Aus der Klinik für Allgemein-, Visceral- und Transplantationschirurgie der
Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The urine proteome of kidney transplant patients: A new approach for the identification of non-invasive rejection markers

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 04.02.2011

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2-DE	Two-dimensional Electrophoresis
ALG	Anti-Lymphocyte Globulin
AMBP	Alpha-1-Microglobulin/Bikunin Precursor
APC	Antigen-Presenting Cell
aRx	Acute Rejection
ATG	Anti-Thymocyte Globulin
BL	Borderline Rejection
CAN	Chronic Allograft Nephropathy
CE	Capillary Electrophoresis
CE-MS	Capillary Electrophoresis Mass Spectrometry
CHAPS	3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate
CIT	Cold Ischemia Time
CMV	Cytomegalovirus
CsA	Cyclosporine A
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ELISA	Enzyme-linked Immunosorbent Assay
ESRD	End Stage Renal Disease
Eta-1	Early T lymphocyte activation 1
FasL	Fas Ligand
FOXP3	Forkhead Box P3
GFR	Glomerular Filtration Rate
HLA	Human Leucocyte Antigen
IAA	Iodoacetamide
ICAM-1	Intercellular Adhesion Molecule 1
IEF	Isoelectric Focussing
IFNγ	Interferon gamma
IL-2	Interleukin-2
kDa	kiloDalton
LFA-1	Lymphocyte Function-associated Antigen 1
LOX-1	Lectin-like Oxidized Low-Density Lipoprotein Receptor-1
MALDI-TOF MS	Matrix-associated Laser Desorption Ionization Time-of-flight Mass Spectrometry
MHC	Major Histocompatibility Complex

MIG ..... Monokine Induced By IFN-gamma
mRNA ..... Messenger Ribonucleic Acid
MS ..... Mass Spectrometry
MW ..... Molecular Weight
NF-κB ..... Nuclear Factor κB
NGAL ..... Neutrophil-Gelatinase-Associated Lipocalin
OPN ..... Osteopontin
PBMC ..... Peripheral Blood Mononuclear Cells
PI-9 ..... Serine Proteinase Inhibitor-9
PMF ..... Peptide Mass Fingerprinting
RANTES ..... Regulated On Activation Normal T-cell Expressed And Secreted
RBP ..... Retinol-binding Protein
RNA ..... Ribonucleic Acid
RT-PCR ..... Reverse Transcriptase Polymerase Chain Reaction
SDS ..... Sodium Dodecyl Sulphate
SELDI-TOF-MS . Surface-enhanced Laser Desorption Ionization Time-of-flight Mass Spectrometry
SIBLING ..... Small Integrin-binding Ligand N-linked Glycoprotein
sICAM-1 ..... Soluble Intercellular Adhesion Molecule-1
sIL-2R ..... Soluble Interleukin-2 Receptor
TBM ..... Tubular Basement Membrane
TCR ..... T Cell Receptor
TEMED ..... Tetramethylethylendiamin
TLR ..... Toll-like Receptor
TNFα and β ..... Tumour Necrosis Factors α and β
Tris ..... Tris(hydroxymethyl)aminomethane
UTI ..... Urinary Tract Infection
VCAM-1 ..... Vascular Adhesion Molecule 1
VLA-4 ..... Very Late Antigen-4

1 Introduction

Physicians have tried for centuries to use patients' urine for non-invasive assessment of disease. History of urine examination from ancient times until nowadays is fascinating as it mirrors the history of medicine itself. The importance of urine in diagnosis was recognized by early civilizations in Egypt and Babylon more than 6000 years ago. Visual inspection of urine, called uroscopy, was used for thousands of years as the first laboratory test by physicians assuming that appearance of the excreted liquid would represent the body's inner working condition. In the Middle Ages, progress in analysis and its clinical significance was hampered by charlatanism and physicians who relied more on urine than on patient examination to diagnose disease.

Today, uroscopy has developed to urinalysis and physicians use urine to diagnose selective disease states. Dipstick testing has become a basic element in the routine clinical assessment of renal and urogenital diseases. Human urine is an easily obtainable biological fluid and contains relevant biomarkers. In case of damage or compromised function of kidney and urogenital tract, the anatomical proximity to the sites of urine excretion and the particular functions of these organs increase the probability to detect changes in the urine proteome. The establishment of more sophisticated methods for analysis of specific urinary proteins has revealed several biomarkers that made the way from bench to bedside. And, the changes in urinary protein patterns are not only detected in nephrological disease; for example, all commercially available pregnancy kits are based on the measurement of β-human chorionic gonadotropin in the urine. Newer technologies, in particular mass spectrometry, have emerged and allow an even more detailed insight into physiological and disease processes. With regard to kidney transplantation, there is a growing clinical interest in the identification of urinary markers that appear during episodes of acute rejection.

1.1 Kidney transplantation

Kidney transplantation can justifiably be regarded as one of the great achievements of modern medicine. It requires a unique integration of knowledge from surgery, nephrology and immunology. No therapeutic option for patients with end-stage kidney disease was available until Joseph Murray successfully transplanted the first kidney in 1954, and until the advent of hemodialysis, whose development was contemporaneous. Since then, many important discoveries in immunology and surgical improvements have paved the way for today's successful transplantation medicine. The different types of transplants are depicted in table 1.

Table 1: ***Characterization and terminology of transplantations***

Genetic relationship between donor and recipient	
Autograft	Donor and recipient is the same individual
Isograft/syngeneic graft	Donor and recipient are genetically identical
Allograft/homograft	Donor and recipient belong to the same species
Xenograft/heterograft	Donor and recipient belong to different species
Anatomical site of implantation	
Orthotopic	Donor organ is placed in the anatomically correct position in the recipient
Heterotopic	Implant placed at a site different from normal anatomy

Beginnings of kidney transplantation are intimately connected with the advances made in vascular surgery. Alexis Carrel (1875-1944) is regarded as a founding father of experimental organ transplantation as he made a substantial contribution to progress in vascular sutures. In 1912, he received the Nobel prize in acknowledgment of his work described in "The transplantation of veins and organs". Simultaneously, the world's first technically successful experimental kidney transplant from dogs and goats was performed in Vienna by Emmerich Ullmann in 1902 [1]. Although the transplants produced urine for a few days, most experiments failed due to thrombosis of the organ.

Very soon it became evident, that surgical improvements alone could not ensure a successful outcome of transplantation. Most transplants did not even start to function, and if they did, it was only for some days until they got rejected by the recipient. However, the idea of successful organ transplantation was not given up. Several scientists of the time already suggested that unknown "biochemical barriers" impeded allograft survival. The Viennese pathologist Karl Landsteiner had discovered the AB0 blood group system in 1901 [2] and paved the way for clinical blood transfusions based on his compatibility concept. Already in 1912, Georg Schöne postulated that immunological phenomena are responsible for transplant failure [3]. But the British zoologist Sir Peter Brian Medawar was the first to describe the elementary processes of the immunological response against foreign tissues based on his observations in rabbit skin transplantations in the 1940s [4]. He was awarded with the Nobel prize in 1960 for the "discovery of the acquired immunologic tolerance".

Until the mid-1950s, results from kidney transplantations in humans were disappointing. Attempts by Mathieu Jaboulay (1906, first xenotransplantation) [5], Yuri Voronoy (1933, first cadaveric donor transplantation) [6], David Hume (1947) [7] and Jean Hamburger (1952, first living donor transplantation) [8] all failed, ending up in rejection or thrombosis of

the graft after few days. Then, on December 23rd in 1954, Joseph Murray performed the first successful kidney transplantation between identical twins [9]. He implanted the kidney extraperitoneally into the iliac fossa and anastomosed its vessels to the iliac artery and vein, a technique which has remained standard in transplantation medicine until today. Subsequent attempts by Murray and Merrill led to 7 successful transplantations between identical twins at the Peter Bent Brigham Hospital in Boston.

Four years later in Paris, Jean Dausset described for the first time a leucocyte antigen, which led to the development of the Human Leucocyte Antigen (HLA) system in the 1960s and 1970s. Major Histocompatibility Complex (MHC) molecules on the surface of graft cells were found to be the principle targets of the immune response. Table 2 shows the relationship of MHC and HLA and their rough functions.

Table 2: ***Major Histocompatibility Complex (MHC) molecules and the HLA system***

MHC class	HLA molecules	Presented antigen	Expressed on	Recognition by
MHC class I	HLA-A, HLA-B, HLA-C	intracellular antigens, like cell owned or viral peptides	most somatic cells	CD4+ T cells
MHC class II	HLA-DR, HLA-DQ, HLA-DP	extracellular antigens, like apoptosis related or bacterial peptides	professional antigen presenting cells	CD8+ T cells

Dausset assumed that an individual's biological identity is defined by its MHC genes. Further research aimed to establish better tissue-typing techniques for cross-matching. Van Rood in Leiden showed that the number of HLA-mismatches between donor and recipient has a major impact on graft survival. In consequence, he founded Eurotransplant in 1968 to provide a broad basis for organ exchange in Europe. Besides the progress in immunological knowledge about the mechanisms of rejection, discovery of new immunosuppressive agents allowed transplantations to become a routine clinical practice.

Nowadays, kidney transplantation is the treatment of choice for end-stage renal disease (ESRD), which is defined as a drop of glomerular filtration rate to 20-25 % of normal. Main causes for end-stage kidney disease are diabetic nephropathy, hypertension and glomerulonephritis, followed by systemic autoimmune diseases and genetic causes like polycystic kidney disease. Loss of kidney function can lead to hypertension and edema due to fluid

volume overload, disorders of the acid-base-balance and blood electrolytes, accumulation of waste products of metabolism in serum, and anaemia because of decreased synthesis of erythropoietin. Some functions of the kidney can be replaced by dialysis. Patients under dialysis treatment are heavily restricted in their daily life, as it is a time-consuming procedure (several hours at least 3 times per week) and normally binds them to the center where dialysis is performed. Successful kidney transplantation offers freedom from dialysis and the restrictions that it imposes, a better quality of life, decreased mortality and it is less expensive. Patient survival one year after living-donor transplantation is 95% and comparably high when the organ comes from a deceased donor. Graft survival ranges between 70-90% after 3-5 years [10]. Long-term survival is better with transplanted patients than with those kept on dialysis [11]. Therefore, most patients under dialysis treatment get listed for transplantation. The demand for suitable organs exceeds the supply by far. In 2007, 2340 deceased-donor kidney transplants were performed in Germany [12], whereas 8207 dialysis patients are still on the waiting list. Up to one third of the organs come from living, genetically related or unrelated donors with bilateral renal function. 2/3 of the related living donors are the patient's parents. More than 2/3 of kidney allografts are from deceased donors [10]. Donors with vascular diseases, diabetes, and malignancies are excluded. The transplant surgery is relatively simple and takes about 3 hours. The donor kidney is placed retroperitoneally in the iliac fossa, vascular anastomosis connects its blood vessels to the iliac artery and vein and the ureter is implanted in the recipient's bladder. A kidney allograft of good quality normally begins to work immediately. Several factors can lead to slow or delayed graft function, which itself is associated with a worse prospect of graft survival and higher acute rejection rates. In contrast to slow graft function, patients with delayed graft function need dialysis in the first week after transplantation due to high serum creatinine levels [13, 14]. Many post transplant problems including viral infections, recurring disease, increased incidence of malignancies and nephrotoxicity are related to the immunosuppressive drugs. But since the major complication in the first months after transplantation is acute rejection (aRx) of the organ, their application remains inevitable [15].

1.2 Immunosuppression

Total-body high-dose radiation was the first attempt to control rejections in renal transplant patients in the 1950s. The outcome was poor, only two successful cases in Paris and Boston were reported [16]. First improvements of transplant survival could be achieved by the discovery of azathioprine between 1957 and 1963 by Hitching and Elion [17], the recognition of its immunosuppressive potential by Schwartz, Stack and Damashek [18] and its introduction into transplantation medicine by Murray and Calne [19, 20] in Boston. Azathioprine

inhibits the synthesis of DNA/RNA in dividing lymphocytes, but also in all other proliferating cells. The toxicity on proliferating tissues may cause side-effects like diarrhea, hair loss and cytopenia. Mycophenolate mofetil has the same mechanism of action and is widely used instead of azathioprine in organ transplantation. It is associated with a lower incidence of acute rejection, less bone marrow suppression and fewer opportunistic infections [21].

Also in 1963, Starzl et al. described the supportive immunosuppressive action of prednisolone in azathioprine therapy [22]. Cortisone had been discovered in 1936 as an adrenal gland steroid with immunosuppressive properties. A combination of azathioprine and steroids became the standard anti-rejection regimen for many years. The work of Starzl ushered in the proliferation of kidney transplantation programs worldwide.

In 1975, Dreyfuss et al. extracted a substance with immunosuppressive, antilymphocytic properties from *Tolypocladium inflatum*, called cyclosporine A (CsA) [23]. Experiments by Jean Francois Borel at Sandoz laboratories in Basel brought CsA to the world's attention [24]. Clinical introduction of CsA by Calne [25] revolutionized transplantation medicine, yielding a strong immunosuppressive potency and less myelotoxic side-effects. It enabled the routine transplantation of organs, a therapy which until then only had been done experimentally. CsA and the later discovered tacrolimus/FK506 inhibit the protein phosphatase calcineurin in T cells. Calcineurin activates the transcription of interleukin-2 (IL-2), which stimulates the differentiation and growth of T cell response. Ironically, calcineurin inhibitors are nephrotoxic and cause damage of the transplanted organ; therefore, their plasma levels must be kept within a narrow range (drug monitoring). Studies have reported that the benefit of tacrolimus is greater than that of cyclosporine in kidney transplant patients [26].

Since the mid-60s, mono- and polyclonal antibodies against immune cells have been developed for clinical use. Their application prevents the immune response against a kidney transplant quickly and very specifically. Because of their immunogenicity, their use can cause strong allergic reactions. Patients might get sensitized and form neutralizing antibodies, which makes a second application inefficient. There are attempts to diminish the allergic reactions by using chimeric or humanized immunoglobulins. The first monoclonal antibody approved for clinical use was OKT3, a murine antibody against the T cell receptor complex. It leads to a quick elimination of T cells from circulation and a depletion of pre-activated T cells. Other monoclonal antibodies like daclizumab and basiliximab target the IL-2 receptor CD25, which is expressed on the surface of already activated T cells. Polyclonal antibodies are obtained from the serum of rabbits or horses, which were immunized with human lymphocytes (ALG) or thymocytes (ATG). They target a broad spectrum of antigens on lymphocytes and cause lysis of the attacked cells.

Quiet soon it was clear that the cocktail approach which combines cyclosporine with steroids and azathioprine was the most effective approach to immunosuppression for transplant patients. Doses and therewith toxicity of the individual agents could be lowered. This combination has lately been replaced by regimens that include newer immunosuppressive drugs. All patients involved in the present study were treated according to a standard protocol with basiliximab as induction agent, followed by tacrolimus, mycophenolate mofetil and tapered steroids as maintenance immunosuppression.

1.3 Graft rejection

Rejection is the recipient's immune response against the transplant, which is recognized as foreign tissue. The molecular mechanisms of acute transplant rejection are complex and not completely understood yet. Rejections are mediated through both humoral and cellular immune mechanisms, which can be active at the same time. The B cell mediated humoral rejection is based on (pre-) existing antibodies against donor HLA molecules expressed by the transplant and usually manifests itself as severe dysfunction with high risk of allograft loss [27]. The T cell mediated cellular rejection is characterized by an inflammatory infiltration of the transplant tissue by mononuclear cells. In the pioneer era of transplantation, most renal allografts were lost during the first year after transplantation due to acute rejection episodes. The causes of renal allograft loss have changed with the introduction of new immunosuppressive agents. Nowadays, the most relevant cause of allograft loss after the first year of transplantation is a chronically-progressive scarring process of renal tissue, called "chronic allograft nephropathy", or "chronic rejection" [28]. Acute rejection episodes, even if subclinical [29], are the main cause of this scarring process and determine long-term graft function and survival in renal transplant patients [30]. Other causes include tubular injury, toxic effects of drug treatments –especially from calcineurin inhibitors–, viral graft infections, and factors related to the graft quality, such as prolonged cold ischemia time [31], old donor age [32], or graft origin from deceased donors. Because of the central role of acute rejection for long-term allograft survival, timely diagnosis and therapy is an utmost task in the care of renal transplant patients. Reliable and early detection of rejection is particularly important in the efforts to spare patients from over-immunosuppression by using minimized individual immunosuppressive protocols.

Rejections are clinically classified by their time of manifestation into hyperacute, accelerated, acute and chronic rejection. This classification does not take into account the underlying immunological and pathological mechanisms.

Hyperacute rejection occurs within minutes or hours after transplantation. It is triggered by pre-formed antibodies against donor HLA or AB0 antigens, which bind to the vascular endothelium of the transplant. Activation of the complement system follows, and platelet thrombi form in capillaries and small arterioles, which finally results in necrosis of the tissue. The transplant must be immediately removed to prevent a systemic inflammatory response. New cross-match techniques have drastically reduced the incidence of hyperacute rejections.

