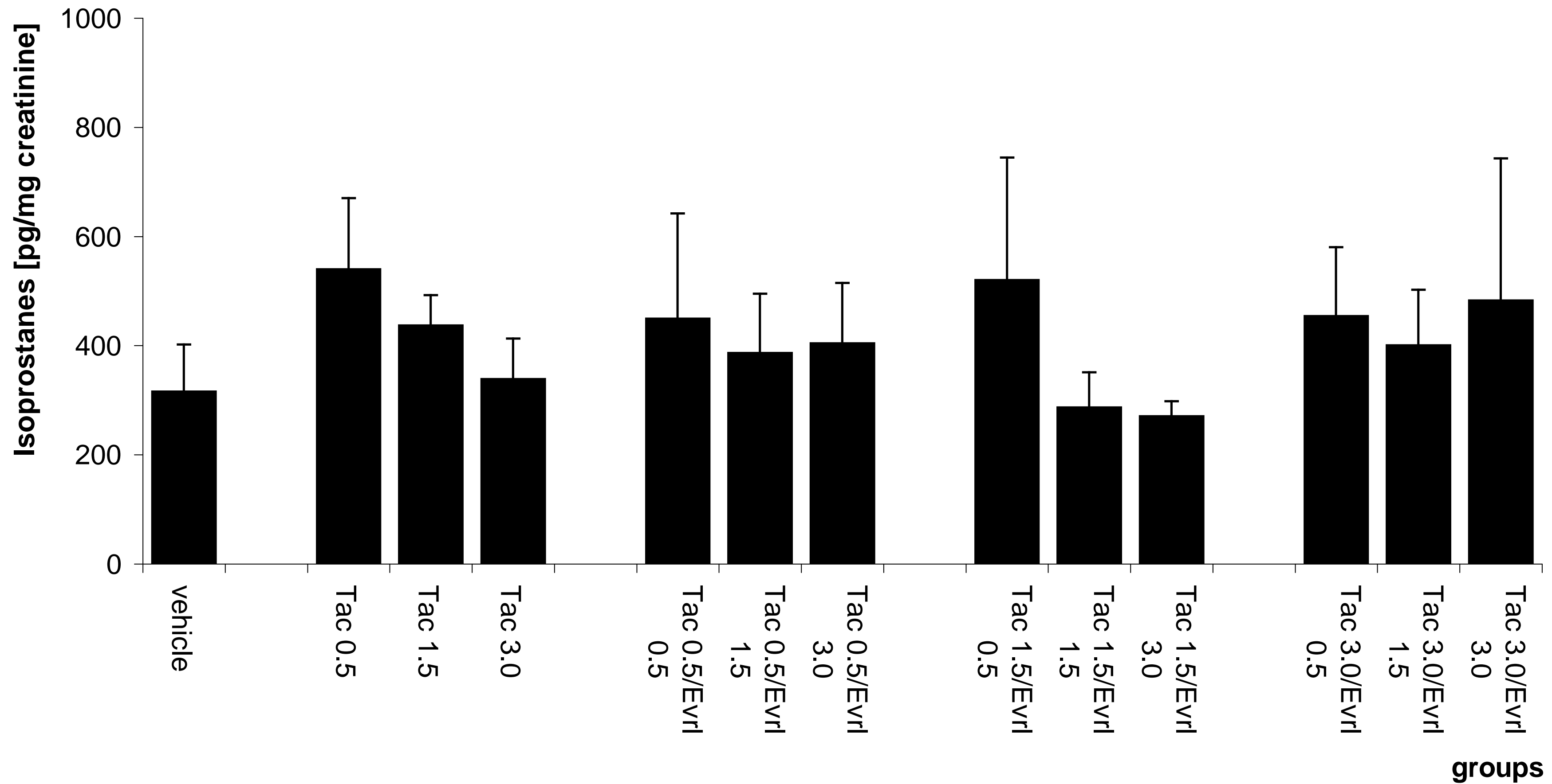
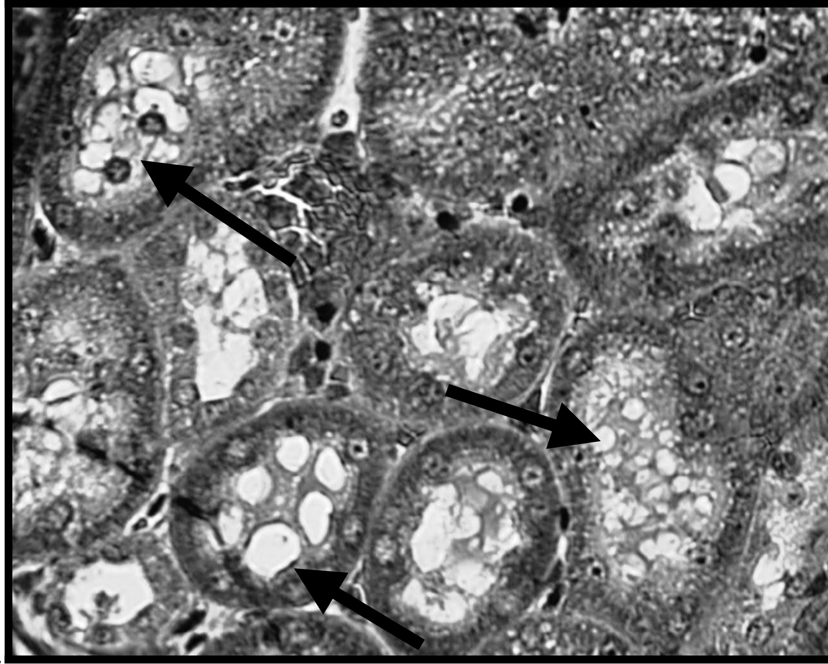
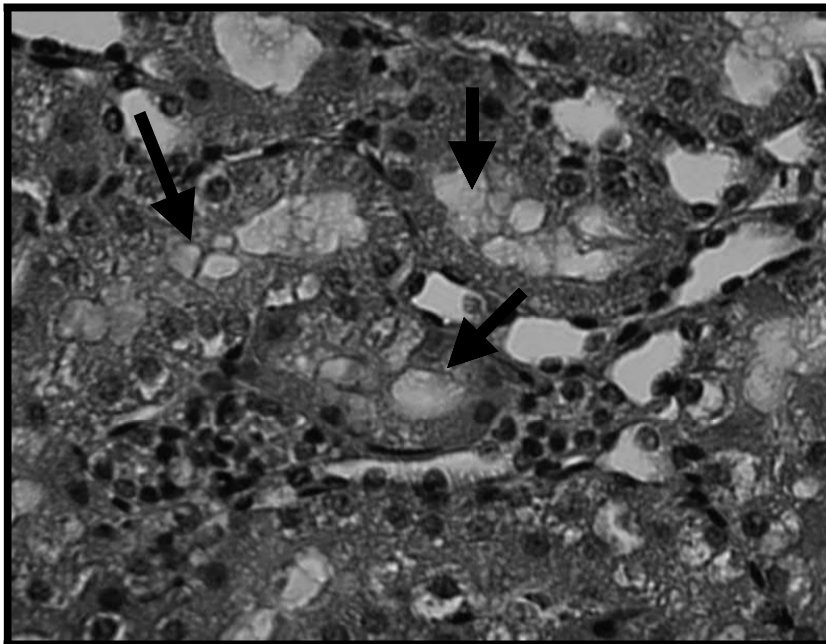


Figure K.3. *15-F_{2t}-Isoprostane concentrations in urine after 28 days of treatment with tacrolimus and everolimus. [One-way-Anova showed no statistically significant differences between groups. Vehicle: control, Tac: tacrolimus, Evrl: everolimus. Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.]*

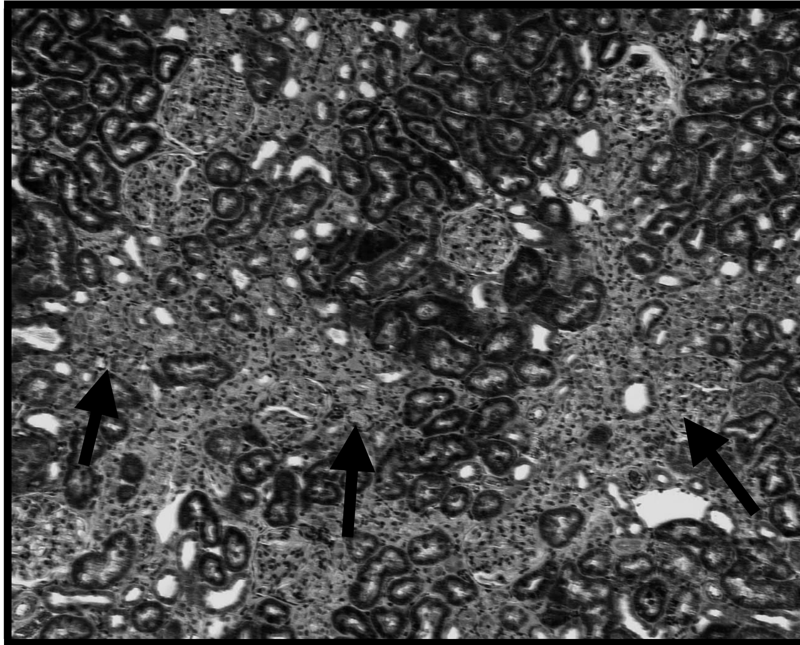


L.1.



L.2.

L.1. and L.2. *Tubular vacuolization is shown. (L1: TC, treatment with tacrolimus and sirolimus, each 0,5 mg/kg body weight. L2: HE, treatment with tacrolimus and sirolimus each 3mg/kg body weight.) 10x40-fold magnification by light microscope. N. Brunner, 2008.*



L.3

L.3. *Tubular atrophy is shown after treatment with tacrolimus and sirolimus, each 3mg/kg body weight.: HE, 10x10-fold magnification by light microscope. N. Brunner, 2008.*

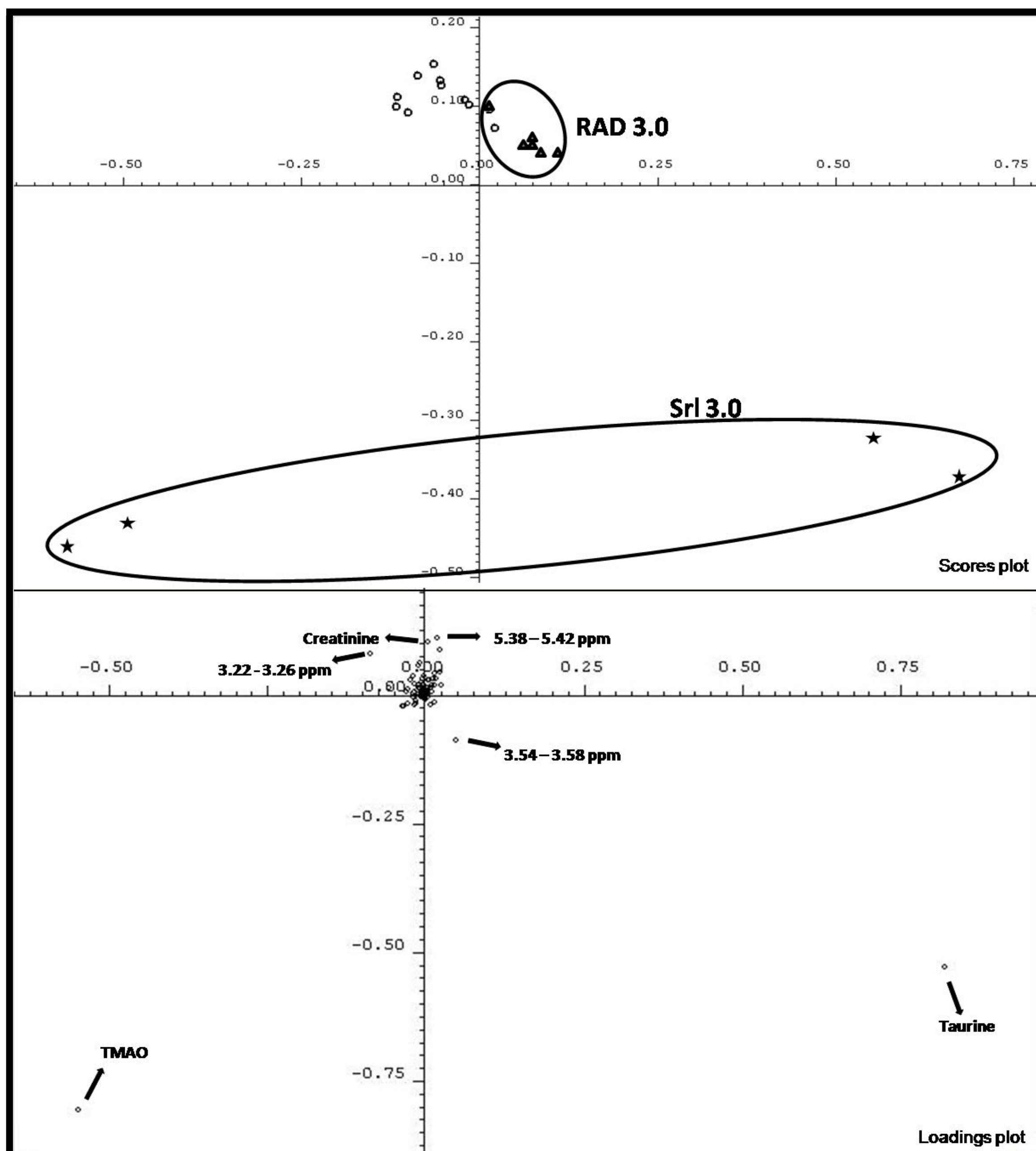


Figure M.1
Principal Component Analysis of rat urine ^{1}H-NMR spectra after 28 days of drug treatment. After "binning" (conversion of NMR spectra into histograms), NMR spectra were loaded into the principal component analysis (M.1.) When rats were treated with everolimus (RAD) or sirolimus (SRL) (both 3.0 mg/ kg/ day), the principal component analysis separated spectra after sirolimus treatment from those in the vehicle control group and after everolimus treatment.