Accelerated rejection occurs 2-5 days after transplantation. It is caused by prior sensitization to donor antigens through blood transfusions, pregnancy or prior transplantations and represents an induced memory reaction leading to a quick accumulation of antibodies and cytotoxic T cells against donor-specific antigens. The principal finding on pathologic examination is fibrinoid necrosis of the small vessels. Due to sensitization, re-transplanted recipients are exposed to a greater risk.

Episodes of **acute rejection** commence 5-7 days after transplantation and occur with decreasing frequency after 3 months. However, acute rejection can appear months to years after transplantation, frequently associated with the withdrawal of immunosuppressive medication. It is a heterogeneous process with regards to the renal structures involved, the mechanisms of immunological injury, and the clinical presentations. Both humoral and cellular mechanisms can be involved in the process.

Antibody-mediated, humoral acute rejection is mediated by B cell populations. Circulating antibodies mainly attack the smaller vessels of the graft, which led to the abandoned term “vascular rejection”. It is detected by the complement fragment C4d at the level of peritubular capillaries. New findings suggest that antibodies are formed not only against donor HLA antigens but also against the angiotensin-II receptor, which could result in severe hypertension [33].

Acute cellular rejection is characterized by mononuclear cells infiltrates of the interstitium, which is composed predominantly of activated T-lymphocytes but also of different subsets of T cells, B cells, NK cells, macrophages and neutrophils [35]. The first step towards an immune response against the graft is the recognition of the transplanted tissue as foreign material by the recipient’s T cells. Their T cell receptor (TCR) binds to MHC molecules on the surface of donor antigen-presenting cells (APC) and detects both the MHC molecule and the peptide that it presents as “foreign”. This mechanism is called allorecognition, and represents the trigger to cell-mediated rejection. Allorecognition occurs by two distinct mechanisms, called the direct and indirect pathways. The direct pathway results from the recognition of MHC molecules, intact, on the surface of donor cells. In addition, donor APCs disseminate to the host lymphoid system and present alloantigens on

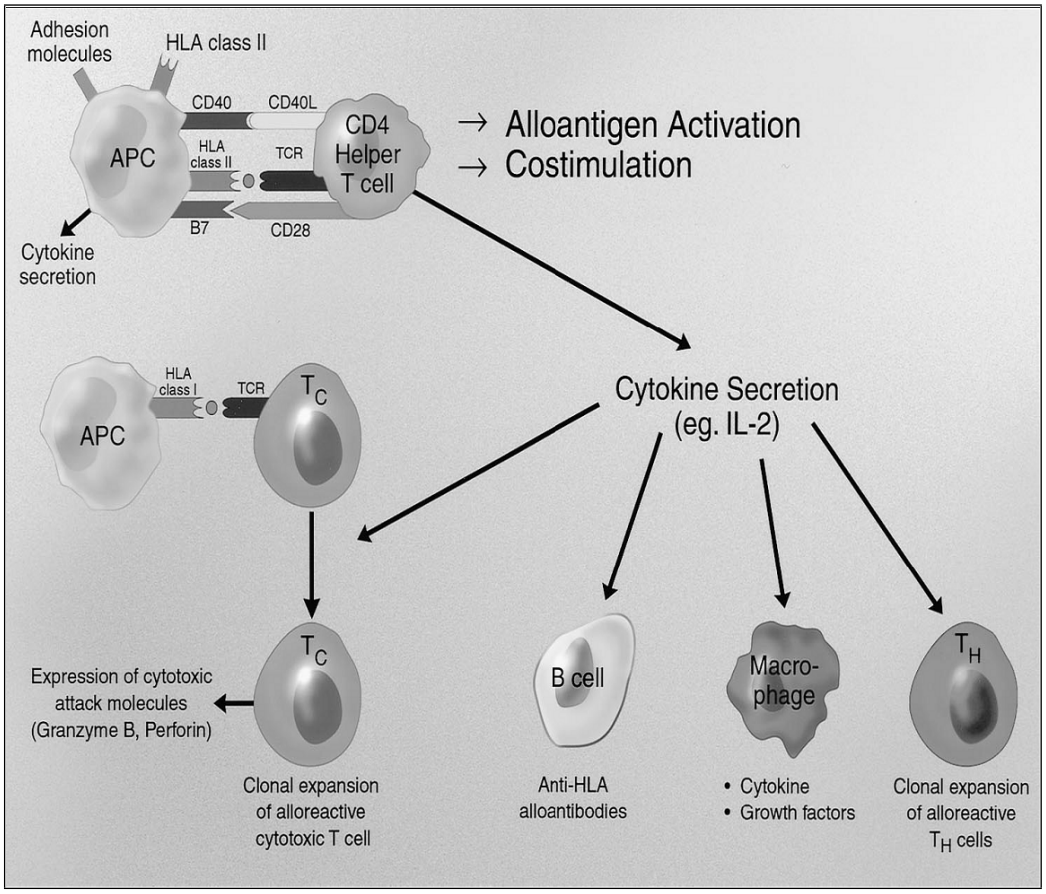


Figure 1: ***Most important co-stimulatory molecules on APC and their ligands on T cells.*** *Allorecognition through the T cell receptor is necessary but not sufficient to activate alloreactive T cells. The B7-CD28 interaction triggers a crucial co-stimulatory signal for T cells and promotes full T cell activation. The absence of a second signal results in functional inactivation of the cell. CD40-CD40L interactions mostly affect the APC, including up-regulated expression of inflammatory cytokines, adhesion molecules and B7. The APC activated via its CD40 receptor has improved T cell activation capacities (from [34]).*

foreign MHC molecules. This mechanism is so immediate that it seems to be primarily involved in acute graft rejection. Indirect allorecognition occurs when donor histocompatibility molecules are internalised, processed, and presented as peptides by host antigen presenting cells. This mechanism is even immediate, but since APCs of donor origin are depleted with time, limiting the influence of the direct pathway, the indirect pathway takes over to be the only mediator of allorecognition in chronic rejection [36]. Under the influence of other co-stimulatory signals (see figure 1), the T cells differentiate and get activated and secrete a certain pattern of cytokines [34]. Those cytokines boost the proliferation rate, accelerate the differentiation of naive T cells into effector cells, modulate cytotoxic activity of other cells and activate macrophages. Production of chemokines leads to a selective recruitment of T cells and monocytes to the transplanted organ. Monocytes infiltrate the tissue with the help of adhesion molecules (see figure 2), develop to macrophages and release cytolytic proteases and free radicals that cause tissue damage. Furthermore, they secrete cytokines that enhance the mobilisation of immune cells and the inflammation process. Activated cytotoxic T cells contribute to tissue damage by directly attacking donor cells. Once they recognize foreign peptides by the TCR-MHC complex, they release their granules containing cytotoxic molecules like granzymes and perforin. Perforin forms pores in the donor-specific target cell, granzymes are transferred to induce fragmentation of intracellular DNA and apoptosis. The second major cytotoxic pathway is initiated by the ligation of Fas and its ligand. Fas ligand (FasL) is expressed on T cells being activated in the inflammation process during rejection. The Fas receptor (CD95) is expressed on donor cells and linked with an intracellular death domain. In case of Fas-FasL ligation, caspases are activated to initiate apoptosis in the target cell [37]. The majority of acute cellular rejection episodes can be treated with immunosuppressants, especially when they appear within the first 6 months of transplantation. Late occurring acute rejections carry a worse prognosis for the allograft and respond less to rejection treatment. Good clinical practice for the treatment of rejections is high-dose corticosteroids over several days, in case of non-responders a mono- or polyclonal antibody might be added. However, every rejection episode contributes to the development of chronic rejection and restricted long-term function of the transplant.

Chronic rejection was the term used to describe a chronically-progressive scarring of renal tissue with deterioration of graft function, occurring at least 3 month post-transplant. It has widely been substituted by the term chronic allograft nephropathy (CAN), as both immune and nonimmune mechanisms cumulatively injure the kidney. CAN has become the dominant cause of kidney-transplant failure and allograft loss. In biopsies, CAN is characterized by interstitial fibrosis and tubular atrophy, representing a final common pathway of injury and its consequent fibrotic healing response. Early causes for tubulointerstitial damage are ischemia-reperfusion injury, acute clinical and subclinical rejection episodes or acute tubu-

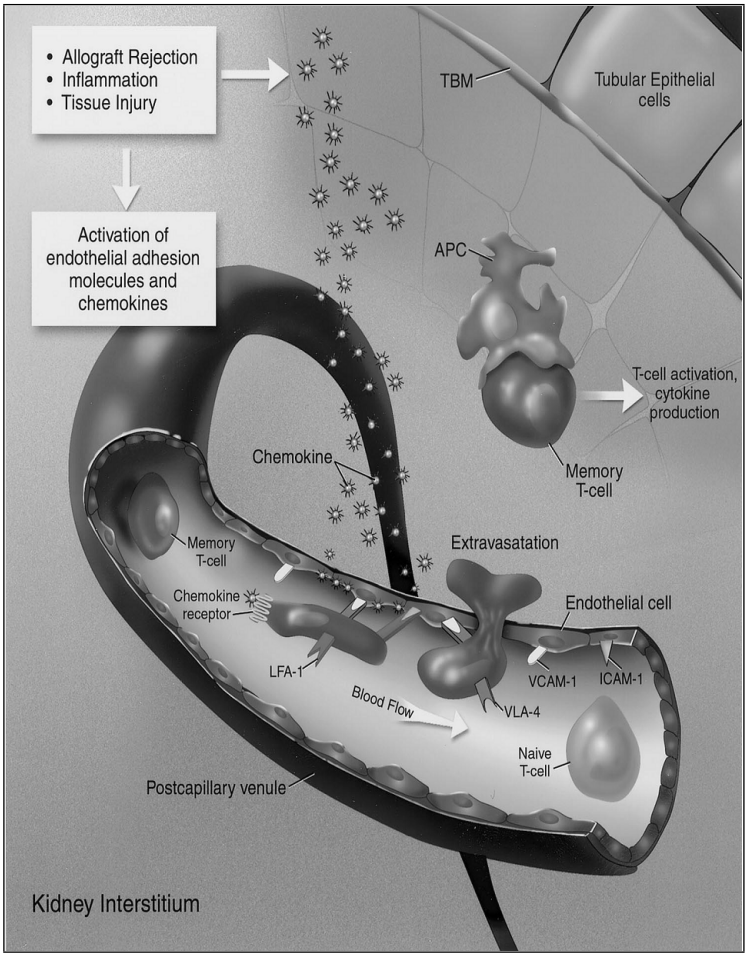


Figure 2: ***Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) are up-regulated due to tissue injury and inflammation in the rejection process.*** *The interactions between lymphocyte counter receptors and endothelial adhesion molecules mediate the extravasation of alloreactive T cells into the kidney interstitium during allograft rejection. Initially, lymphocytes roll over the endothelial cells through low affinity interactions. Chemokines that are produced at the site of inflammation are displayed on the surface of the endothelium and become exposed to T cells. Ligation of chemokines to specific T cells receptors results in a modification of the structure of the integrins LFA-1 and VLA-4 on the lymphocytes so that they can bind tightly to ICAM-1 and VCAM-1, respectively, on the endothelial cells. This binding leads to a stable arrest of lymphocytes and allows their extravasation. Once extravasated, T cells become activated when they encounter alloantigens and release cytokines that will promote the inflammatory infiltrate leading to the destruction of tubular cells. TBM denotes tubular basement membrane (from [34]).*

lar necrosis. Ongoing cellular and humoral immune reactions are said to be responsible for causing a transplant vasculopathy with fibrous intimal thickening of the arteries and variable glomerular lesions. Later, microvascular and glomerular injury increases frequently as a result of calcineurin inhibitor nephrotoxicity, but also from hypertension, polyoma virus infection and occasionally from recurrent or de novo glomerulonephritis. Donor-related factors (brain death, age and pre-existing disease) and procurement-related factors (warm and cold ischemia, perfusion quality and transport) also influence the development of CAN. The newly revised Banff classification system published in 2007 has renamed chronic allograft nephropathy, "Interstitial fibrosis and tubular atrophy, without evidence of any specific etiology" [38].

1.4 Rejection diagnosis

During the first days after transplantation, patients are intensively monitored with respect to their general condition, temperature, blood pressure, pulse, urine output, and weight. Blood parameters including blood cell counts, electrolytes, serum creatinine, urea, and glucose are determined daily to notice every change that could be suspected of rejection. Fever and leukocytosis might be signs of an ongoing immune response against the allograft. But most clinical symptoms of acute rejection episodes are consequences of impaired function of the kidney allograft. If the attacked organ fails to produce urine, patients will gain weight due to fluid retention. Urea levels in blood and calculation of GFR give an impression of the filtration capacity. Disorders in serum electrolyte composition are also considered as aberrations from normal kidney function. Creatinine is an important parameter to determine the kidney's clearance of body waste products. Anaemia could rely on a lack of erythropoietin, a hormone produced in healthy kidneys. Ultrasound examination is performed to reveal disturbances in the graft, although specificity and sensitivity for acute rejection is limited, even with the use of echo enhancers [39]. If a patient is suspected of having a rejection episode, a needle biopsy will be carried out to confirm the diagnosis. Histologic assessment of the graft remains the gold standard for rejection and most differential diagnoses.

1.4.1 Serum creatinine

Creatinine is a degradation product of creatine phosphate in muscle and is produced at a fairly constant rate depending on the body's muscle mass. It is mainly filtered in the glomerulus of the kidney, though a small amount is actively secreted. The tubular reabsorption is marginal. This makes creatinine a useful parameter to calculate the glomerular

filtration rate (GFR) via its serum and urine levels. If the filtering function of the kidney is impaired, serum levels will rise, and therefore, creatinine is commonly used as an indicator of renal function. However, surveillance of post-transplant patients by this approach implies recognition of an immunological injury at a relatively late stage. A rise in serum creatinine will not be seen until the kidney is markedly damaged. The relative lack of sensitivity of serum creatinine in detecting dysfunction has been discussed extensively [40]. As serum levels also depend on the body's muscle mass, the trend of serum levels over time is more important than the absolute serum level.

1.4.2 Glomerular filtration rate (GFR)

GFR is the total volume of fluid filtered from the renal glomerular capillaries into the Bowman's capsule per unit time. It can be calculated by measuring a parameter that has a constant level in the blood, and is freely filtered but neither reabsorbed nor secreted by the kidneys. Creatinine meets these requirements fairly well, and as it is a physiologic compound and can be measured easily, it is widely used to determine the GFR. The Assumption that the amount of creatinine filtered in the glomerulus per unit time is the same amount which appears in urine leads to the following formula:

$$GFR = \frac{\text{Urine concentration} \times \text{Urine flow}}{\text{Plasma concentration}}$$

It implies that urine flow, urine concentration, and plasma concentration of creatinine are known, hence a 24-h urine collection is necessary. To allow comparison of results between people of different sizes, the GFR is often corrected for the body surface area and expressed in comparison to the average sized person as $\frac{\text{ml}}{\text{min} \times 1.73 m^2}$. Estimating GFR by serum creatinine measurement alone did not suffice to get reliable values [41]. A number of more sophisticated formulas have been conceived to estimate GFR by serum creatinine levels, e.g. the widely used Cockroft-Gault formula implies the patient's age, gender, weight and serum creatinine level to calculate the GFR [42].

1.4.3 Serum albumin

Albumin is the major plasma protein. Its main functions are transport of different substances and the regulation of the blood colloidal osmotic pressure. Under normal physiological conditions, small amounts of protein (<150 mg/day) are excreted with the urine. The kidney restricts passage of plasma proteins in the mass range above ca. 40 kDa during the filtration process in the glomeruli. Proteins below ca. 40 kDa are reabsorbed from the glomerular

filtrate and degraded in the proximal renal tubules. Diseases, which adversely affect the function of glomeruli and tubuli, including renal malignancies, inflammation and nephrotoxic agents, cause excessive losses of proteins in the urine, called proteinuria. Historically, proteinuria of more than 150 mg/day was regarded as abnormal. However, it is nowadays accepted that low-level albuminuria (between 30 and 300 mg/day) is often characteristic for early renal disease [43]. As this concentration of albumin is below the detection threshold of traditional assays, the condition is termed microalbuminuria. In contrast, overt proteinuria or macroalbuminuria is reached if protein or albumin excretion is greater than 300 mg/day and becomes accessible for dipstick testing. Urine dipstick testing is essentially albumin-specific.

Pathological proteinuria is classically divided into 3 categories: glomerular proteinuria, tubular proteinuria and overload proteinuria. Glomerular proteinuria results from an increase in the permeability of the glomerular capillary wall to macromolecules (particularly albumin) and usually results from glomerular disease. Tubular proteinuria can be due to 2 mechanisms: reduced reabsorption of normally present proteins from the glomerular filtrate and excretion of proteins due to injured tubular epithelial cells. It is usually caused by diseases of the tubulointerstitium. Overload proteinuria results from an excess of low-molecular-weight proteins that are reabsorbed by the proximal tubules under physiological conditions. These proteins are most often immunoglobulin light chains (in plasma cell dyscrasias), but hemoglobin (in intravascular hemolysis), myoglobin (in rhabdomyolysis), and lysozyme (in myelomonocytic leukemia) have also been identified.