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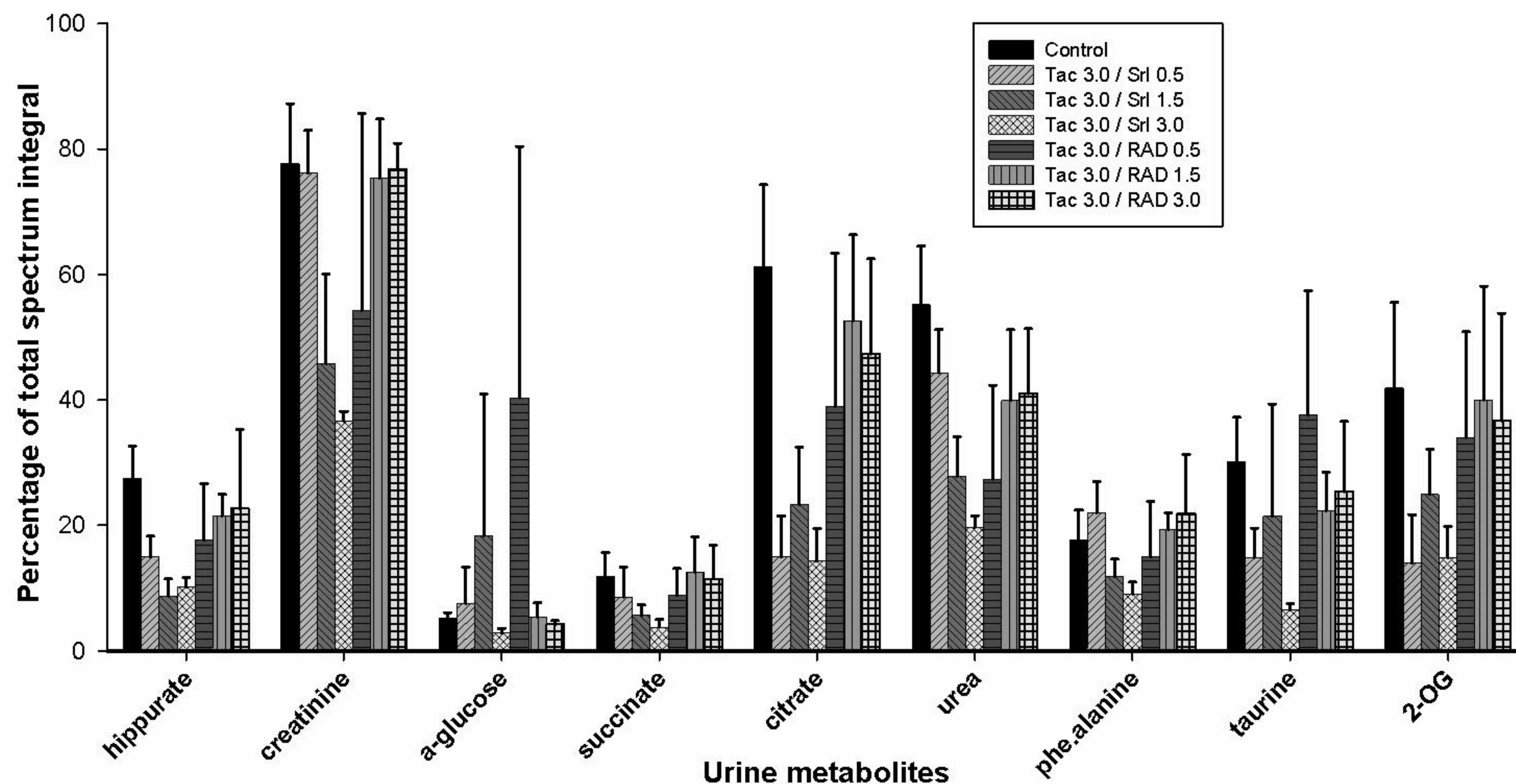

Figure M.2. *Changes in urine metabolite pattern after 28 days of treatment with tacrolimus and its combination with sirolimus and everolimus. All urine metabolites were determined semi-quantitatively by 1H- NMR, all values were normalized based on the total integral and are presented as means + standard deviations (n=minimum 3 for all groups). Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days. Group comparison by One-way-Anova, significances are not shown in figure.*

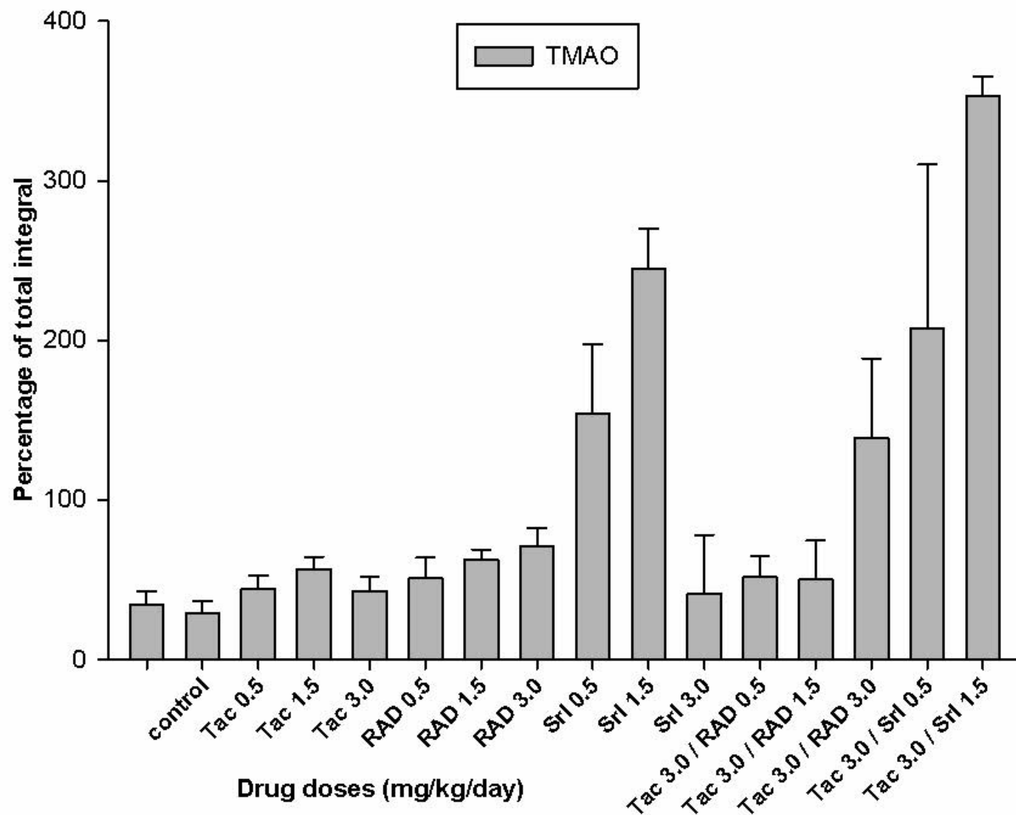

Figure M.3. *Dose-dependency of the effects of tacrolimus and its combination with sirolimus or everolimus (RAD) on the proximal tubule. Trimethylamine oxide (TMAO), an established proximal tubule toxicity marker is used as a surrogate marker. The relative concentrations of TMAO were determined using ^{1}H-NMR and were normalized based on the total integral of the NMR spectra. All data are presented as means + standard deviations (n= 3 or 4). Numbers behind treatment groups: Dose of specific immunosuppressant in mg/kg body weight, applied over 28 days.*

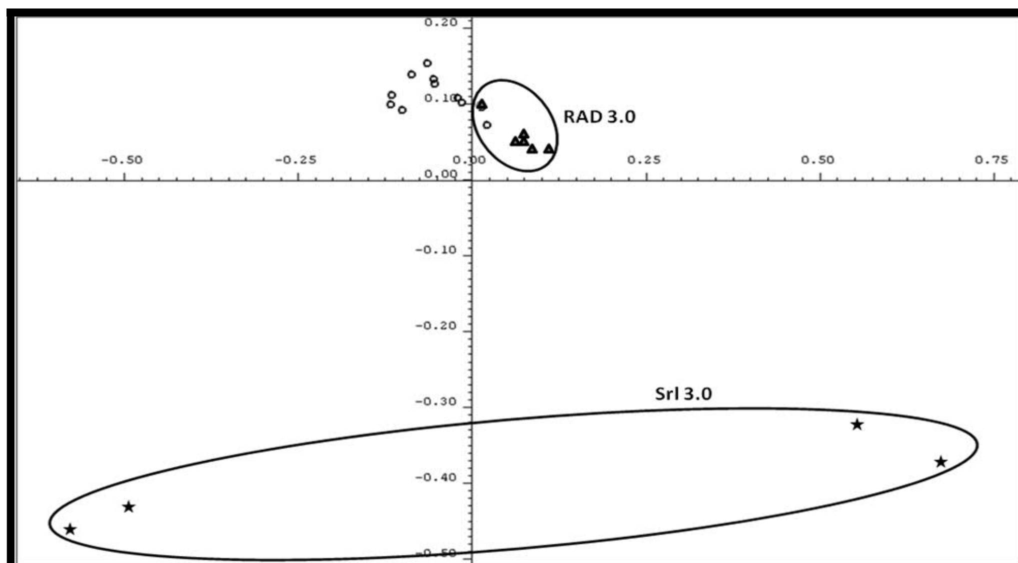

Figure M.4.

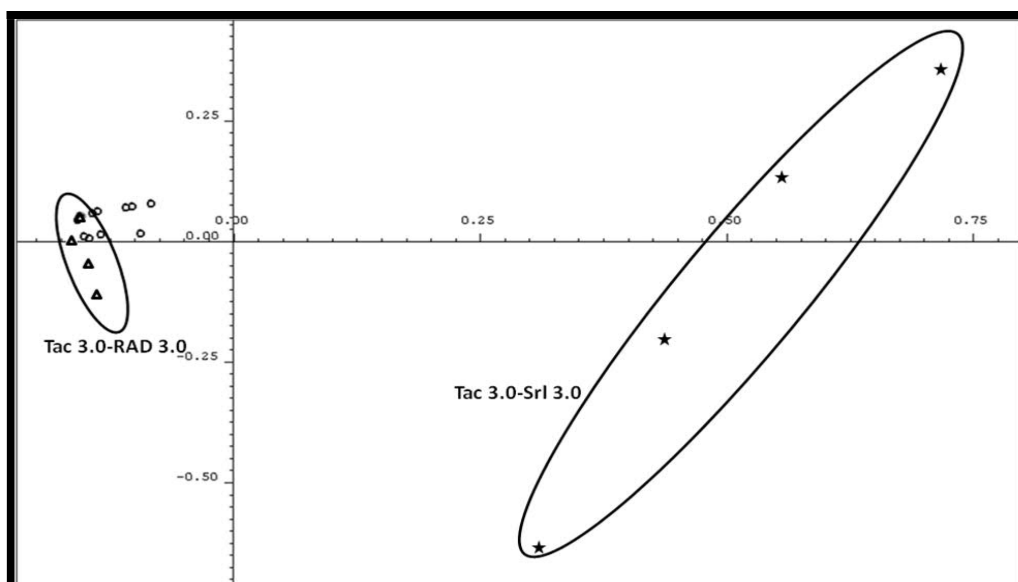

Figure M.5.

Figures M.4. and M.5. *Principal Component Analysis of rat urine ^{1}H-NMR spectra after 28 days of treatment with tacrolimus and its combination with sirolimus or everolimus. After "binning" (conversion of NMR spectra into histograms), NMR spectra were loaded into the principal component analysis. (M.4.) The principal component analysis separated spectra after sirolimus treatment from those in the vehicle control group and after everolimus treatment (M.5.). The urine NMR spectra after treatment with tacrolimus and sirolimus were separated from those after treatment with tacrolimus and everolimus, while the latter were not found being different from the vehicle controls.*

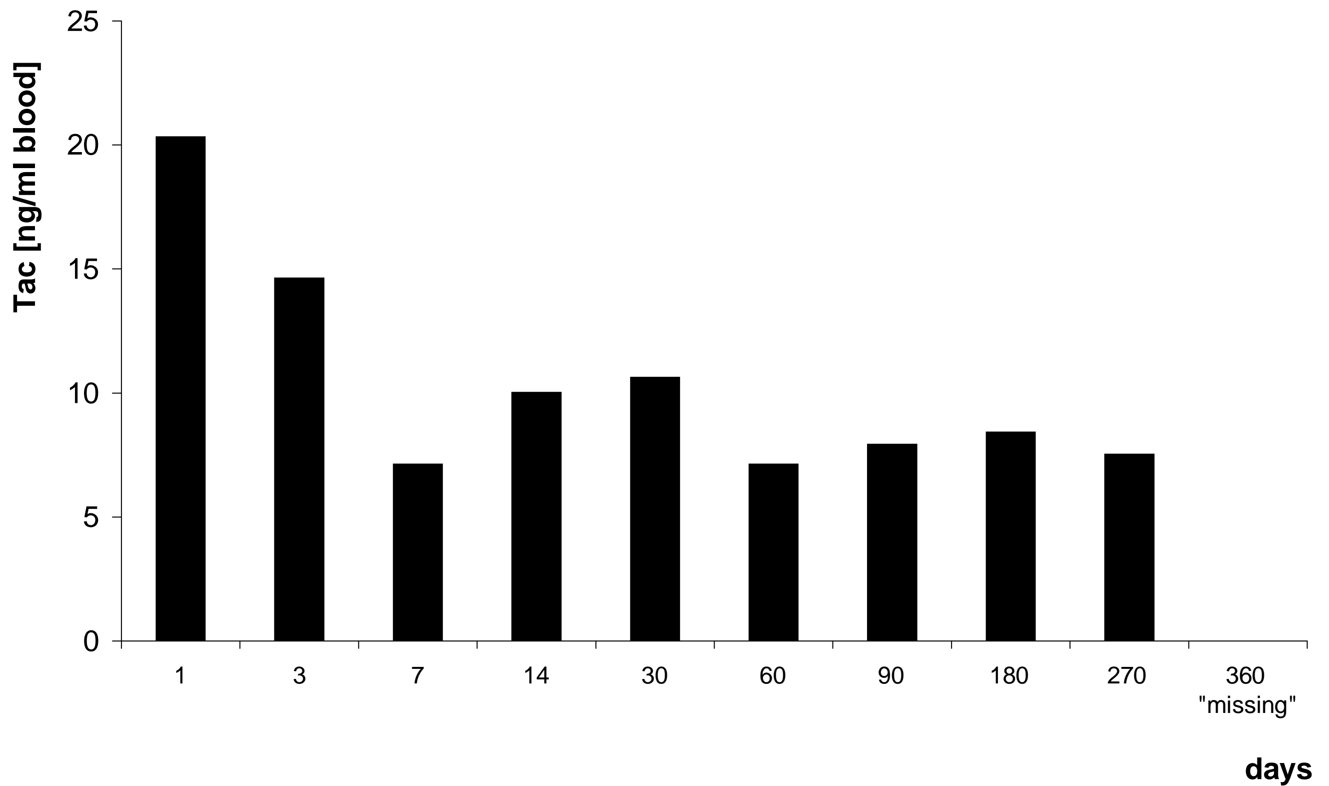

Figure N.1. Tacrolimus blood concentrations of patient # 10 observed over 360 days. No event of toxicity or rejection happened under treatment with tacrolimus. [Tac:tacrolimus. Value day 360 was missing.].