Albumin is still the principal urinary protein measured in clinical diagnostics. Microalbuminuria is a clinically important marker of early diabetic nephropathy and concomitant cardiovascular disease. The gold standard for quantification of microalbuminuria is analysis of a 24-hour urine collection by the Biuret method. But as 24-hour collections are inconvenient for the patient, and as there is good correlation between the protein-creatinine ratio of a single spot urine sample and results obtained by 24-hour urine collection, many guidelines now recommend the measurements of urine protein-creatinine ratio to 24-hour urine collection [44]. The correlation was also shown for renal transplant patients [45]. Halimi et al. measured urinary albumin excretion in 616 renal allograft recipients at variable time points after transplantation. About half of them had normoalbuminuria, the other half had micro- or macroalbuminuria. Microalbuminuria and macroalbuminuria were found to be powerful predictors of ESRD and death, with microalbuminuria being a risk factor for graft loss even in patients without proteinuria [46]. In our study, we observed no altered urinary excretion of albumin during episodes of rejection, as depicted in chapter 4.2.1.

1.4.4 Allograft biopsy

Kidney biopsy is the gold standard procedure for the assessment of allograft dysfunction. A small sample of renal tissue is obtained by percutaneously puncturing the transplant under local anaesthesia and ultrasound guidance. An "adequate" specimen is a biopsy with 10 or more glomeruli and at least 2 arteries. Histological evaluation of the sample by an experienced pathologist can discriminate between most differential diagnoses. The multiplicity of acute rejection has been categorized by the BANFF classification [47], which is widely used for the evaluation of renal allograft biopsies. Histological findings are categorized topographically into tubular ("t"), vascular ("v"), interstitial ("i") and glomerular ("g") changes and rated by their severity. The cardinal features of acute/active rejection are infiltration of mononuclear cells in tubules (tubulitis) and the intima of arterial walls (arteritis). Table 3 shows the criteria for categorization of acute/active rejection in 6 types by the BANFF classification.

Table 3: ***BANFF classification for acute/active rejection of renal allografts***

Type (Grade)	Histopathological findings
Borderline	Mild tubulitis, no intimal arteritis - "suspicious for acute rejection"
Ia	Significant interstitial infiltration, foci of moderate tubulitis (>4 mononuclear cells/tubular cross section or group of 10 tubular cells)
Ib	Significant interstitial infiltration, foci of severe tubulitis (>10 mononuclear cells/tubular cross section or group of 10 tubular cells)
IIa	Significant interstitial infiltration, mild to moderate intimal arteritis
IIb	Significant interstitial infiltration, severe intimal arteritis comprising >25% of the luminal area
III	Transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells

In most centers, post-transplant surveillance primarily relies on monitoring diuresis, serum creatinine and urea levels and the estimated GFR. Anuria or a persistent rise in serum creatinine levels will trigger a biopsy. However, this reactive strategy may fail to detect acute

rejection at its very beginning. Some transplant centers have therefore turned to regularly performing control biopsies to detect acute rejection earlier. High frequencies of so called "subclinical rejections" have been reported in protocol control biopsies [48]. This approach, however, is limited for different reasons: sampling error due to focal nature of infiltrates and limited sample size, high costs, procedural risks like bleeding or arterio-venous fistulas, and the stress for the patient [49]. Therefore, it is essential to look for additional tools that detect acute rejection in the allograft by non-invasive means.

1.5 New rejection markers

Many attempts have been made to establish alternative non-invasive markers of rejection. Yet, none of these markers has made its way into today's clinical practice, suggesting that the "ideal marker" is still to be discovered. Many expectations of new markers need to be considered. 1. Sample collection should be easy and repeatedly possible which applies for blood and urine. 2. The marker should be stable over time and insensitive against pH changes and other sample constituents like interfering substances. 3. Analysis of the marker should be methodologically simple, reproducible, and time and cost efficient. 4. The practical value of the marker relates to its sensitivity and specificity, in order to differentiate between rejection and non-rejection, to determine the severity of rejection and to discriminate between rejection and other causes of graft injury. It is unlikely that one marker alone will fulfil all these expectations. A combined set of markers could better reflect the heterogeneous process of rejection. From a mathematical point of view, combination of markers with their individual sensitivities and specificities increases the overall sensitivity and specificity. Present approaches are based on two principles. One is to monitor aspects of the immune system which includes allorecognition/activation and the effector pathway of inflammation. The other principle is to detect injury to tubular epithelial cells, the renal interstitium and to vascular and glomerular structures. Traditionally, selection and establishment of new markers has been based on their known role in rejection. A novel avenue to define rejection markers is characterized by a reverse approach, i.e. by the technologies of mRNA expression profiling and proteomics. These techniques use the entire information of mRNA expression, proteins and peptides that is detectable in the sample. Applying bioinformatics, the entire molecular information is then used to establish sets of markers which are representative of rejection compared to samples without rejection. The major challenge of this approach is to exactly define rejection phenotypes and to be aware of confounding factors like infectious, toxic and ischemic injury.

1.5.1 Blood-derived markers

Assuming that immune processes towards the allograft are reflected by circulating leukocytes and their mediators, blood tests appear suitable for monitoring the immune system. In case of graft injury -especially endothelial injury-, local factors from the graft might pass over into blood circulation and could be detected there. Many studies used reverse transcriptase polymerase chain reaction (RT-PCR) to examine gene expression in peripheral leukocytes of renal transplant patients. Up-regulation of mRNA expression of cytokines and cytotoxic effector molecules like granzyme B, perforin and FasL in peripheral blood mononuclear cells (PBMCs) was shown to be associated with acute rejection in renal transplant patients [50, 51]. In addition to granzyme B and perforin, Sabek et al. also found HLA-DRA to be up-regulated [52]. CD40L gene expression of peripheral CD4+ T cells was also found to be increased in acute rejection [53]. Using flow cytometry, Posselt et al. demonstrated that expression of CD69 on CD3+ and CD8+ peripheral T cells correlated closely with the presence of acute rejection in renal allograft recipients [54]. Serum and plasma are also sources for soluble factors that might appear before or during rejection. Although a few authors reported increased serum cytokine levels during rejection [55, 56], other authors could not confirm those results with the same or other cytokine/chemokines or their soluble ligands/receptors [57, 58]. Interleukin-2 and its soluble receptor (sIL-2R) were suggested as predictors of allograft rejection by various authors [59, 60]. Elevated plasma concentrations of tumour necrosis factors α and β (TNFα and β) and of soluble intercellular adhesion molecule-1 (sICAM-1) two or three days prior to rejection diagnosis were reported in one study [61]. Burkhard et al. investigated serum levels of myeloid-related protein, a molecule secreted by monocytes/neutrophils during transendothelial migration, in renal transplant recipients. They found positive serum levels preceding rejection episodes by a median of 5 days [62]. Also, soluble CD30 (sCD30) was considered to be a promising marker to identify patients at risk for acute rejection by some authors [63, 64].

1.5.2 Urine-derived markers

Urine seems to be an obvious choice for evaluating immune activity and immune injury in the kidney. Markers can originate from nephron structures by secretion, transcellular migration, and release or shedding from injured cells. Rejection-associated dysfunction and injury of podocytes and tubular epithelial cells, which are in close contact with the urine, might be reflected by certain markers in urine. It has to be considered that the entire urogenital tract contributes to the urine composition, which is particularly important when allograft or urinary tract infection is present.

The transcriptional approach

Many investigators have explored RT-PCR detection of gene transcripts that are relevant to immune activation markers using mRNA from cells that are excreted into the urine. Significantly increased levels of granzyme B and perforin were found in cells that were harvested from rejection-associated urine compared to samples obtained in the absence of rejection, chronic allograft nephropathy, toxicity or acute tubular necrosis [65]. Other effector molecules of the cytotoxic T cell pathway that are up-regulated in urine pellets during acute rejection episodes include the serine proteinase inhibitor-9 (PI-9) [66] and CD103 [67]. In addition, protein and transcript expression of the IFNγ-inducible chemokine IP-10 and the chemokine receptor CXCR3 are elevated in the urine sediments of patients with acute rejection [68]. Our group assessed multiple cytotoxic markers simultaneously and prospectively in renal allograft recipients and found elevated urinary mRNA expression for CD3, granulysin and RANTES (regulated on activation normal T-cell expressed and secreted) during rejection episodes. Prospective sampling suggested that granulysin expression most frequently preceded the development of acute rejection [69]. Our group also found significant elevated levels of NKG2D mRNA in urine sediment prior to rejection [70]. Urinary FOXP3 has also been associated with biopsy-proven rejection [71]. Soluble adhesion molecules and the complement degradation product C4d were found to indicate rejection episodes [72]. Measurements of urinary monokine induced by IFN-gamma (MIG, CXCL9) could predict acute rejection with a sensitivity of 93% and a specificity of 89% [73].

Urine proteomic approach

In comparison to the transcriptional approach, proteomics examine the abundance of finally translated proteins and peptides. There are different techniques of proteome analysis, but they generally share several common steps. Starting with a biological sample containing a complex mixture of proteins, the most important tasks are to

- separate the proteins into smaller fractions, e.g. by two-dimensional electrophoresis (2-DE) or capillary electrophoresis(CE),
- digest the proteins into peptide fragments,
- ionize the peptide fragments,
- determine the masses of the peptide fragments, e.g. with mass spectrometry (MS),
- compare the peptide masses with protein databases to recognize peptide patterns that are associated with known proteins in the database.

The order of these steps may vary.

Wittke et al. used capillary electrophoresis mass spectrometry (CE-MS) to establish a urine peptide pattern from stable renal transplant patients without rejection, and compared this to the patterns of patients with acute rejection episodes [74]. They found a pattern of 16 peptides (range 1.0-8.1 kDa), which could classify correctly 16/19 patients with acute rejection. Interestingly, two of the misclassified cases had vascular rejections. The identity of the peptides remained unclear. Reichelt et al. used surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) with 2 different protein chip arrays and found 2 biomarkers at 25.71 kDa and 28.13 kDa that gave a diagnostic sensitivity of 90% and 93% and a specificity of 80% (SAX2) and 85% (CM10), respectively [75].

Using surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS), Clarke et al. analyzed urine samples from 17 patients with biopsy-proven rejection and 15 non-rejecting patients. They found a set of 45 protein that allowed to differentiate between the two patient groups. The best diagnostic performance was found for peaks at 6.5, 6.6, 7.1 and 13.4 kDa. A separate analysis using another software package showed that two proteins (3.4 and 10.0 kDa) distinguished the two groups with a sensitivity of 83% and a specificity of 100%. However, the identity of the proteins was not published by the group, and they did not validate their results with an independent dataset [76]. Yet another study used SELDI-MS to compare urine samples from 22 stable patients and 23 patients showing biopsy-proven acute rejection. Samples from rejecting patients were identified by 4-7 protein peaks (2003.0, 2802.6, 4756.3, 5872.4, 6990.6, 19018.8, and 25665.7 Da), with a sensitivity of approximately 90% and a specificity of approximately 80% [77]. In a follow-up paper, two of the proteins were identified as human β-Defensin-1 (4.7 kDa) and α1-antichymotrypsin (4.4 kDa) by tandem mass spectrometry and ProteinChip immunoassay [78]. Schaub et al. used SELDI-TOF MS and very rigid patient selection criteria to detect candidate proteins in urine and found a "rejection pattern" by visual inspection of software-generated gel views of the spectra. The rejection pattern included three peaks with m/z of 5270-5550, 7050-7360 and 10530-11100 Da. These protein peaks were seen in 17/18 patients with acute rejection but were absent in the majority of non-rejecting patients [79]. In a follow-up study, the group characterized these protein peaks as cleavage products of β_2-microglobulin [80]. The association of urine β_2-microglobulin with acute rejection episodes was confirmed by another study. Using matrix-associated laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS), a protein peak at 11.7 kDa was found to correlate strongly with acute rejection and was identified as β_2-microglobulin [81]. Recently, Schaub et al. investigated in a rigid clinical setting the role of urinary β_2-microglobulin and three other candidate markers (retinol-binding protein (RBP), neutrophil-gelatinase-associated lipocalin (NGAL), and α_1-microglobulin) in detecting subclinical tubulitis found in protocol biopsies. Although none

of the markers could distinguish between stable transplants with normal tubular histology and stable transplants with subclinical tubulitis, urinary levels of all markers *except* β_2-*microglobulin* were significantly elevated in patients with clinical tubulitis (grade Ia/Ib) [82].

In 2008, Mao et al. published a study which aimed to find a diagnostic peptide pattern for subclinical rejections in otherwise stable renal transplant recipients using SELDI-TOF-MS and their own data analysis system. They found 4 characteristic protein peaks with m/z of 2761, 10762, 11729 and 11940 Da, which could detect subclinical rejections with a sensitivity and specificity of 81.5% and 81.8%, respectively [83].

2 Aims of the study

Acute rejection represents a major complication after kidney transplantation, since its occurrence significantly affects long-term graft survival. Non-invasive and timely diagnosis of acute rejections, even when subclinical, is essential for minimized, individual immunosuppression and could be realized by the identification of specific and sensitive rejection markers in urine. Examination of the regulation of such markers could also lead to new insights and a better understanding of rejection processes on a cellular and molecular level.

Transplantation medicine stands to benefit from new proteomic techniques in identifying rejection markers. In the present study, a method for the analysis and identification of protein candidate markers in urine was developed. The markers should serve for prediction and non-invasive diagnosis of acute rejection episodes in the early post-transplant period. In detail, the following aspects were covered:

1. Reproducibility and simplicity are important features for analytical methods in clinical studies. Methods for extraction and separation of urinary proteins are not yet standardized and differ widely, and therefore, we raised the questions: Do our precipitation method and 2-DE MALDI-TOF represent a reliable approach to visualize possible changes in urinary proteins during rejection episodes? Is it possible to follow expression profiles of single marker proteins, even those of low abundance and low molecular weight.

2. The characterization of the human urine proteome could significantly contribute to a better understanding of physiological and pathological processes in the kidney. With our proteomic approach, we tried to answer the following questions: Which proteins are regularly present in urine of renal transplant patients? Which of them are high-abundance and which ones are low-abundance? Where do those proteins originate from?

3. Urinary proteins are a potential source of new rejection markers and are therefore intensively studied. Following their expression profiles in the early post-transplant period, we aimed to solve the questions: Are rejection processes in the kidney reflected by alterations in the urinary protein pattern? Which proteins are differentially expressed in urine of rejecting patients? Do these proteins have a known function, which could play a role in or be a result of the rejection process?

4. The value of a differentially expressed protein as rejection marker in a diagnostic test depends amongst others on its availability for easy and reproducible laboratory test.

Is it possible to measure the urinary concentrations of selected, differentially expressed proteins by the ELISA method? Are their concentrations elevated/lowered in urine of rejecting patients?

3 Materials and methods

3.1 Transplanted patients

All 66 patients that participated in this prospective study were recruited between July 2007 and May 2008 from the Department of Surgery, Universitätsmedizin Charité, Berlin. Most patients received their first kidney allograft, only 11 were undergoing re-transplantation. The study was approved by the Ethical Committee of the Universitätsmedizin Charité, Berlin. All patients were informed about the aims and gave written consent to the study.

Out of 66 patients, 54 could be released from the hospital in a good condition within 4 weeks after transplantation, 12 of them had to stay hospitalized for a longer period. This was due to complications including infection, bleedings or allograft dysfunction because of acute rejection or toxic drug effects. 16 patients had to undergo re-laparotomy. If there were clinical signs for acute rejection, a kidney graft biopsy was performed with a 1.4mm needle under ultrasound control. All biopsies taken were analyzed by an independent and qualified pathologist of the Department of Pathology, Charité Universitätsmedizin Berlin. Histological findings were classified according to the BANFF '97 criteria, and confirmed clinical diagnosis of acute allograft rejection. Around 20% of the patients transplanted between July 2007 and May 2008 were diagnosed with borderline or more severe rejection within the first 4 weeks post transplantation. Treatment upon rejection consisted of methylprednisolone bolus over 5 days and Thymoglobulin or mAb OKT3 if necessary. Some patients showed signs of toxic drug effects in their biopsy, and in those cases the dosis of calcineurin inhibitors was reduced.

All patients were treated according to a standard protocol. They were induced with one perioperative shot of an anti-IL-2 receptor monoclonal antibody, Simulect (Behring, Marburg, Germany), and a second on day 4 after transplantation. Maintenance immunosuppression consisted of either cyclosporine A or tacrolimus, in addition to both mycophenolate mophetil and tapered steroids. Blood concentrations of tacrolimus ranged between 8 and 10ng/ml.

Urine specimens were collected in frequent intervals during the postoperative period, as described in section 3.2. 2-D electrophoresis gels were prepared at two different gel concentrations (12% and 18%). Urine specimens of rejecting and non-rejecting patients were analyzed at both gel concentrations and the results were compared using the Delta2D software package (Decodon, Greifswald, Germany). The tight sampling schedule gave us a sufficiently broad sample pool for two complementary evaluations: First, we tried to find interesting biomarker candidates by comparing groups of rejecting and stable patients. Our aim was to find out which proteins would be differentially expressed in samples from patients rejecting

their allograft compared to those with a stable clinical course without signs of rejection. And secondly, we had the possibility to follow expression profiles of interesting spots in the postoperative course of the individual patients.