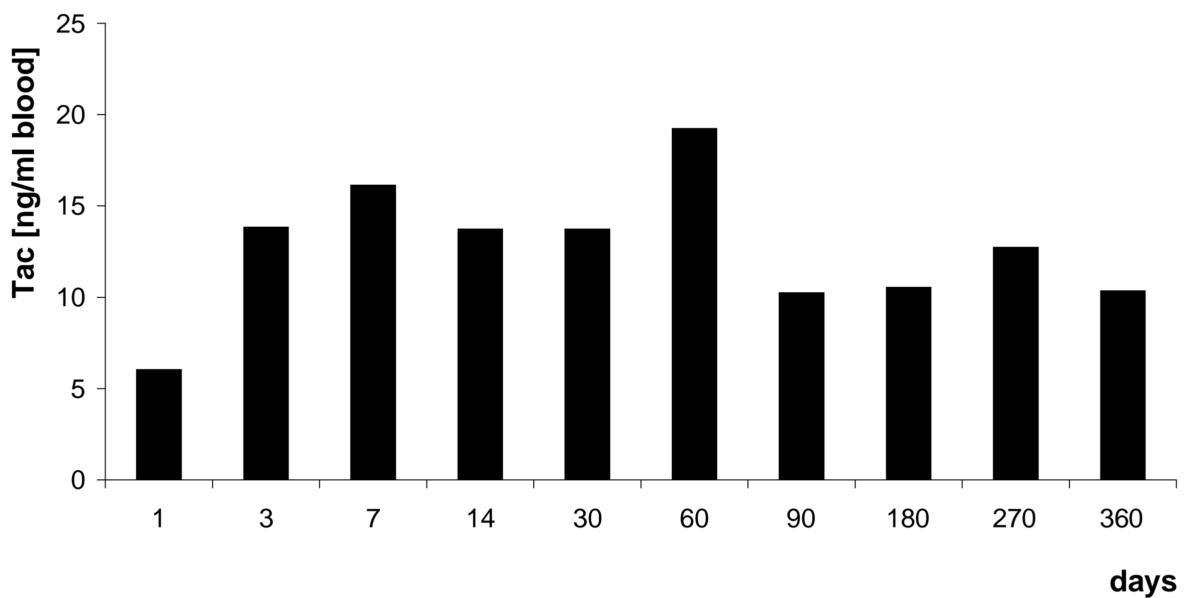

***Figure N.2.** Tacrolimus blood concentrations of patient # 31 observed over 360 days. No event of toxicity or rejection happened under treatment with tacrolimus. [Tac:tacrolimus.].*

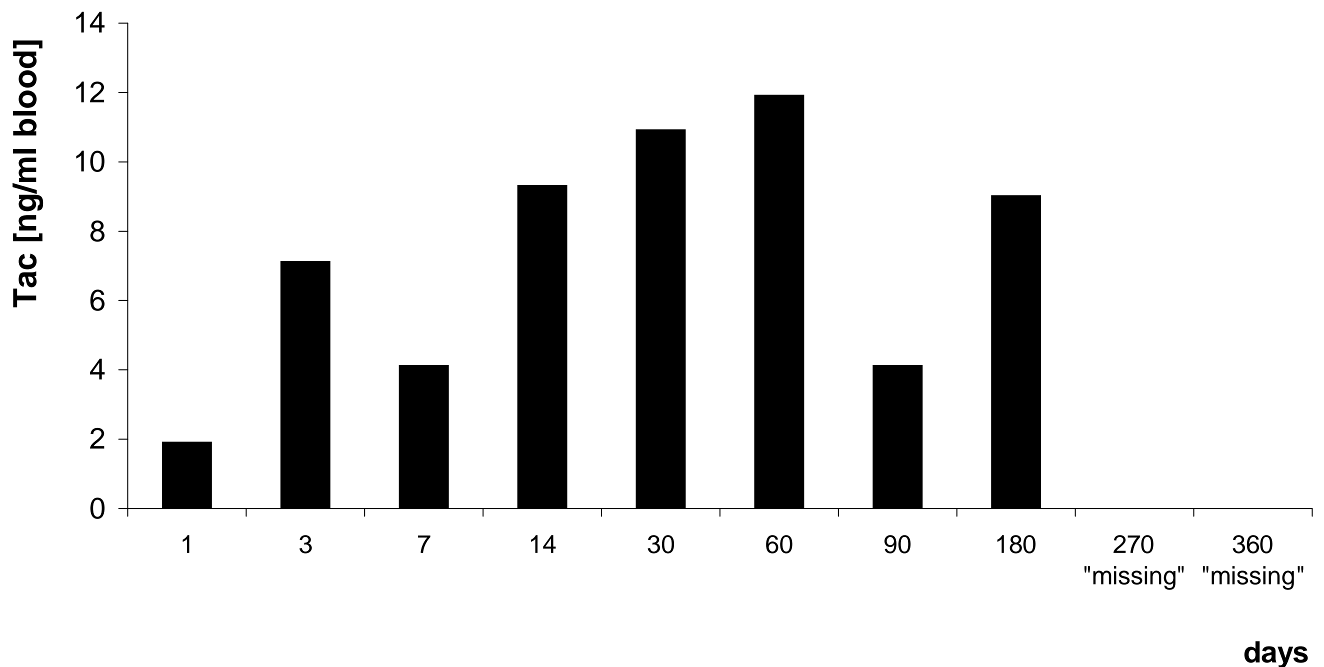

Figure O.1. *Tacrolimus blood concentrations of patient # 9 observed over 360 days. Events of toxicity happened on days 41 and 53 under treatment of tacrolimus. [Tac tacrolimus. Values days 270 and 360 were missing.].*

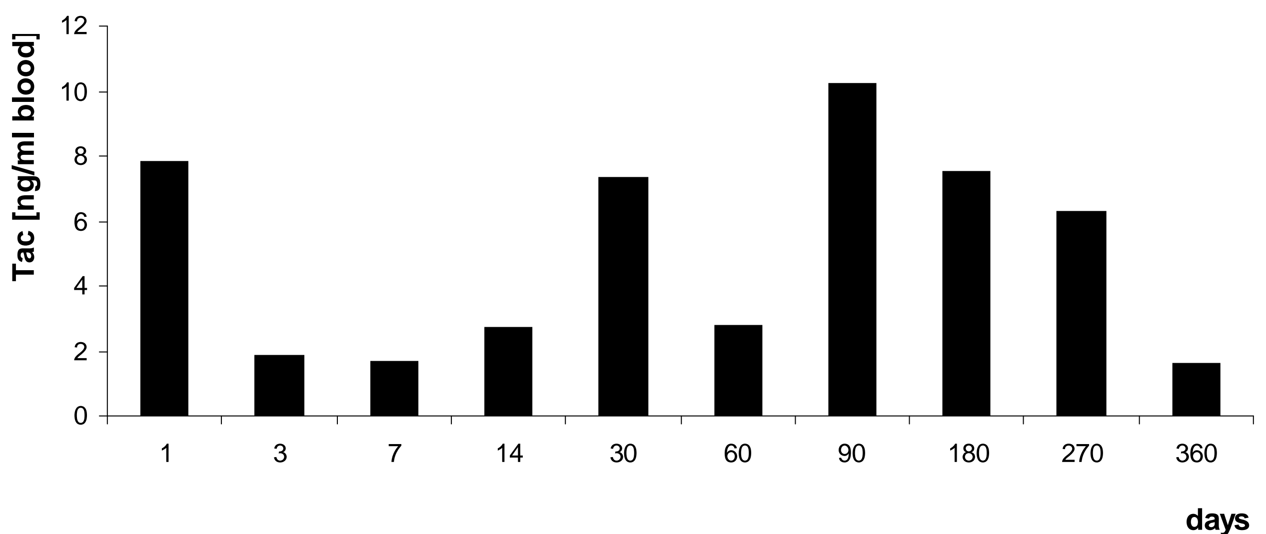

Figure O.2. *Tacrolimus blood concentrations of patient # 4 observed over 360 days. Event of toxicity happened on day 11 under treatment with tacrolimus. [Tac:tacrolimus.].*