In order to realise the first approach, we defined a stable and a rejecting patient group. From all 288 samples available, we chose the ones where we expected the most striking differences. We therefore defined rigid inclusion criteria for both groups:

1. In the stable group, samples were included only from first-transplant patients who had a stable clinical course post-transplantation, no UTI, no need for dialysis and a decrease in serum creatinine levels of at least 75% after the first five days. Most of them had a 6-month GFR of >40 ml/min. Samples were collected during the first 12 days post transplantation.

2. From rejecting patients, only samples were chosen that were taken 1-6 days **before** biopsy-confirmed rejection diagnosis and rejection treatment. Biopsies revealing acute cellular rejections were performed on days 7, 9, 16 and 27 post transplantation.

3. Furthermore, urine samples were of first or second voided morning urine and exclusively obtained from the double-j catheter collection bag, that is free from contamination from the urogenital tract.

For 12% gels, we selected 18 representative samples (13 stable vs. 5 rejection samples) from 6 renal transplant patients (4 stable vs. 2 rejecting patients). Table 4 gives an overview over the most relevant clinical parameters of the included allograft recipients.

For 18% gels, 22 representative samples (13 stable vs. 9 rejection samples) from 14 renal transplant patients (9 stable vs. 5 rejecting patients) were selected for analysis. Table 5 shows the patient demographics of the included allograft recipients for analysis in 18% gels.

Obviously, the first evaluation aimed at identifying differentially expressed proteins during rejection episodes. In the second approach, we followed the change of concentrations of promising biomarkers over time using the ELISA method (for more details, see 3.6). For this approach, we chose samples according to the criteria set for 12% and 18% gel analysis from the same patients as listed in tables 4 and 5. Additionally, we analyzed samples from 4 patients that were diagnosed with biopsy-confirmed borderline rejection. Averaged patient demographics for ELISA analysis are shown in table 6.

Table 4: ***Clinical information of renal transplant patients analyzed in 12.5% gels.*** *aRx = acute rejection, shown as grade according to the BANFF classification; MM = HLA mismatches; UTI = urinary tract infection; CIT = cold ischemia time in hours*

Case	Sex	Age	aRx	MM (broad)	MM average	UTI	CIT	CIT average
Stable group								
NTX003	f	49	no	4	3.5	yes	13	10.4
NTX004	m	50	no	3		no	9.5	
NTX005	f	39	no	3		no	12.5	
NTX007	m	56	no	4		no	6.75	
Rejection group								
NTX009	m	54	III°	4	4.5	no	15.6	12.8
NTX018	f	60	I°	5		no	10	

3.2 Urine collection and processing

Urine specimens (50-100ml) were collected during hospitalization of the patients after transplantation. As specimen collection was prospective, we tried to follow a tight sampling schedule. If available, 3 samples were gathered in the first week after transplantation and one sample every following week. In total, 288 urine specimens from 66 patients were collected.

From each sample, 2 x 2ml aliquots were directly frozen in Eppendorf tubes at -20°C until further analysis. The rest was mixed with 10% trichloroacetic acid and left in the refrigerator for at least 20 hours at 4°C. Then it was centrifuged at 15 000 rpm for 60 minutes. The pellet was resuspended in 100% ethanol and centrifuged for another 20 minutes at 15 000 rpm. In this manner, the pellet was washed 4 times. Finally, it was frozen at -20°C in a 1.5ml tube until proteomic analysis.

3.3 2-D electrophoresis

2-D electrophoresis is a method for separating and identifying proteins. A mixture of proteins is separated in two orthogonal dimensions which correspond to two protein properties. In

Table 5: ***Clinical information of renal transplant patients analyzed in 18% gels.*** *aRx = acute rejection, shown as grade according to the BANFF classification and BL = borderline rejection; MM = HLA mismatches; UTI = urinary tract infection; CIT = cold ischemia time in hours*

Case	Sex	Age	aRx	MM (broad)	MM average	UTI	CIT	CIT average
Stable group								
NTX007	m	56	no	4		no	6.75	
NTX011	f	41	no	0		no	9.4	
NTX013	f	65	no	3		no	18.2	
NTX014	f	54	no	3		no	10.1	
NTX022	m	33	no	5	3.1	no	10.8	9.6
NTX024	m	26	no	3		no	7.75	
NTX034	m	44	no	2		no	7.5	
NTX035	m	43	no	5		no	7.2	
NTX046	f	36	no	3		no	8.9	
Rejection group								
NTX009	m	54	III°	4		no	15.6	
NTX018	f	60	I°	5		no	10	
NTX019	m	47	I°	2	3.6	no	13	12.2
NTX032	m	34	I°	5		no	8.5	
NTX048	f	40	I°	2		no	14	

a first step and in the first dimension, proteins are separated according to their charge, a process called isoelectric focusing (IEF). Thereby, a gel strip is made up with a (mostly linear) pH gradient (e.g. pH 4-7) and an electric field is applied in the gradient's direction across the gel. As proteins are electrically charged at all pH values other than their isoelectric points, they will move along the field (i.e. they will move in an electrophoresis) and accumulate at positions where the local pH values within the gel equal their isoelectric points.

In a second step and in second dimension, proteins are separated according to their mass. This is done by coating the (usually already denatured) proteins with the negatively charged molecule sodium dodecyl sulphate (SDS). The number of attached SDS molecules is roughly proportional to the protein's mass, resulting in approximately the same mass/charge ratio of each protein. Coating with SDS gives the proteins a negative charge and, applying an

Table 6: ***Patient demographics of renal allograft recipients analyzed with ELISA*** *aRx = acute rejection group, grades of rejection according to the BANFF classification with BL = borderline rejection are shown in the last four columns; 1stTx = recipients receiving their first allograft; reTx = re-transplanted recipients; MM = HLA mismatches; CIT = cold ischemia time in hours; DGF = delayed graft function*

	n	Sex m/f	Age mean (SD)	1stTx	reTx	MM mean (SD)	CIT mean (SD)	DGF	BANFF '97			
									BL	I°	II°	III°
aRx group	5	3/2	47 (10)	3	2	3.5 (1.7)	12.2 (2.91)	2	0	3	1	1
BL group	4	2/2	49 (11)	2	2	3.3 (1.1)	11 (3.5)	1	4	0	0	0
Controls	12	6/6	46 (13)	11	1	2.9 (1.7)	10 (3.2)	1	0	0	0	0

electric field in perpendicular direction to that of the former IEF process, it allows them to migrate in the acrylamide gel towards the anode. The polyacrylamide gel acts like a molecular sieve and retains large proteins much stronger than small ones. In effect, the larger the proteins are the shorter are the distances they migrate during the time period of the second separation step. The result of both steps is that sample proteins are spread out across a 2-D gel on a large area and get separated from each other. Figure 3 shows a typical 2-DE gel made from one of our samples. Finally, the protein spots in the gel are stained with silver or coomassie staining. Scanning of the stained gels produces a digital image and allows software-based analysis.

In summary, the procedure includes the following steps: - Solubilisation and denaturation of the sample proteins - Separation in the first dimension by isoelectric focussing - Separation in the second dimension by SDS-PAGE - Gel staining

3.3.1 Solubilisation and denaturation of the sample proteins

After thawing, samples were resuspended in urea-thiourea solution. Protein concentration was determined by the Bradford method. The proteins were dissolved in rehydration buffer,

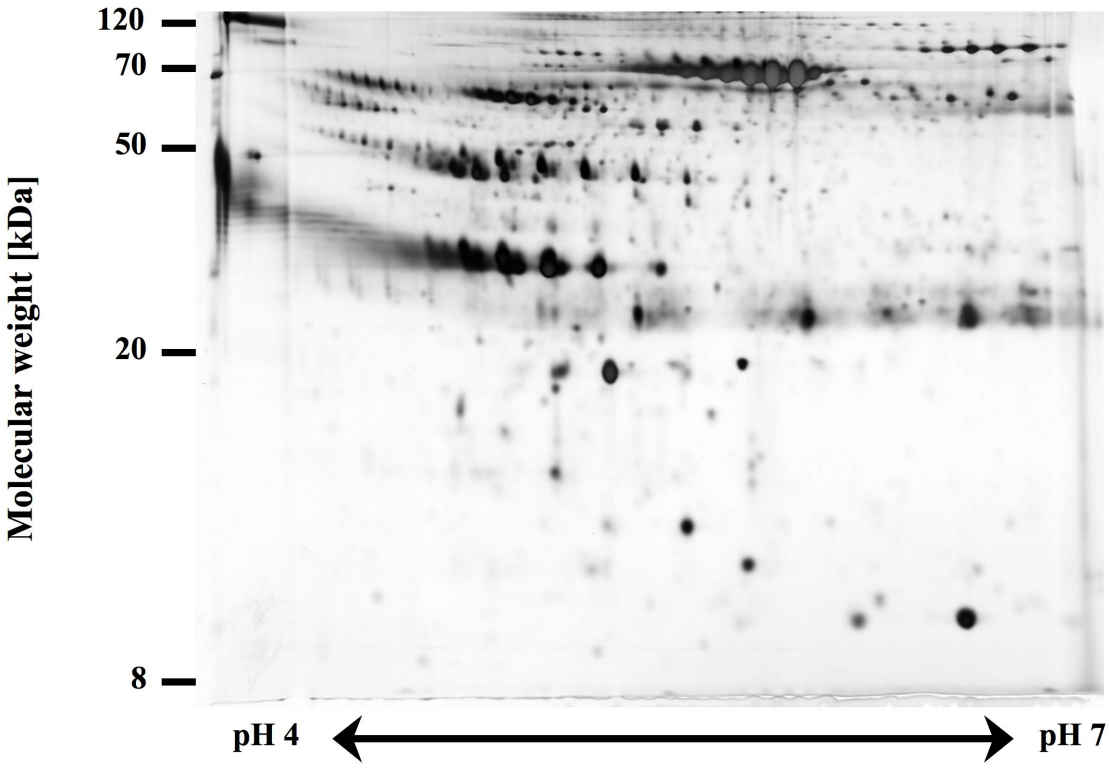


Figure 3: ***Two-dimensional electrophoresis gel from a renal transplant patient.*** *Horizontal separation: proteins are first separated on basis of the isoelectric point by isoelectric focusing. Vertical separation: SDS-PAGE follows to separate proteins by their molecular weight. Protein spots can be excised from the gels and analyzed in a mass spectrometer.*

containing

- urea and thiourea to cleave disulfide and hydrogen bonds and convert all proteins into single conformations; this prevents protein aggregates and modifications
- CHAPS to get hydrophobic proteins into solution
- DTT as reductant to cancel different oxidation steps
- carrier ampholytes as substitute for ionic buffer to keep proteins in solution
- bromophenol blue as a control for start and running conditions.

Sample tubes containing 75µg of protein were filled up to 450µl with rehydration buffer. 50µl of urea-thiourea solution was added. All ingredients were mixed in an Eppendorf Thermomixer for 1 hour at 1 400 rpm and 20°C, and centrifuged afterwards for 15 minutes at 13 000 rpm.

3.3.2 First dimensional separation by isoelectric focussing

We used Immobiline Dry Strips, which contain a preformed pH gradient (pH 4-7) immobilized in homogeneous polyacrylamide gels. They are 24cm long, plastic backed and delivered dehydrated. The samples in rehydration buffer were loaded on the strips, overlaid with paraffin oil as cover fluid and rehydrated for 17 hours. Then the strips were put in an IPGphor horizontal apparatus, and contacted at both ends by wet electrode strips. IEF was performed at 500V for 1 minute, ramping up to 3 500V for 1.5 h and focussing at 3 500V for up to 60 000Vh.

3.3.3 Second dimensional separation by SDS-PAGE

The gel strips with the IEF-separated proteins were then frozen at -20°C for 5 hours. After defrosting, the strips were equilibrated in buffer I containing 6M urea, 375M Tris pH 8.8, 20% glycerol, 40% SDS, and DTT for 15 minutes. Then the strips were equilibrated for another 15 minutes in buffer II (as equilibration buffer I but containing iodoacetamide (IAA) instead of DTT and a small amount of bromophenol blue). After that, strips were loaded upon previously made acrylamide gels and covered with a small agarose stripe. Acrylamide gels were produced in different concentrations to determine which might be most suitable. The gels contained 12.5, 15 or 18% acrylamide (40%), 1.5M Tris pH 8.8, 0.4% SDS, 10% ammonium persulfate and $C_6H_{16}N_2$ (TEMED). The running buffer contained 20% SDS, Tris and glycine. SDS-PAGE was run for 20 h at 230V.

3.3.4 Gel staining

After 2-DE, protein spots in the gel were made visible using the silver staining method, adapted from the method described by Blum et al. [84]. The procedure is accomplished in five steps, with washing steps in between. In the first step, the gels are fixed in a solution containing 50% ethanol, 12% acetic acid and 0.05% formaldehyde (37%). After 1-3 hours of incubation, gels are washed twice in 50% ethanol for 20 minutes. In the second step, gels are pretreated in pretreatment solution for exactly one minute. Pretreatment solution contains 2g/l $Na_2S_2O_3$ in ddH_2O. After washing in H_2O, silver staining is accomplished in the third step. Silver solution contains 0.2% silver nitrate ($AgNO_3$) and 0.075% formaldehyde (37%). The gels were incubated in the silver nitrate solution for 20 minutes in the dark. Stain was developed in the fourth step after the gels had been washed in distilled water. Developing solution was made up of 60g/l Na_2CO_3, 0.05% formaldehyde (37%) and $Na_2S_2O_3$. Development was interrupted in the fifth step as soon as the desired intensity and contrast

of the protein spots was achieved. To interrupt the development, the gels were incubated for 30 minutes in a stop solution containing 1% glycine. After a final washing, the gels were packed in plastic bags and scanned.

Silver staining yields a good sensitivity for the detection of protein spots, but it is hardly compatible with subsequent mass spectrometric analyses. Therefore, gels used for MALDI-TOF analysis were stained with coomassie staining. Coomassie staining also starts with fixing the gels for 30 minutes at room temperature. Fixing solution was made of 40% methanol and 10% acetic acid. After washing, the gels are stained in coomassie blue R350 stain for at least one hour. Coomassie blue stain solution contains 0.25% coomassie blue R350, 20% methanol and 10% acetic acid. In the next step, gels are destained until the protein spots are visible but the background is clear. This can take some hours and destaining solution should be changed several times. Destaining solution contains 50% methanol and 10% acetic acid in ddH_2O. Finally, gels are equilibrated in a storage solution containing 5% acetic acid.

3.4 Image analysis

Stained gels were scanned using a Seiko Epson scanner. Image analysis was performed with the Delta2D software package. The first step of analysis is gel image warping. Even if one and the same protein mixture is separated on two gels according to the same procedure, spots from identical proteins almost never lie on top of each other. However, the overall pattern of the spots from the protein mixture with the relative positions of the individual spots is very reproducible. Hence, some stretching and compressing in one direction or the other is necessary to get rid of differences in spot positions and to align the images of different gels relative to each other. In other words, gel image warping is the essential tool to compensate for running differences between different gels. Warped images can then be overlaid to produce two-color images, where differences (due to differences in the original protein mixtures) become visible. In every group, the optically best image was chosen as reference image and the other images were warped onto it.

In the next step of analysis, proteome maps are built by fusing multiple images of one group into a new, synthetic image. Fused images show all spots on one unified image, serving as a reference map for group comparisons. The spot patterns result from combining the images pixel-by-pixel using a weighted average function.

In a last step, all spots on one fused image are registered to produce a consensus spot pattern with spot boundaries, which can then be transferred back to the original images.

Different strategies were used to discern differences in protein patterns between rejecting and non-rejecting patients:

1. Diagrammatic gel images were produced as master maps by merging gel images from all rejecting and all non-rejecting patients. The master maps were then used to compare visually the degree of protein expression differences seen in the primary gel images from the other group.

2. Statistical comparison of the two groups was based on digital quantification of the single spots. Comparison of the two patient groups required further processing of scanned gel images with the Delta2D software package. According to the manufacturer's manual, the following steps need to be taken to discover differentially expressed spots: All gel images from both patient groups were warped by a group-warping algorithm to provide comparability of spot positions and spot intensities were then quantified. Basically, the data were obtained in three steps: 1. Spot detection: Image segments are identified that are occupied by spots. 2. Spot matching: Spot detection is done on the fused master image (the proteome map) and spots are transferred to the single images. Matching is then inherent to the process of transferring and thus comprises 100% of all detected spots. Quantification is always done using the original image. 3. Spot quantification: Summing up and calibration curve dependent recalculation of the gray values of the pixels belonging to each spot. Background is subtracted, and all quantities are normalized before comparing them between gel images.

3.5 Identification of proteins by MS

3.5.1 In-gel digestion

Protein spots of interest were excised from the gel with a spot cutter with a picker head of 2 mm diameter and transferred into 96-well microplates loaded with 100 μL of water per well. Trypsin digestion and subsequent spotting of peptide solutions onto the matrix-assisted laser desorption-ionization (MALDI) targets were performed automatically in a protein handling station (Ettan spot handling workstation) with a modified standard protocol that has been described before [85]. Peptide solutions were then analyzed by MALDI-TOF MS.