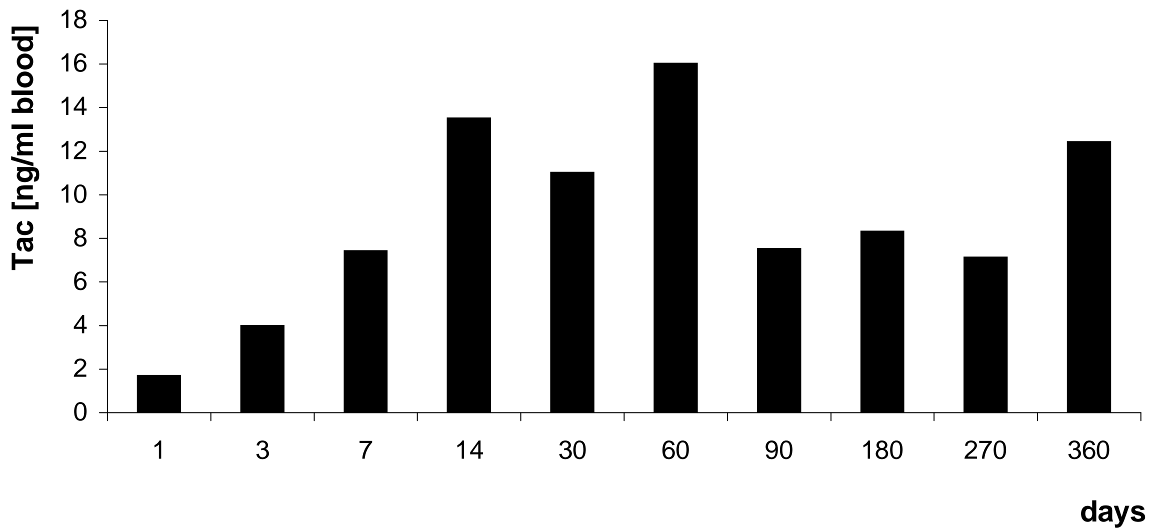

Figure P.1. *Tacrolimus blood concentrations of patient # 23 observed over 360 days. Event of rejection happened on day 106 under treatment with tacrolimus [Tac: tacrolimus.].*

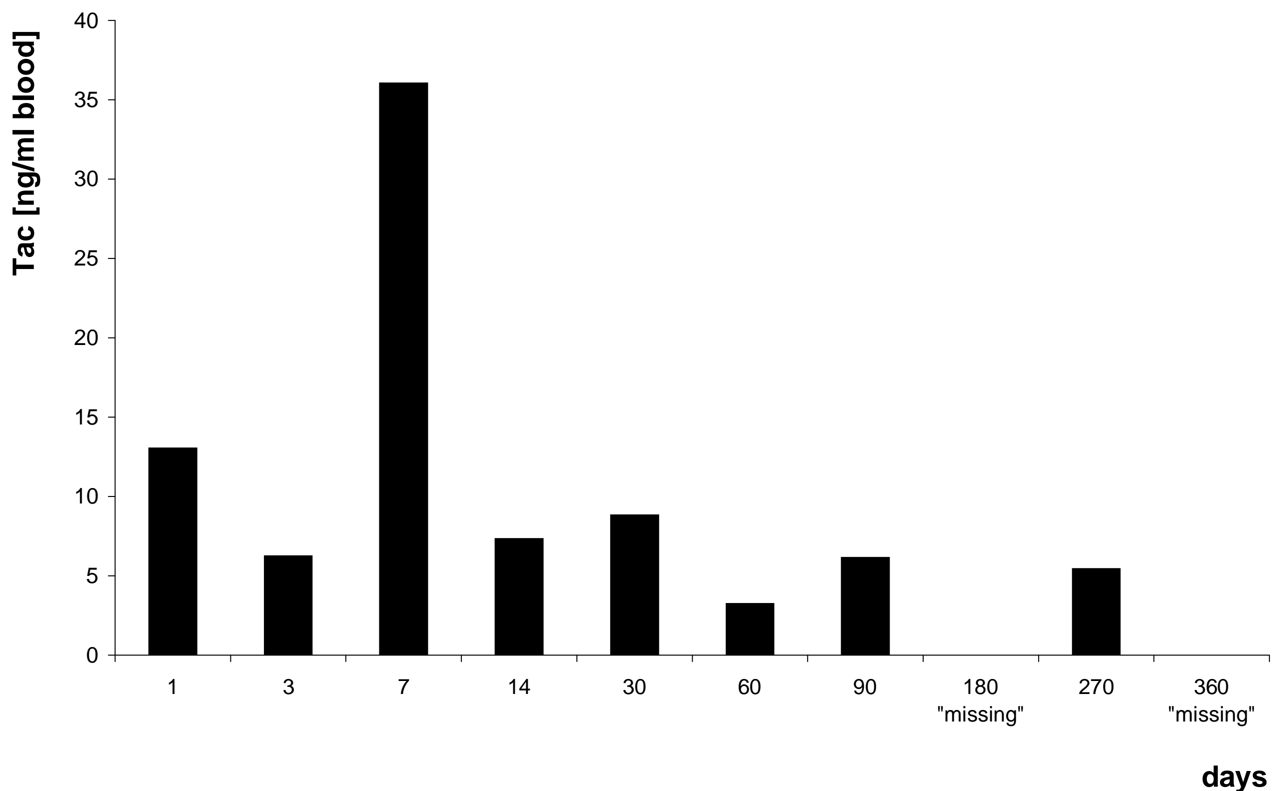

Figure P.2. *Tacrolimus blood concentrations of patient # 42 observed over 360 days. Events of rejection happened on days 270, 287, 298 and 314 under treatment with tacrolimus. [Tac: tacrolimus. Values days 180 and 360 were missing.].*

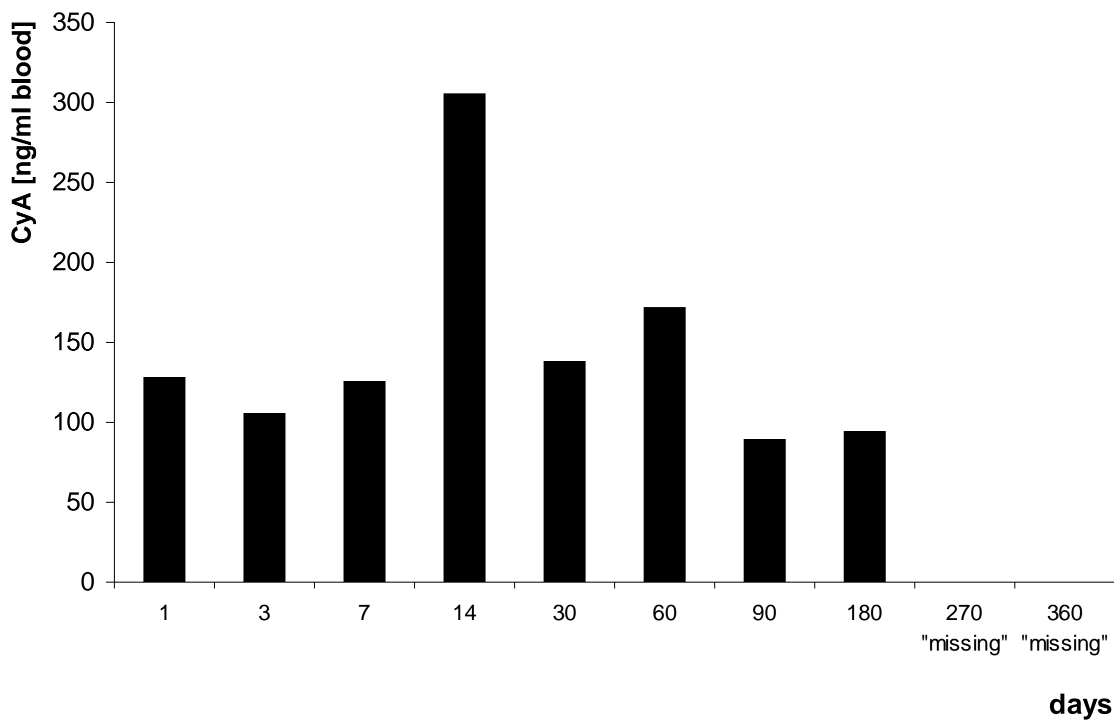

Figure Q.1. Cyclosporine blood concentrations of patient # 49 observed over 360 days. No event happened under treatment with cyclosporine. [CsA: cyclosporine. Values days 270 and 360 were missing.].

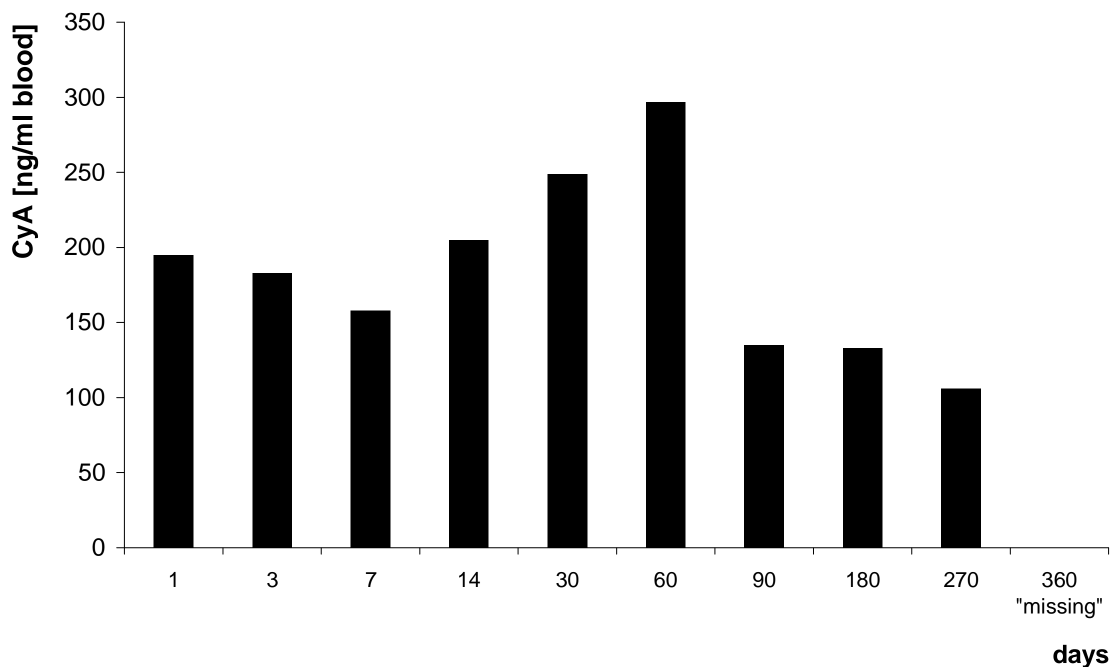

Figure Q.2. Cyclosporine blood concentrations of patient # 33 observed over 360 days. Event of rejection happened on day 222 under treatment with cyclosporine. [CsA: cyclosporine. Value day 360 was missing.].

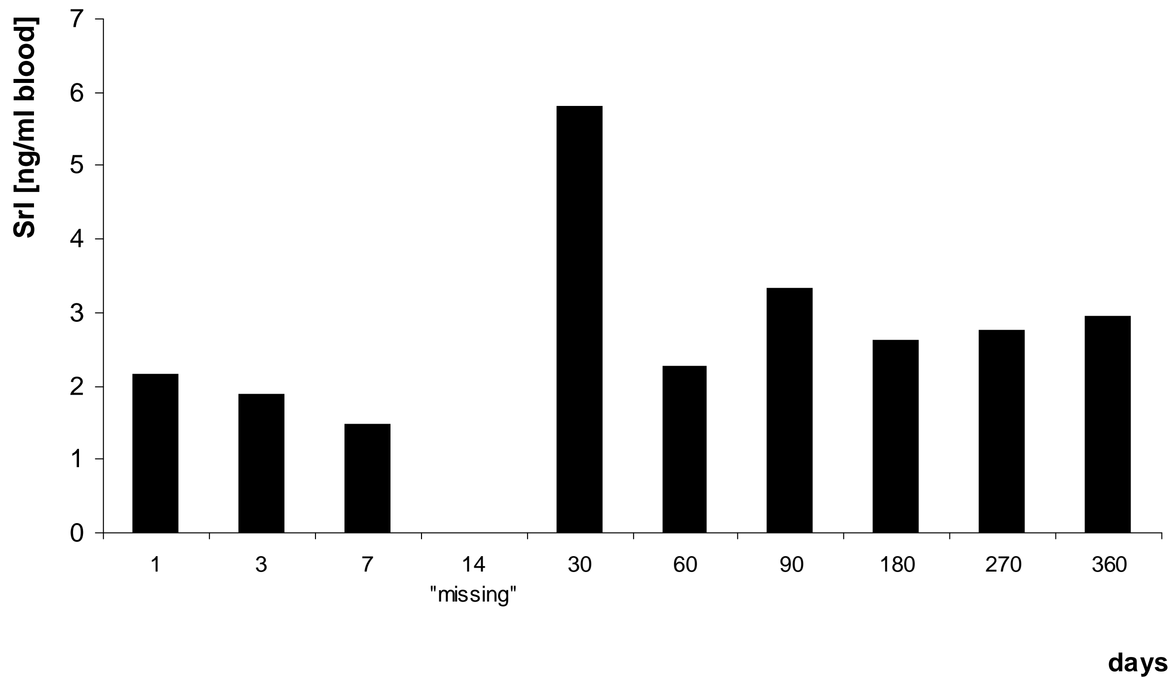

Figure R. *Sirolimus blood concentrations of patient # 1,1 observed over 360 days. No event happened under treatment with sirolimus. [Srl: sirolimus. Value day 14 was missing.].*

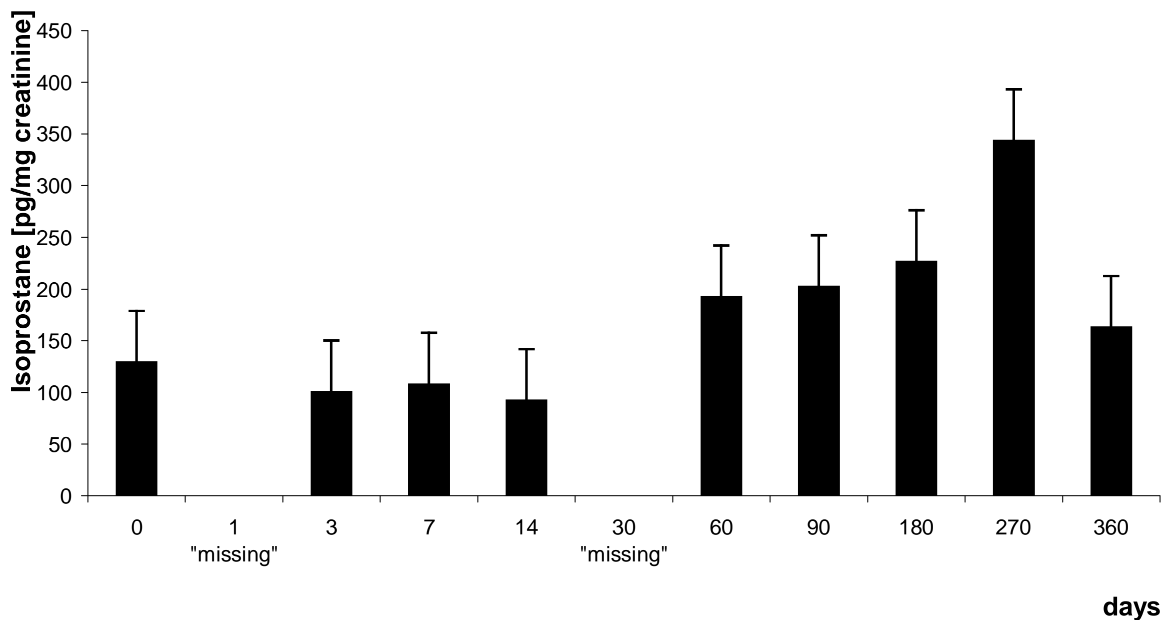

Figure S. *Isoprostanes in urine of patient # 24 undergoing biopsy-proven rejections on days 47,97 and 170. A peak of isoprostane concentrations around the events could be observed. [Crea: creatinine. Values days 1 and 30 were missing.].*