3.5.2 MALDI-TOF MS

MALDI is a sub-method of mass spectroscopy. It is often used to analyze biomolecules which require a particularly gentle ionization. The biomolecules are embedded in a matrix

of crystallized host molecules, i.e. analyte and matrix are co-crystallized on the so-called target plate. Most common matrix molecules are 3,5-dimethoxy-4-hydroxycinnamic acid, alpha-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. By hitting the target with a short laser pulse, a small amount of the matrix/analyte mixture is vaporized. Though the analytes are vaporized, they are protected by the matrix molecules from being extensively cleaved into fragments by the laser pulse. Simultaneously with vaporization, matrix molecules get ionized and transfer a part of their charge to the analytes. The so ionized analytes are accelerated in an electric field and their molecular masses are determined in a time-of-flight mass spectrometer (usually equipped with ion mirrors). The time-of-flight (tof) thereby depends on mass (m) and charge (z) of the analyte, described in the following relationship:

$$\text{tof} \propto \sqrt{\frac{m}{z}}$$

Figure 4 shows a schematic depiction of a MALDI-TOF mass spectrometer.

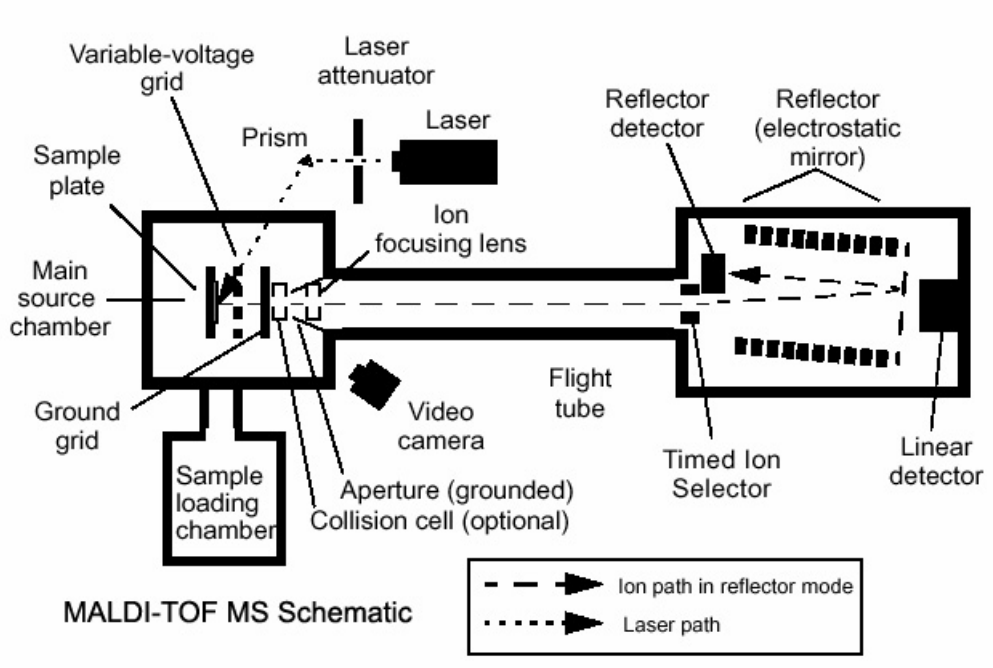


Figure 4: ***Schematic illustration of MALDI-TOF-MS*** *The peptides applied to the sample plate (at the left) are desorbed and ionized by a laser pulse. After acceleration by a variable voltage grid they fly in high vacuum to the detector at the right. The times of flight of the analytes are translated to masses and acquired spectra are used for peptide mass fingerprinting.*

In our study, MALDI-TOF spectroscopy of peptide solutions was carried out on a 4800 proteomics analyzer. Peptide mass spectra were recorded in reflector mode in a mass range from 900 to 3700 Da with a focus mass of 2000 Da. For one main spectrum 25 subspectra with 100 shots per subspectrum were accumulated with a random search pattern. If the autolytic fragment of trypsin with the monoisotopic $(M+H)^+$ m/z at 2211.104 reached a signal-to-noise ratio of at least 10, an internal calibration was automatically performed with this peak for a one-point calibration. MALDI-TOF-TOF analysis was performed for the five strongest peaks of the TOF spectrum after excluding background peaks (e.g. trypsin fragments, chemical noise, keratin background). For one main spectrum, 20 subspectra with 125 shots per subspectrum were accumulated with a random search pattern. The internal calibration was automatically performed as one-point calibration if the monoisotopic arginine $(M+H)^+$ m/z at 175.119 or lysine $(M+H)^+$ m/z at 147.107 reached a signal-to-noise ratio of at least 5. The peak lists were created using the GPS-Explorer software with the following settings: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 10 peaks per 200 Da; and minimal area of 100 and maximal 65 peaks per precursor and a minimal signal-to-noise ratio of 5.

3.5.3 MS data analysis

Peptide mass fingerprinting (PMF) was conducted with the database search against a Swiss-Prot Database (Ver.55.1 restricted to *Homo sapiens* sequences) with the Mascot search engine Ver. 2.1. The identification was considered significant if the Mowse score exceeded a value of 55, which corresponds to a P value of 0.05. Cellular designation was denoted for the respective proteins in descriptions by literature references of Swiss-Prot/TrEMBL and EMBL-EBI.

3.6 ELISA

Osteopontin (OPN) in urine was measured with the Human Osteopontin Quantikine ELISA Kit, which employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for OPN has been pre-coated onto a microplate. Samples and standards were pipetted into the wells and any OPN present was bound by the immobilized antibody. After washing away any unbound substances, a polyclonal antibody against OPN conjugated to horseradish peroxidase was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution containing tetramethylbenzidine is added and color develops in proportion to the amount of OPN bound in the initial

step. The color development is stopped with sulfuric acid and the intensity of the color is measured.

3.7 Used materials

Material	Source
Hardware & Software	
Thermomixer	Eppendorf, Hamburg, Germany
Vortexer	Roth, Karlsruhe, Germany
Centrifuge	Eppendorf, Hamburg, Germany
Fridge	Liebherr, Ochsenhausen, Germany
Freezer	Sanyo, Osaka, Japan
Photometer	Tecan, Crailsheim, Germany
Scanner	Seiko Epson Corporation, Tokyo, Japan
IPGphor horizontal apparatus	Amersham Biosciences, Tokyo, Japan
Protein handling station	GE Healthcare, Uppsala, Sweden
Spot cutter	Bio-Rad, Hercules, USA (CA)
4800 proteomics analyzer	Applied Biosystems, Foster City, USA (CA)
Mascot search engine	Matrix Science Ltd, London, UK
GPS-Explorer software	Applied Biosystems, Forster City, USA (CA)
GraphPad Prism V. 5.00 software	GraphPad Software, San Diego, USA
Delta2D software	Decodon GmbH, Greifswald, Germany

Table 7: *(continued)*

Material	Source
Commercial kits	
Human Osteopontin Quantikine ELISA	R&D Systems, Minneapolis, USA
Immobiline Dry Strips	GE Healthcare, Uppsala, Sweden
Plastics	
Pipet tips	Biozym, Hessisch Oldendorf, Germany
Eppendorf tubes (0.5ml, 1.5ml, 2ml)	Sarstedt, Nümbrecht, Germany
Falcon conical tubes (15ml, 50ml)	BD Biosciences, Heidelberg, Germany
Chemicals	
Ethanol 96%	Biochrom AG, Berlin, Germany
Trichloroacetic acid	AppliChem, Darmstadt, Germany
Urea-thiourea solution	PAA Laboratories, Linz, Austria
CHAPS	Biochrom AG, Berlin, Germany
DTT	Biochrom AG, Berlin, Germany
Carrier ampholytes	PAA Laboratories, Linz, Austria
Bromophenol blue	PAA Laboratories, Linz, Austria
Urea	Biochrom AG, Berlin, Germany
Tris	Biochrom AG, Berlin, Germany
Glycerol	Biochrom AG, Berlin, Germany
SDS	BD Biosciences, Heidelberg, Germany
DTT	BD Biosciences, Heidelberg, Germany
Iodoacetamide	PAA Laboratories, Linz, Austria
Acrylamide	BD Biosciences, Heidelberg, Germany
Tris	BD Biosciences, Heidelberg, Germany
Ammonium persulfate	BD Biosciences, Heidelberg, Germany
TEMED	Biochrom AG, Berlin, Germany
Glycine	PAA Laboratories, Linz, Austria
50% ethanol	Biochrom AG, Berlin, Germany
12% acetic acid	Biochrom AG, Berlin, Germany
0.05% formaldehyde	PAA Laboratories, Linz, Austria
Na2S2O3	AppliChem, Darmstadt, Germany

Table 7: *(continued)*

Material	Source
0.2% silver nitrate	AppliChem, Darmstadt, Germany
Formaldehyde	BD Biosciences, Heidelberg, Germany
Na2CO3	AppliChem, Darmstadt, Germany
Formaldehyde	PAA Laboratories, Linz, Austria
Na2S2O3	AppliChem, Darmstadt, Germany
1% glycine	BD Biosciences, Heidelberg, Germany

4 Results

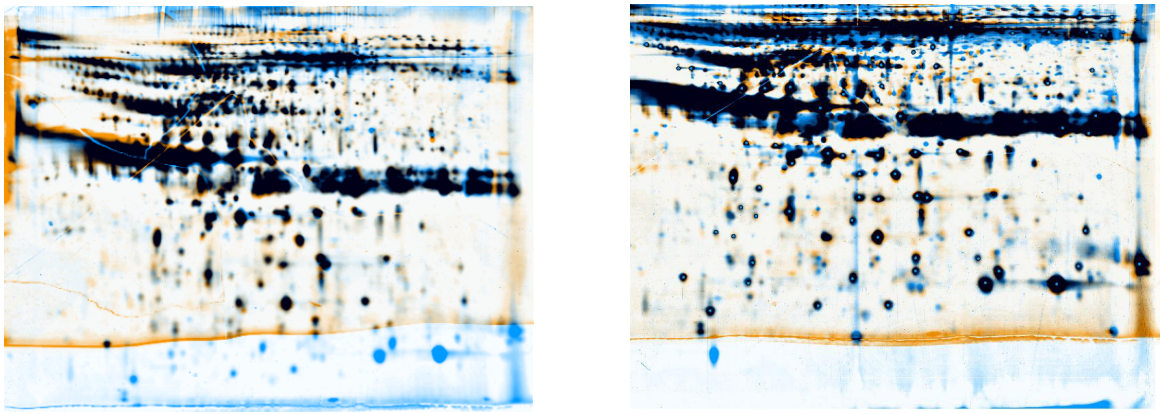
4.1 Establishment of the 2-DE technique: 12.5% vs. 15% vs. 18% gels

No urine proteomic examination of renal transplant patients based on the image analysis of polyacrylamide gels has yet been reported to our knowledge. Therefore, we had to find out the concentrations in polyacrylamide that yield the best comparability and resolution. Initially, 12.5% polyacrylamide gels were used for the electrophoretic separation of several urinary protein samples from rejecting and non-rejecting renal transplant patients. At first glance, major areas of the gels seemed to be occupied by at least 10 broad, converging spot chains. Those areas were constitutively present in all gels and represent high-abundance proteins in human urine, like serum albumin, AMBP protein, zinc-alpha-2-glycoprotein and Ig kappa and Ig lambda chains. Only minor parts of the gels were areas of well separated, single spots. These parts of the gels with peptides of low molecular weight seemed to be particularly interesting with regard to differences between samples from rejecting and non-rejecting patients. In first MALDI-TOF analyses of ca. 250 excised spots, 122 different proteins were identified in a range from about 11 to 1020 kDa (and between pI=4 and 11). As mentioned in the introduction (chapter 1.5.2), biomarkers have been found in former proteomic studies in a mass range from about 1 to 28 kDa, with most of them below 12 kDa. In case of kidney injury it seems to be more likely for smaller proteins to pass over into urine. In addition, a dramatic loss of total protein in urine samples was found when they were dialyzed against dialysis membrane with a molecular weight cut-off of 6-8 kDa [86]. The finding was explained by a high proportion of proteins of low molecular weight in the urinary composition. We therefore tried to improve the resolution those parts of the gels where small proteins should be represented.

In consequence, gels were prepared with 15% polyacrylamide. PAGE was performed with a number of samples. Comparing same samples analyzed in both 12.5% and 15% gels by matching common spots, we found several new low molecular weight proteins. Figure 5 (a) shows exemplarily two digitally overlaid gel images from the same sample.

The resolution of 15% gels for smaller proteins presumably detects molecules with a mass > 8 kDa. We guessed that another increase of the polyacrylamide concentration in the gels to 18% could lower the detection threshold to 5 kDa. A further increase in gel concentration in order to detect even smaller proteins would have required a change in the running buffer system with negative influence on gel quality. Once again, new spots appeared in the 18% gels. Areas with high-abundance proteins appeared to be more condensed in the corresponding

parts of the gels. We therefore decided to prepare some more 18% gels to find out whether the newly appearing spots might disclose interesting biomarker candidates. MALDI-TOF analyses of 65 spots revealed 42 unique proteins in the mass range 8.5-290.7 kDa. Some of them were differentially expressed in rejection samples - a finding which could not be detected in 12% gels. Figure 5 (b) shows the overlaid images of a 15% and a 18% gel from the same sample as shown in figure 5 (a).



(a) 12% vs 15% gel

(b) 15% vs 18% gel

Figure 5: ***Optimisation of 2-D electrophoretic gels: Comparison of one sample in different gel concentrations.*** *(a) shows a two color image of overlaid gels: The same sample was run in a 12% gel (in orange) and in a 15% gel (in blue). The lower part of the image reveals more blue spots representing the increased resolution for smaller proteins. (b) Comparing a 15% (orange) with a 18% (blue) gel image from the same sample we found even more blue spots, i.e. an even better resolution in the lower part of the gel.*

4.2 The 12% and 18% proteome maps of renal transplant patients

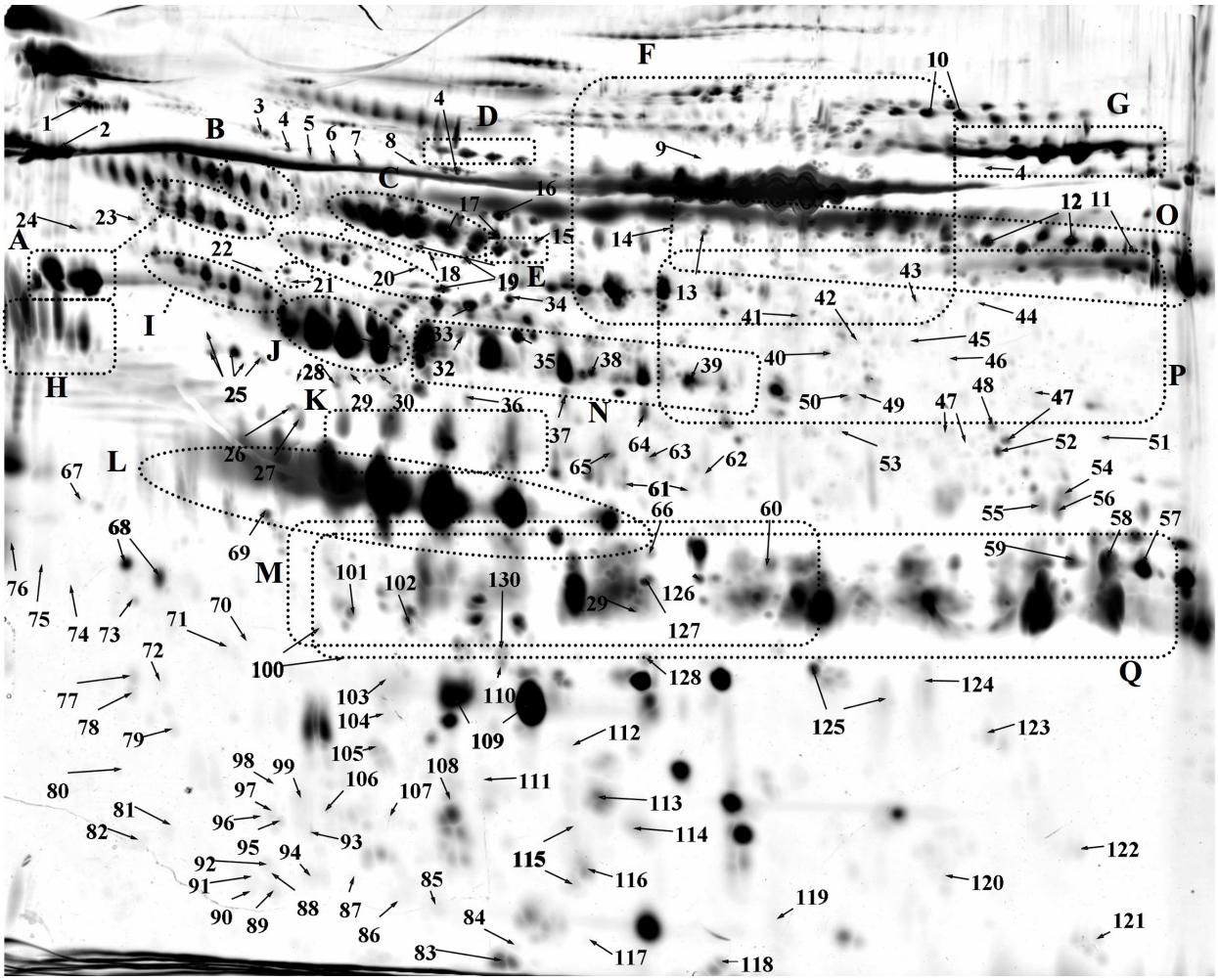


Figure 6: ***The 12% urine proteome map of renal transplant patients.*** *Areas of highly abundant proteins are labeled with letters A-Q, all other identified spots are labeled with ascending numbers (1-130). Corresponding protein annotations are listed in table 10.*