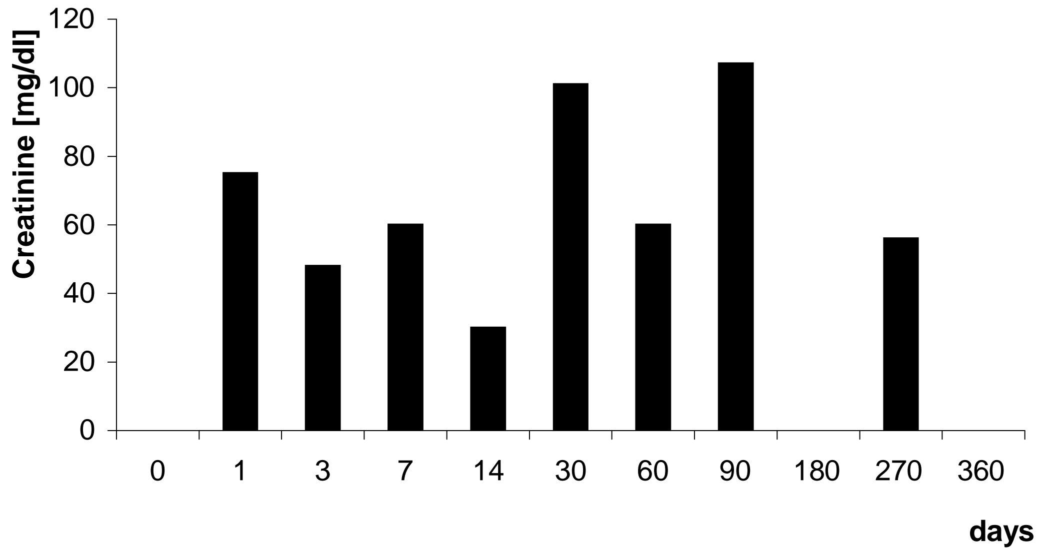

Figure T.1. *Graphic of patient # 37 with biopsy-proven event of toxicity at day 74. An increase of urinary creatinine concentration was observed. [Values days 0, 180 and 360 were missing.].*

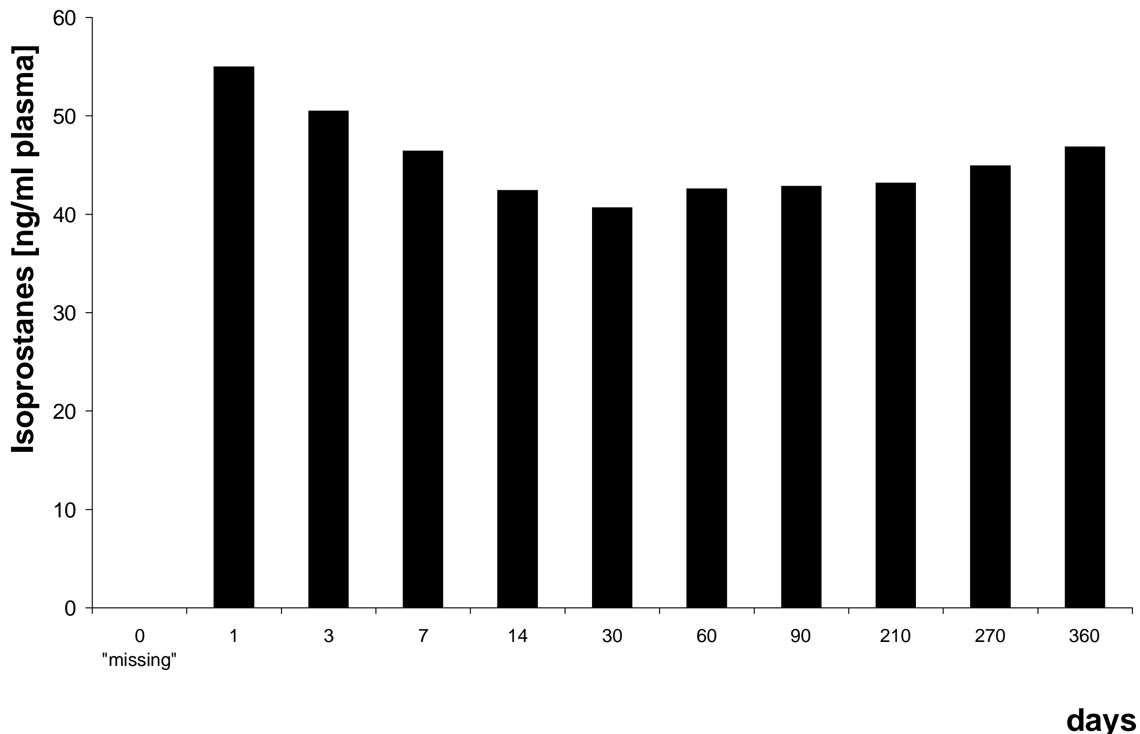

Figure U.1. *Isoprostanes in plasma of patient # 7undergoing biopsy-proven event of rejection on day 7. No peak of isoprostane concentration around time of event could be observed. [Iso: isoprostanes. Value day 0 was missing.] .*

11. Danksagung

Mein Dank gilt PD. Dr. Volker Schmitz für die exzellente Betreuung meiner Dissertation, die Vermittlung der Kooperation mit dem „Department of Anaesthesiology“ und seiner unermüdlichen Unterstützung und Motivation.

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Danke an Prof. C. Röcken für die histologische Expertise und Anleitung.

Danke an die Patienten, die menschlichen und tierischen Probanden, die an der Studie teilgenommen haben.

Mein Dank gilt außerdem meinen Eltern für ihren Glauben an mich und ihre Unterstützung.

Danke Björn für deine Liebe.

12. Curriculum Vitae

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

13. Publikationsliste

Crunk A, Christians U, Bohra R et al. “Sirolimus, but not Everolimus, Enhances Tacrolimus Nephrotoxicity in the Rat.” Poster. American Society of Transplantation meeting, 2009

Schöning W, Bohra R, Brunner N et al. „Everolimus and sirolimus have different effects in the rat kidney when combined with cyclosporine.” Poster. American Transplant Congress, San Diego, CA, 2010

Bohra R, Schöning W, Schmitz V et al. „Sirolimus and everolimus have different effects in rat kidney when combined tacrolimus.” Poster. National Predoctoral Clinical Research Training Program, 2010

Christians U, Klawitter J, Klawitter J et al. “Biomarkers of immunosuppressant organ toxicity after transplantation: status, concepts and misconceptions.” Expert Opin Drug Metab Toxicol 2011; 7(104): 175-200.

Klawitter J, Klawitter J, Schmitz V et al. „Low salt diet and cyclosporine toxicity: the role of PDZK1, CD147, NEMO, JAK/STAT and AKT signaling.” J Proteome Res. 11(11):5135-44. 10/2012.

Klepacki J, Klawitter J, Christians U et al. “Novel combinatorial biomarker panel in human urine displays ability to monitor kidney function in renal transplant recipients.” . 60th ASMS Conference on Mass Spectrometry, Vancouver, Canada May 20-24, 2012.

Bohra R, Schoning W, Klawitter J et al. “Everolimus and sirolimus in combination with cyclosporine have different effects on renal metabolism in the rat.” PLoS One; 7(10): e48063. 10/2012

Klepacki J, Brunner N, Schmitz V et al. „Development and validation of an LC-MS/MS assay for the quantification of the trans-methylation pathway intermediates S-adenosylmethionine and S-adenosylhomocysteine in human plasma.” Clin Chim Acta; 421C: 91-97 03/2013.

14. Erklärung gemäß der Promotionsordnung

Ich erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

Metaboliomics –
New biomarkers for early detection of immunosuppressive-induced nephrotoxicity and chronic rejection after KTX.
A translational analysis from animal model to kidney transplant patients.

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, 01.12.2012

Nina Brunner