Urinary samples from renal transplant patients were chosen according to the criteria mentioned in chapter 3.1 and electrophoretic gels were done thereof. Gels were scanned and the resulting images were analyzed with the software package. 12% and 18% proteome maps were created by fusing all gel images in both concentrations into one synthetic image, unifying all spots and the spot identifications from the single sample gels. Figure 6 and figure 7 show the 12% and 18% urine proteome map of renal transplant patients that we created by fusing gel images of 18 (in 12% gels) and 22 (in 18% gels) urine samples. More than 1400 discrete spots were present in those proteome maps between 8 kDa and 80 kDa, and between isoelectric points of pI=4 and 9. It has to be considered, that we found discrep-

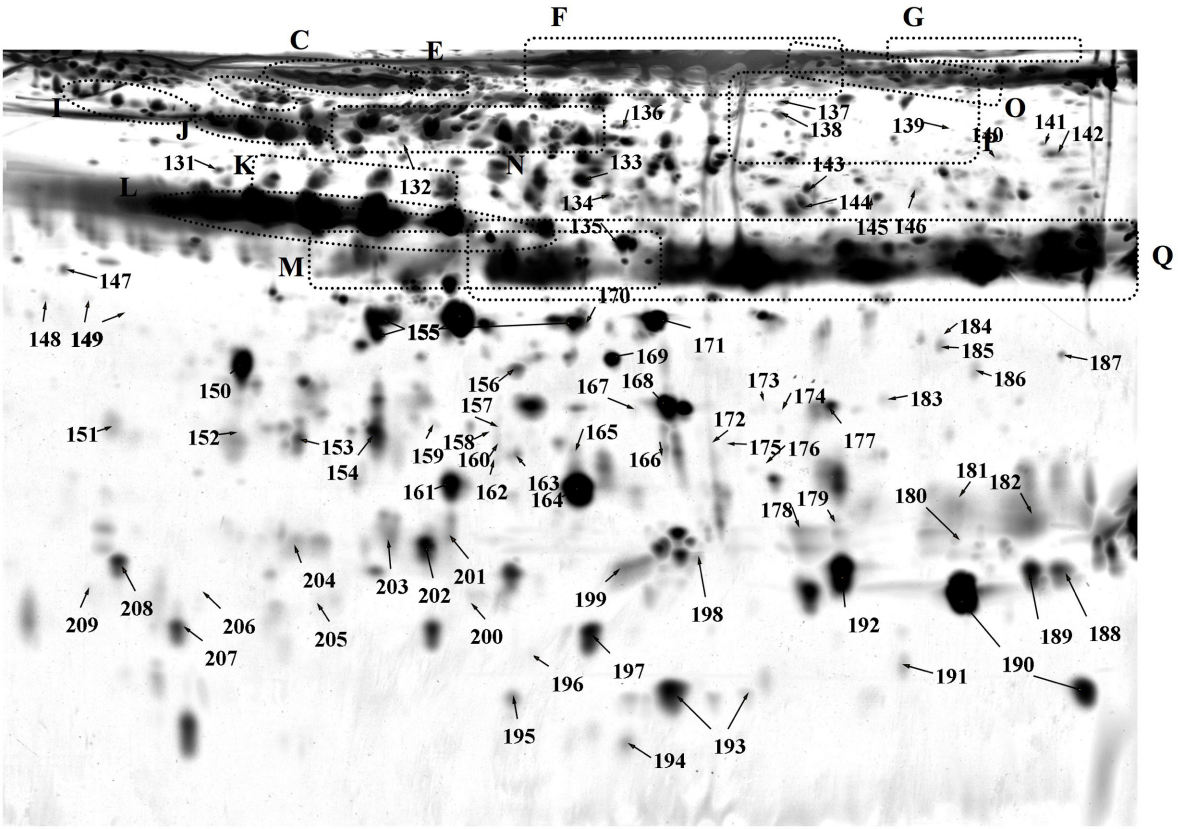


Figure 7: ***The 18% urine proteome map of renal transplant patients.*** *Areas of highly abundant proteins are labeled with the same alphabetic characters as in figure 6, all other identified spots are labeled with ascending numbers (131-209). Corresponding protein annotations are listed in table 10.*

ancies between the theoretical and the apparent MW as deduced from the spot positions in the gels. For example, we identified replicase polyprotein 1ab with a theoretical MW of 790 kDa at an apparent MW of ca. 18 kDa. Most of these differences can be explained by post-translational modifications, as the theoretical MW of a protein is computed only from its amino acid sequence. Furthermore, the effect of gel filtration is not only due to mass values, but protein shape and protein-matrix interactions also play a role. The same applies for theoretical and apparent pI as a protein migrates to different pH values in IEF depending from the degree of its previous glycosylation.

Table 10 in the appendix lists all identified proteins, their theoretical MW and pI, as well as their cellular designation and their accession number in the Swissprot database. As expected from high levels of post-translational modifications in most urinary proteins and the presence of proteolytic products, ca. 400 identified spots collapsed to 178 unique protein annotations. All identified spots in the proteome maps are consecutively numbered as listed in table 10.

In several spots we identified more than one protein. On the other hand, some proteins were represented by several spots. This explains why several spot numbers in the list have more than one corresponding protein assignment.

4.2.1 High-abundance proteins

Frequently, high-abundance proteins were found in many neighbouring spots. In order to get a better overview in the proteome map, we defined 17 constant areas of high-abundance proteins that were constitutively present in all urinary samples:

- Area A: large multispot train containing Alpha-2-HS-glycoprotein
- Area B: large multispot train containing Alpha-1-antichymotrypsin and Kininogen-1
- Area C: large multispot train containing alpha-1-antitrypsin and Kininogen-1
- Area D: a single spot chain containing Alpha-1B-glycoprotein
- Area E: 2 bigger spots containing Vitamin D-binding protein
- Area F: a large multispot trains containing Serum albumin
- Area G: large multispot trains containing Serotransferrin
- Area H: a spot train containing Alpha-1-acid glycoprotein 1
- Area I: a spot train containing Leucine-rich alpha-2-glycoprotein
- Area J: large multispot train containing Zinc-alpha-2-glycoprotein
- Area K: 4 big spots containing Complement factor B
- Area L: large multispot train containing AMBP protein (containing alpha-1-microglobulin)
- Area M: diffuse distribution of Prostaglandin-H2 D-isomerase
- Area N: a single spot chain containing Haptoglobin
- Area O: a broad spot band containing Ig gamma-2 chain C region and Ig gamma-4 chain C region
- Area P: diffuse distribution of Fibrinogen beta chain [containing Fibrinopeptide B] and Fibrinogen gamma chain

- Area Q: a broad spot band containing Ig kappa and Ig lambda chains

All of them except prostaglandin-H2 D-isomerase are typical components of blood plasma and are probably filtrated by the glomerulus since their molecular weight (deduced from their positions in the gel) is equal or less than 70 kDa. Despite reabsorption by tubular cells, or if reabsorption is hampered, they may be present in final urine. Although prostaglandin-H2 D-isomerase (a protein that is widely expressed in several subcellular compartments and might act as a scavenger for harmful hydrophobic molecules) is not a typical plasma protein, it was also among the more abundant urinary proteins. All the abundant proteins were already identified in human urine of healthy individuals in previous reports [87, 88].

Of course, we detected serum albumin – the main plasma protein – in all samples. Its main functions and its role as indicator of renal function are already described in detail in chapter 1.4.3. In the present study, it was detected semi-quantitatively, and no difference could be observed between stable and rejecting patients (also see figure 9). It turned out to be neither sensitive nor specific for episodes of acute rejection in the early post transplant period.

AMBP protein (containing alpha-1-microglobulin) was also found in high abundance in all gels. AMBP protein is proteolytically processed into distinctly functioning proteins: alpha-1-microglobulin, which belongs to the superfamily of lipocalin transport proteins and may play a role in the regulation of inflammatory processes, and bikunin, which is a urinary trypsin inhibitor belonging to the superfamily of Kunitz-type protease inhibitors. If not bound in high molecular mass complexes, monomeric alpha-1-microglobulin passes through the glomerulus over into primary urine, where it is reabsorbed by the proximal tubule and catabolized. Although serum and urinary levels have been investigated in kidney transplantation and in several disease states of the kidney for decades [89], the major clinical application remains the use of urinary alpha-1-microglobulin as marker for proximal tubular damage [90]. Since tubular function is impaired in first days post-transplant and broad protein areas containing alpha-1-microglobulin were found in all urine samples of kidney recipients, it was among the high-abundance proteins as mentioned above. Interestingly, three out of numerous spots identified as AMBP protein were found to be differentially expressed in rejection vs. stable samples (see 4.2.3). However, the sensitivity and specificity of this protein as a rejection marker was strongly limited in our analytical setup.

Kininogen-1 is the inactive precursor of kinins, a badly definable group of blood proteins that play important roles in coagulation, blood pressure, inflammation and pain. Kininogen was reported to be down-regulated in urinary samples of a renal carcinoma patient after nephrectomy in one study [87]. We found broad protein chains of Kininogen-1 together with

alpha-1-antichymotrypsin and alpha-1-antitrypsin to be constitutively present in merely all samples.

Alpha-1-antitrypsin is an acute phase protein that inhibits trypsin, elastase and other serine proteases and thereby protects body tissues from enzymes involved in inflammatory reactions. Its plasma concentration rises in inflammatory states, e.g. during episodes of acute rejection in kidney transplantation. Urinary excretion has been evaluated as a diagnostic tool in essential and secondary hypertension [91], but not yet in kidney transplantation. Although we identified one differentially expressed spot in 18% gels as alpha-1-antitrypsin, all other alpha-1-antitrypsin spots were present in all gels and not specific for rejection samples. Moreover, the position of the differentially expressed single spot in the gel did not correspond to the expected position of alpha-1-antitrypsin. We therefore consider the single differential expression to be an accidental finding.

Furthermore, complement factor B appeared to be constantly present in all gels. Maybe this protein is highly expressed in urine from renal transplant patients and transplantation-specific. In addition to 4 constantly expressed spots (area K), we identified complement factor B in an intensive protein spot that was over-expressed in most rejection samples (see 4.2.3). Factor B is part of the alternate pathway of the complement system whose activation appears to mediate ischemia/reperfusion injury. Mice deficient in complement factor B showed protection from ischemia/reperfusion damage in one study [92]. The complement system is part of the innate immune defense system, and although most studies about organ rejection have put emphasis on cellular rejection, the humoral component of rejection has lately become more popular among immunologically oriented investigators. Proximal tubular cells are not only target, but also source of complement factors. Locally produced complement also plays important roles in inflammatory and immune responses against the graft. It has become good practice to stain allograft biopsies for C4d along peritubular capillaries, a degradation product of the complement factor C4, to reveal humoral rejections.

Finally and as expected, we found large spots of immunoglobulin light chains in our samples as those are secreted by the tubules.

4.2.2 Low-abundance proteins

In addition to the areas with high-abundance proteins, 209 single spots were identified, labeled in the proteome maps with consecutive numbers, and listed in table 10 in the appendix. 178 unique protein annotations belong to the 209 spots. Figure 8 shows the distribution of the proteins identified according to their subcellular location. In both, 12% and 18% gels,

approximately 50% of the identified proteins originate from the plasma. This is not surprising, since urine is a plasma filtrate. Many of them are expressed by the liver and secreted into plasma. They have a variety of functions as enzymatic, transport, coagulation, complement and immunomodulatory proteins.

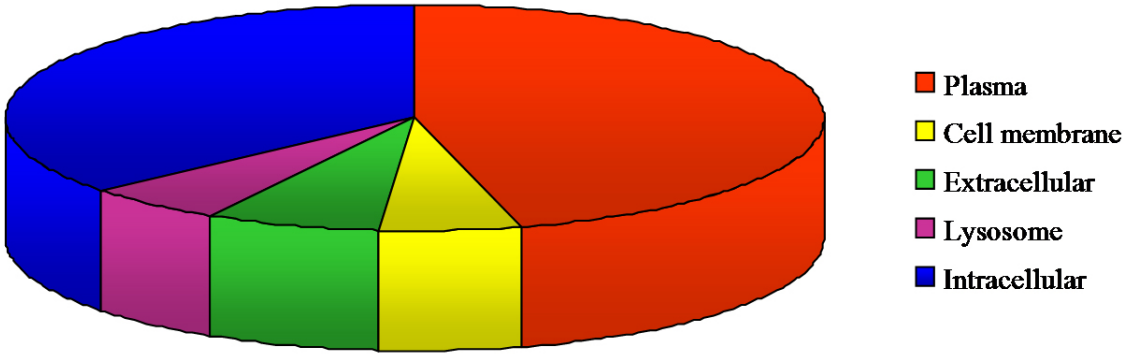


Figure 8: ***The composition of urine proteins.***

Ten of the identified proteins are associated with the **extracellular** matrix and body fluids other than plasma. Among those, we found the extracellular basement membrane proteins perlecan (basement membrane-specific heparan sulfate proteoglycan core protein, spot 1) and entactin (nidogen-1, spot 25), that play important roles in the maintenance of the glomerular filtration barrier. In addition, the extracellular small molecular weight proteins dermatopontin (spot 78) and lumican (spot 7) were detected. Dermatopontin mediates adhesion, interacts with TGF-beta and was lately suggested for new functions in early immune system [93]. Lumican, a leucin-rich small proteoglycan, is also a binding partner of TGF-beta and its involvement in diabetic nephropathy is currently under investigation. Another identified protein being secreted into the extracellular space by vascular endothelial cells is connective tissue growth factor (CTGF, spot 71), which was recently found to be an interesting biomarker and mediator of chronic allograft nephropathy [94]. Multimerin-2 (spot 174) was only seen in 18% gels.

Ten **cell-membrane** associated proteins were identified. Among those, we found the glycosyl-phosphatidyl-inositol (GPI) membrane-anchored CD59 glycoprotein (spots 72, 88, 96, 98, 126) as a very abundant urinary protein. CD59 is a potent inhibitor of the complement membrane attack complex. Together with monocyte differentiation antigen CD14 (spots

19) from this category, CD59 is highly expressed on the cell surface of leukocytes, but was also found to be expressed on glomerular and tubular epithelia. A soluble form of CD59 exists. The urinary and tubular expression of CD59 and CD14, respectively, was investigated in different renal pathologies [95]. Cadherin (spot 3) has been identified as an urinary protein in several reports [96, 86, 87], whereas trem-like transcript 1 protein (spot 83) is an example for a, to our knowledge, not previously detected protein in human urine. The two-cell membrane-associated proteins found in 18% gels were the above mentioned CD59 glycoprotein (spots 151 and 152) and meprin A, a brush-border enzyme that was reported to be involved in chronic allograft nephropathy (CAN) [97].

Ten **lysosomal** proteins were identified, all of them being enzymes. Lysosomal alpha-glucosidase (spot 96) is essential for the degradation of glycogen to glucose in lysosomes. Proteins like cathepsin Z (spot 39), cathepsin D (spot 66), dipeptidyl-peptidase 1 (spot 124) and tripeptidyl-peptidase 1 (spot 41) are lysosomal serine/thiol proteases, that are involved in degradation and rearrangement processes of the extracellular matrix and in immune responses. Ganglioside GM2 activator (spot 150) is another enzyme from this family and was only found in 18% gels. Arylsulfatase A (spot 14) catalyzes degradation processes of membrane glyco- and sphingolipid components and has been suggested to be a rejection biomarker in 1980 by Cavallini et al. [98].

Sixty-three **intracellular** proteins were identified, among those 43 of cytoplasmatic, 14 of nuclear, 5 of endoplasmatic and 1 of mitochondrial origin. Most likely, these proteins or their proteolytic peptides are released into the urine as a result of cell or tissue leakage. Most of these proteins have not yet been identified in human urine to our knowledge, such as elongation factor 1 and 2 (spots 79 and 81), crk-like protein (spot 101) and programmed cell death 6-interacting protein (spot 76). Other identifications confirm previous findings [87], including carbonic anhydrase 1 (spot 55), heat shock cognate 71 kDa protein (spot 56) and skeletal muscle alpha-actin (spot 35). Seven intracellular proteins were only detected in the 18% gels. Among those, we found fatty acid-binding protein, that was shown to be over-expressed in acute rejection of rat renal allografts [99], and napsin A, whose presence in urine was observed previously [100]. Ubiquitin was also found in considerable abundance.

Several isoforms of keratin were identified for many spots. As this is most likely derived from post-separational contaminations, they were withdrawn from spot annotations. Table 11 shows the types of keratin that were identified and taken out from table 10.

4.2.3 Rejection-associated proteins

To determine the differentially expressed urinary proteins of rejecting patients, fused images of all 2-DE gel images from the stable controls and the rejecting patients were created and used as a reference map for visual comparison of each analytical 2-DE image with the other group. In addition, we compared the spot intensities of the two groups after having translated them into average grey values as described in 3.4. Averages were calculated of % volume and average gray values of each spot. To reveal differentially expressed spots that could serve as rejection markers, we first compared rejection group samples to stable group samples. The ratio of rejection group's mean % volume and control group's mean % volume for each of the 1467 detected spots was calculated. Next, we looked for spots that showed increased or decreased mean intensity by a factor of at least 5.

12% gels

In 12% gels, 129 spots were found to be differentially expressed in this way. Among them, 42 were under-expressed, and 87 over-expressed in the rejecting patients. 32 of them were identified spots. Table 8 lists the identified, differentially expressed spots in 12% gels.

Table 8: ***Differentially expressed spots with protein identifications in the 12% proteome map.*** *The ratio of average % volume of the rejection group and average % volume of the control group is shown as fold change.*

Label	Protein Name	Accession Number	MW	pI	Fold change
19	Monocyte differentiation antigen CD14 precursor	P08571	40050.74	5.84	7.342
43	Septin-1	Q8WYJ6	41944.24	5.56	-6.547
44	Serum albumin precursor	P02768	69321.49	5.92	-290.108
46	Structural maintenance of chromosomes protein 1A	Q14683	143144.1	7.51	12.368
48	Hemopexin precursor	P02790	51643.27	6.55	18.056
52	Angiopoietin-related protein 2 precursor	Q9UKU9	57068.44	7.23	10.367
54	Protein disulfide-isomerase precursor	P07237	57080.67	4.76	-6.313
55	Carbonic anhydrase 1	P00915	28852.39	6.59	-5.265

Table 8: *(continued)*

Label	Protein Name	Accession Number	MW	pI	Fold change
56	Heat shock cognate 71 kDa protein	P11142	70854.22	5.37	-7.199
67	Carbonic anhydrase 1	P00915	28852.39	6.59	22.196
68	AMBP protein precursor [Contains: Alpha-1-microglobulin	P02760	38973.98	5.95	12.090
	Immunoglobulin J chain	P01591	15584.55	4.62	12.090
70	Antithrombin-III precursor	P01008	52568.86	6.32	5.241
71	AMBP protein precursor [Contains: Alpha-1-microglobulin	P02760	38973.98	5.95	9.265
	Connective tissue growth factor precursor	P29279	38064.85	8.43	9.265
	Lysozyme C precursor	P61626	16526.28	9.38	9.265
72	CD59 glycoprotein precursor	P13987	14167.79	6.02	11.222
	Galectin-3-binding protein precursor	Q08380	65289.28	5.13	11.222
76	Programmed cell death 6-interacting protein	Q8WUM4	95963.12	6.13	9.263
77	Hemopexin precursor	P02790	51643.27	6.55	18.952
	Vitelline membrane outer layer protein 1 homolog precursor	Q7Z5L0	21520.3	4.9	18.952
81	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	P31930	52612.43	5.94	5.267
85	3-mercaptopyruvate sulfurtransferase	P25325	33157.64	6.13	6.158
88	CD59 glycoprotein precursor	P13987	14167.79	6.02	8.902
	Kininogen-1 precursor	P01042	71912.15	6.34	8.902
	Osteopontin precursor	P10451	35401.24	4.37	8.902
94	Tryptophanyl-tRNA synthetase, cytoplasmic	P23381	53131.61	5.83	10.287
98	AMBP protein precursor [Contains: Alpha-1-microglobulin	P02760	38973.98	5.95	5.318
	CD59 glycoprotein precursor	P13987	14167.79	6.02	5.318

Table 8: *(continued)*

Label	Protein Name	Accession Number	MW	pI	Fold change
100	Plasma retinol-binding protein precursor	P02753	22995.26	5.76	-16.265
110	Plasma retinol-binding protein precursor	P02753	22995.26	5.76	-16.231
122	Cytochrome b5	P00167	15320.51	4.88	5.740

Seven of the identified protein spots were found to be **under-expressed** in urine samples of renal transplant recipients undergoing acute rejection. Heat shock cognate 71 kDa protein is among the identified proteins, an ubiquitously expressed cytoplasmic protein that belongs to the phylogenetically highly conserved heat shock protein 70 family. Heat shock proteins (Hsps) are induced in pro-inflammatory responses, but there is evidence that they have – if released extracellularly – a range of immunoregulatory functions. Their role in vascular disease, autoimmunity and allograft rejection has been investigated by several groups. It has been proposed that expression of Hsps in allograft tissue promotes acute and chronic graft rejection [101, 102, 103], however, several findings indicate that the presence of Hsps and anti-Hsp reactivity might reflect an anti-inflammatory, protective response [104, 105, 106]. Urinary excretion of heat shock proteins has not been studied in this context, but our preliminary findings of under-expressed heat shock cognate 71 kDa protein in rejecting patients support this suggestion.

Furthermore, we were surprised to find plasma retinol-binding protein (RBP) to be underexpressed in rejection samples. This is in contradiction to the observation of enhanced expression of a spot identified as RBP in 18% gels. RPB delivers retinol from the liver to peripheral tissues and belongs to the lipocalin family, a group of rising interest as urinary protein biomarkers. Urinary excretion of RBP was reported to be significantly increased in renal transplant patients with clinical tubulitis grade Ia/Ib, compared to patients with normal tubular histology by Schaub et al. [82]. In previous reports, urinary excretion of RBP correlated with the cold ischemia time of renal allografts [107].

Twenty-five identified protein spots were **over-expressed** in 12% gels of rejection group patients. Around one third of them were identified as **plasma** proteins, including AMBP protein (containing alpha-1-microglobulin), angiopoietin-related protein 2, connective tissue growth factor and antithrombin-III (AT-III). Enhanced expression of angiopoietin-related

protein 2 was found in microvascular lesions of diabetic glomerulopathy [108] and in human renal cell carcinoma tissues. To our knowledge, no association to acute allograft rejection has been reported previously. That applies also to connective tissue growth factor (CTGF, spot 71), but this protein was, as mentioned above, recently described as an interesting biomarker and mediator of chronic allograft nephropathy (CAN) [94]. AT-III inhibits several serine proteases of the intrinsic pathway and thereby regulates the blood coagulation cascade. It is the most important molecule to prevent intravascular blood coagulation. Already in the '80s, a number of authors suggested a connection between elevated plasma AT-III levels and renal allograft rejection [109]. Its function is greatly enhanced in the presence of heparin. As vascular thrombosis plays a considerable role in the rejection process, it was recently shown that the application of heparin or recombinant human AT-III had protective effects for transplant patients with thrombophilic risk factors [110] and in pig-to-primate renal xenotransplantation [111]. Increased urinary loss of AT-III might enhance the risk of vascular thrombosis in renal allografts.

Two **cell membrane** proteins were among the over-expressed protein spots identified. Those were CD59 and CD14, two proteins with high expression on leucocyte surfaces. However, over-expression of CD59 and CD14 is not well established since some CD59- and CD14-spots were found to be differentially expressed whereas others were equally expressed in both rejection and control samples.

Among the over-expressed **intracellular** proteins, we found programmed cell death 6-interacting protein and carbonic anhydrase 1. Programmed cell death 6-interacting protein might play a role in apoptosis and cell proliferation. Carbonic anhydrase 1 catalyzes the rapid conversion of carbon dioxide to bicarbonate and protons. No relation to acute allograft rejection was found in literature.

Two over-expressed spots contained **extracellular** proteins, osteopontin and vitelline membrane outer layer protein 1 homolog precursor. Osteopontin (spots 87 and 88), also known as early T lymphocyte activation 1 (Eta-1), is a secreted glycoprotein and expressed by bone, kidney and epithelial tissues. Furthermore, it can be found in endometrial tissues, endothelial cells, T cells, macrophages, smooth muscle cells, and many tumor types as well as in several biological fluids including human plasma, serum, breast milk, and urine. Based on gene structure and chromosomal location, osteopontin is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. It is a highly acidic, multi-domain protein with a predicted MW of approximately 33 kDa, although it might range up to 75 kDa due to extensive glycosylation and phosphorylation. Several conditions including atherosclerosis, valve stenosis, myocardial infarction, and rheumatoid arthritis are accompanied by an upregulation of osteopontin in tissues. Despite its role in bone metabolism, osteopontin modulates

inflammatory processes by regulating macrophage differentiation and recruitment. Another function is co-stimulation of T cells and chemotaxis. Several inflammatory mediators including TNF-α, IL-1β, NO and LPS stimulate OPN expression [112]. A recent study by Alchi et al. suggests a potential role in acute renal allograft rejection [113].

18% gels

In 18% gels, 47 spots were found to be differentially expressed proteins in rejecting and non-rejecting patients. Among them, 42 spots were over-expressed, and 5 under-expressed in the rejecting patients. Of the 47 differential protein spots, 15 were identified by MS analyses, representing 12 unique proteins (Table 9). All 15 identified spots were over-expressed in rejection samples.

Table 9: ***Differentially expressed spots with protein identifications in the 18% proteome map.*** *The ratio of average % volume of the rejection group and average % volume of the control group is shown as fold change.*

Label	Protein Name	Accession Number	MW	pI	Fold change
140	Ester hydrolase C11orf54	Q9H0W9	35094.61	6.23	5.459
141	Retinol-binding protein 4	P02753	22995.26	5.76	9.300
144	Complement factor B	P00751	85478.52	6.67	12.386
147	Immunoglobulin J chain	P01591	15584.55	4.62	8.423
186	Serum albumin precursor	P02768	69321.49	5.92	7.671
187	Serum albumin precursor	P02768	69321.49	5.92	30.268
188	Cystatin-M	Q9H112	16500.35	8.31	5.643
190	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3]	P61769	13705.91	6.06	12.297
191	Alpha-1-antitrypsin	P01009	46707.02	5.37	8.170
193	Ubiquitin	P62988	8559.62	6.56	6.114
195	Ubiquitin	P62988	8559.62	6.56	17.805
196	Meprin A subunit alpha precursor	Q16819	84314.31	5.42	5.304
200	Ig gamma-1 chain C region	P01857	36083.17	8.46	5.208
200	Ig gamma-3 chain C region	P01860	32309.82	7.89	5.208
200	Ig gamma-2 chain C region	P01859	35861.76	7.66	5.208
200	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	94914.41	5.7	5.208

Table 9: *(continued)*

Label	Protein Name	Accession Number	MW	pI	Fold change
201	SH3 domain-binding glutamic acid-rich-like protein	Q9H299	12766.38	5.22	8.336
205	Ig gamma-1 chain C region	P01857	36083.17	8.46	5.449
205	Ig gamma-3 chain C region	P01860	32309.82	7.89	5.449
205	Ig gamma-2 chain C region	P01859	35861.76	7.66	5.449

Seven of the 12 differentially expressed, identified proteins are **plasma** proteins. Among them was retinol-binding protein 4 which was found to be under-expressed in 12% gels. In addition, we identified complement factor B in some differentially expressed spots. As complement factor B is part of the innate immune system, an involvement in the process of acute rejection appears probable.

We were not surprised to identify β_2-microglobulin in a large spot prevalently observed in rejection samples. β_2-microglobulin is the beta-chain of MHC class I molecules that are present on almost all body cells. It is non-covalently attached to the heavy α_3-chain, in case of degradation and metabolism of the MHC molecule it dissociates from the heavy chain and appears as free monomer in the extracellular fluid. Free β_2-microglobulin is filtered by the glomerulus and almost completely reabsorbed by proximal tubule cells. In healthy subjects, urinary excretion of β_2-microglobulin is limited to 370μg/24 hours, but increases in case of proximal tubular malfunction. In contrast, if glomerular filtration rate decreases, urinary excretion will decrease and serum levels will increase correspondingly. Serum levels of β_2-microglobulin have been extensively investigated in renal transplantation for decades [114, 115], but although determination of urinary and serum levels represent a sensitive assessment of glomerular and tubular function of the allograft, the value of β_2-microglobulin as a rejection marker remains unclear. As serum β_2-microglobulin levels cannot differentiate rejection from cyclosporine toxicity or infection [115], they were considered as an adjunct to, not as a replacement for, classical methods for detecting acute rejection. However, recent studies have found strong correlations between urinary β_2-microglobulin excretion and episodes of acute rejection [79, 80, 81].

One **cell membrane** protein, the brush-border enzyme meprin A, was detected as differentially expressed in 18% gels. Meprin A is a member of the metzincin families, which were reported to be differentially regulated in chronic renal allograft rejection by Berthier et al.

[97]. It is the major matrix degrading enzyme of renal tubules and is increasingly excreted in urine in the case of tubular cell injury.

One **extracellular** protein was among the differentially expressed proteins in 18% gels. Cystatin M is a member of the cystatin family that reversibly inhibit lysosomal proteases (e.g. cathepsins). Another member of this family, cystatin C, is a sensitive marker for the estimation of GFR. Cystatin M differs significantly from other cystatins in biochemical properties, chromosomal localization, and tissue specificity. Recent findings revealed that the cystatin M gene expression is differentially regulated in breast and oral cancer. There is no reported relation to kidney diseases or urinary excretion of cystatin M.

Three **intracellular** proteins were found to be differentially expressed in 18% gels: Ubiquitin, SH3 domain-binding glutamic acid-rich-like protein, and ester hydrolase C11orf54. SH3 domain-binding glutamic acid-rich-like protein was lately reported to be over-expressed in brain cancer, but no association with kidney disease or kidney transplantation was found in literature. The same applies to ester hydrolase C11orf54. We were surprised to identify a relatively large spot highly represented in rejection samples as ubiquitin. As the name says, ubiquitin is an ubiquitously expressed protein, which can be covalently linked to lysin either as monomer or as polymer. Attachment to proteins as lysin-linked polymer usually leads to their degradation by proteasomes. Linkage to proteins as monomer is required for numerous functions, including regulation of gene expression, stress response, maintenance of chromatin structure, DNA repair, and the incorporation of plasma-membrane proteins into vesicles for urinary excretion by *exosomes*. Recently, a proteomic study on urinary proteins in diabetic nephropathy revealed a lack of processed ubiquitin in urine of diabetic nephropathy patients compared to a healthy control group [116]. Ubiquitination and the ubiquitin-proteasome pathway control many processes and are involved in formation of urinary exosomes. The involvement of ubiquitin in immune responses underlines its potential as a rejection marker.

4.3 Literature search

All identified proteins were subjected to an extensive literature search in order to evaluate their potential role in the allograft rejection process. Scientific literature sources, SwissProt and PubMed entries were used for gathering information about the molecules. Several proteins were found to be related to immunologic processes, and some could be directly linked to renal allograft rejection. Among those were:

1. **Osteopontin**: Secreted glycoprotein, chemoattractant for mononuclear cells, up-regulated in inflammatory states. Osteopontin activity is mediated by different receptors including the hyaluronan receptor CD44, which is involved in the activation, proliferation,

adhesion, and extravasation of lymphocytes. OPN was related to cyclosporine toxicity. A recent study found OPN expression to be enhanced in rejection biopsies, especially in tubules surrounded by inflammatory cells [117, 113, 118, 119, 120, 121].

2. **β_2-microglobulin**: Beta-chain of MHC class I molecules, present on almost all body cells. Urinary excretion was found to be elevated in renal allograft rejection, but also in cyclosporine-induced nephrotoxicity and infections, in particular CMV infections. However, recent proteomic studies identified β_2-microglobulin as a marker for acute rejection [122, 123, 124, 125, 79, 80, 81, 82].

3. **Hsp71**: Induced in pro-inflammatory responses, involved in binding of antigens and delivery to APCs. During ischemia, reperfusion and transplantation itself it is supposed to protect cells from injury and necrosis. Later on, this effect might change to activation of rejection [104, 105, 106, 101, 102].

4.4 Profiles of candidate markers

After putative rejection biomarkers had successfully been identified by group comparisons, we tried to evaluate the single markers by following their expression profiles in selected example patients. In addition, some markers were analyzed quantitatively in free urine by the ELISA method. Our extensive sampling approach allowed us to follow the spot expression kinetics of identified proteins in the postoperative course of the patients that had undergone transplantation.

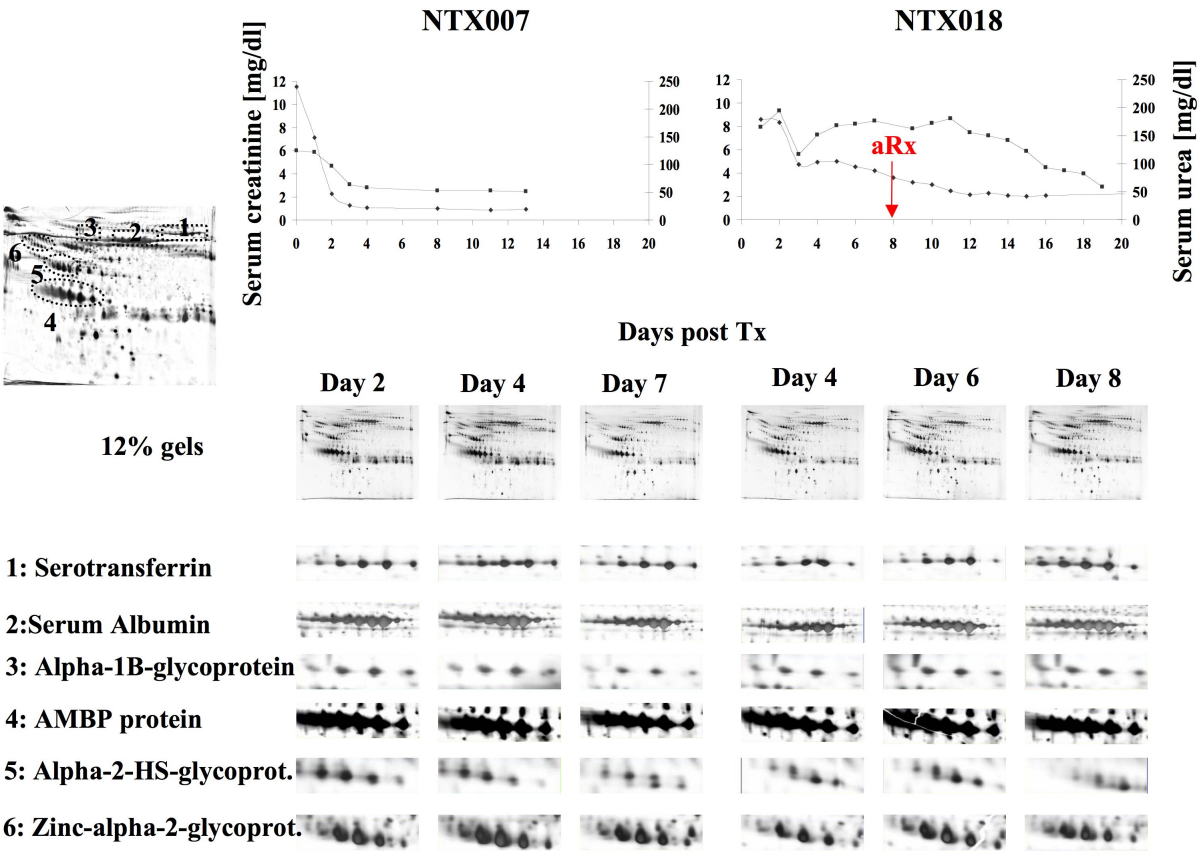


Figure 9: ***Individual spot expression kinetics of high-abundance proteins in a control and a rejecting patient.*** *NTX007 showed high pre-transplant serum creatinine levels that steadily fell below 1 mg/dl in the first days post-transplant. There was no need for dialysis or biopsy, and the patient was released on day 14pt. Serum creatinine levels of NTX018 also decreased in the early post-transplant period, as the allograft started to function right after implantation. Because urea levels stayed above 150 mg/dl, a biopsy was performed on day 9 post Tx and revealed a cellular rejection grade Ia. After administration of prednisolone bolus over 5 days, serum creatinine and urea levels normalized, and the patient could be released on day 26pt.*

Figure 9 exemplarily shows the spot expression kinetics of selected high-abundance proteins in samples from a patient of the control group (NTX007) and from a patient of the rejection group (NTX018). Shown in the top part of the figure are serum creatinine and urea levels of the patients on the days following transplantation (day 0). Urine samples were taken on day 2, 4 and 7 post-transplant from patient NTX007 and on days 4, 6 and 8 post-transplant from patient NTX018. The 2-DE gels prepared from those samples are shown in the first line below. The high similarity of the gels underlines the reproducibility of our method. On

top left part of the figure, positions of the depicted proteins are shown in a sample gel image. Enlarged images of the gel regions occupied by these high-abundance proteins 1-6 are shown in the lines below. No difference in spot expression of serotransferrin, serum albumin, alpha-1B-glycoprotein, AMBP protein, alpha-2-HS-glycoprotein and zinc-alpha-2-glycoprotein can be seen. Spot trains of those major urinary proteins seem to be equally expressed in both patients.

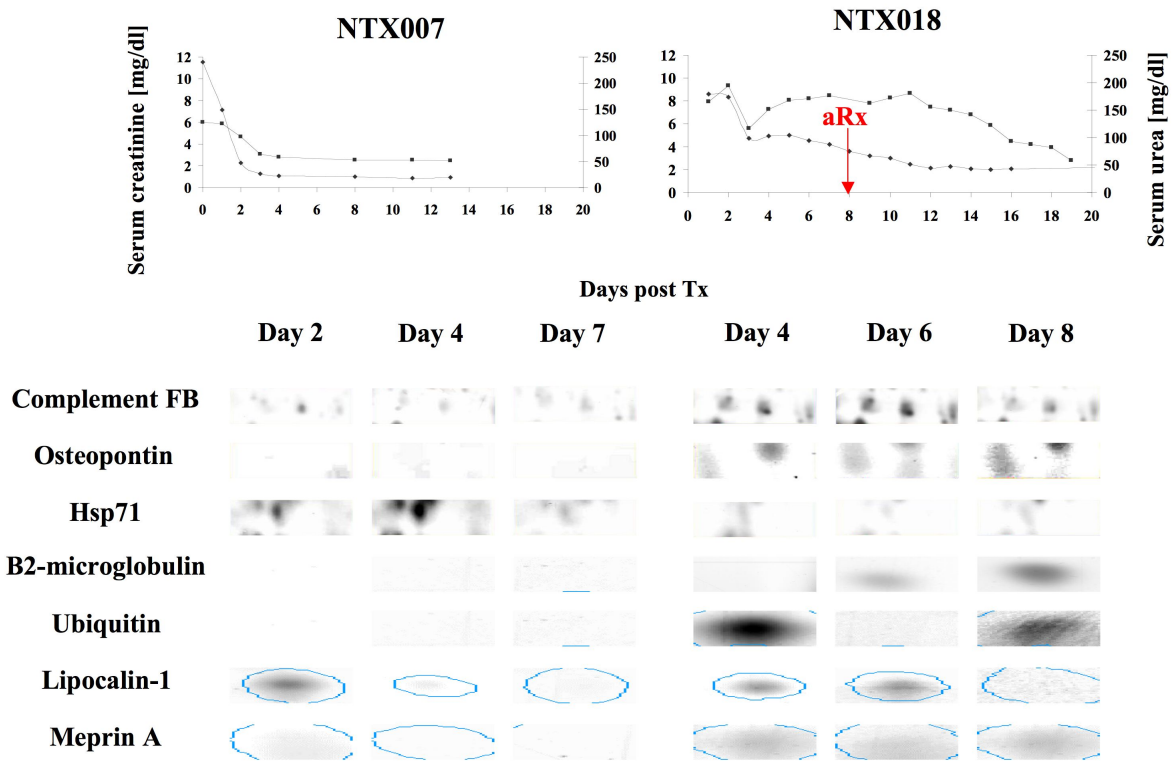


Figure 10: ***Individual spot expression kinetics of rejection-associated proteins in a control and a rejecting patient.*** *Differences in spot expression were detected in several low-abundance proteins. Complement FB = Complement factor B; Hsp71 = Heat shock cognate 71 kDa protein; B2-microglobulin = β_2-microglobulin.*

However, differences were observed in several low-abundance protein spots in sample gels from those patients. Figure 10 shows the spot expression profiles of several rejection-associated proteins in patients NTX007 and NTX018. Complement factor B and osteopontin spots are more intense in the gels of the rejecting patient. Osteopontin spot intensity even seemed to increase until the day of rejection diagnosis. This is also true for β_2-microglobulin. No β_2-microglobulin spots were observed in gels from the control patient, neither were any

ubiquitin spots. In the rejecting patient, ubiquitin spots were of great intensity but were not detected on all days before transplantation. Meprin A and lipocalin-1 spots seemed to be more strongly expressed in gels from patient NTX018, whereas heat shock cognate 71 kDa protein was more intense in samples from patient NTX007.

Osteopontin

Osteopontin was among the over-expressed proteins in rejecting patients in 12% gels. An extensive literature search was performed and several cross-references to possible roles in the allograft rejection process were found. We therefore decided to examine its urinary concentration in rejecting and non-rejecting patients with the ELISA method.

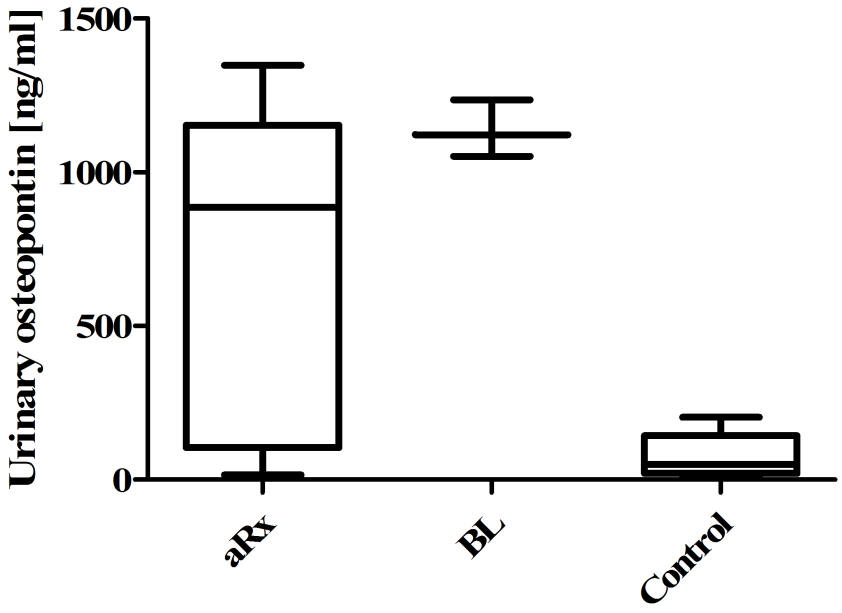


Figure 11: ***Urinary concentration of osteopontin of renal transplant patients is associated with acute rejection.*** *Mean urinary osteopontin concentrations of patients undergoing borderline and more severe rejections were measured and compared to values of the control group.*

Urinary osteopontin concentrations in healthy individuals were found to range from 122 to 8796 ng/ml in literature. In our study, raw urinary samples were diluted in a 1:150 ratio with purified water and osteopontin concentrations were then determined using the Quantikine Osteopontin ELISA kit (see section 3.6). Urinary osteopontin concentrations in renal transplant patients ranged from 12.2 to 2062.5 ng/ml. It has to be considered that high concentrations of calcium and calcium oxalate cause inaccuracies in the measurement of

urinary osteopontin [126]. Figure 11 shows mean osteopontin concentrations in the different patient groups. Patients with episodes of acute allograft rejection (n=5) showed higher levels of urinary osteopontin than stable patients (n=12). Interestingly, also patients that were diagnosed with borderline rejections (n=4) showed equally high concentrations of urinary osteopontin. With regard to the small number of examined individuals, no statistical significance could be determined.

To follow the dynamics of urinary osteopontin expression, 16 samples from 5 rejection patients were analyzed. Three of them showed elevated osteopontin levels days before biopsies were carried out and diagnosis was confirmed by histologic examination. Figure 12 shows a representative example of one patient.

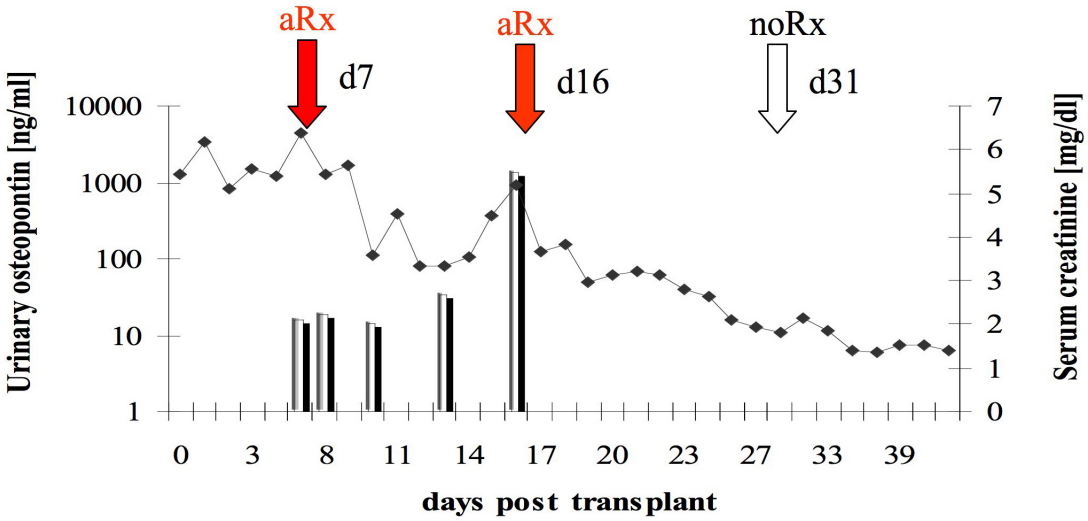


Figure 12: ***Individual kinetics of urinary osteopontin concentration*** *in a patient suffering from episodes of acute rejection. With delayed allograft function and serum creatinine levels (line) above 5 mg/dl, the patient had to undergo re-laparotomy because of ureteral necrosis on day 7. During re-laparotomy a biopsy was taken that revealed a rejection grade III in the histologic examination. Under treatment with the monoclonal antibody OKT3 and six plasmapheresis sessions for five days, serum creatinine levels decreased. But as the retention parameters increased again during the following days, allograft re-biopsy was performed on day 16. Elevated urinary osteopontin levels (columns) were detected prior to biopsy, that revealed a cellular rejection grade IIb. Another rejection-free biopsy was taken on day 31.*

5 Discussion

5.1 The need for rejection markers

The development of new immunosuppressive agents as well as the actual practice in the histopathological evaluation of biopsy material and the refined therapeutic options to control opportunistic infections have contributed to an improved short-term graft survival in kidney transplantation in recent years. Incidence of acute rejections after renal transplantation has also been reduced by highly developed clinical monitoring. However, each episode of acute rejection compromises the long-term graft survival and is a main risk factor for the development of chronic rejection. Timely diagnosis of acute rejection episodes is necessary for a prompt adaption of the immunosuppressive therapy. Clinical symptoms for acute rejections are fatigue, decrease in diuresis, fever, increasing blood pressure, transplant pain, and a rise of serum creatinine. Current strategies for monitoring and diagnosis of acute rejections may lead to false-positive or false-negative results and they are not capable of detecting acute rejections at an early stage, which means that the allograft is already damaged at the time of diagnosis. Furthermore, under intensive immunosuppressive therapy, acute rejection episodes might occur without clinical signs. Those so called subclinical rejections do not exhibit a deterioration of graft function. Subclinical rejections can be diagnosed by control biopsies according to a center-specific schedule. Early treatment of subclinical rejections might contribute to a prolongation of graft survival. Despite highly developed immunosuppressive therapies and clinical monitoring, the prevalence of subclinical rejection has not been reduced. That could also explain the stagnation in chronic rejections in kidney transplant recipients. Although histologic examination of graft tissue allows a clear diagnosis in most cases, the highly invasive procedure of a needle biopsy impedes its frequent application.

For all these reasons, reliable and easily accessible rejection markers are attractive and may lead to improved diagnostic standards and different monitoring strategies. Based on high sensitivity and specificity, the predictive value of a new marker should be high, and the analytical procedure should allow easy and non-invasive monitoring of patients after transplantation and in the post-clinical course. Moreover, the diagnostic tests should have a good cost-benefit ratio and should be carried out using established methods to identify patients at risk for a rejection episode. New markers could also reveal insights into the pathogenesis of the rejection process. In the present study, the urine proteome was investigated for changes during episodes of acute rejection after kidney transplantation and marker proteins associated with acute rejection were identified.

5.2 Non-invasive sources for markers

Allograft tissue obviously exhibits the best source of material for diagnosing acute rejection. But for reasons already discussed, serial examination of allograft tissue is not suitable for the monitoring of kidney transplant patients. Analysis of cell free body fluids, on the other hand, is advisable, not only because of its homogeneity. Peripheral blood serum shows a highly complex composition. In a rodent model it was shown that considerable changes in the serum protein profile during rejection were only seen in case of strongly restricted allograft function. In this study it was suggested, that changes in the urinary protein composition might be detected at an earlier stage of the rejection process [127]. The extraordinary complexity of the serum proteome hampers the analysis of correlations with rejections. Urine is an ideal source of material for non-invasive diagnostic and monitoring purposes, as it is easily obtainable in large amounts. A shortcoming of clinical studies is the heterogeneity of the patients. Among other aspects, they differ in age, medical history, and individual immunosuppression. Also, the donor's organs may have different extents of HLA-mismatches with the recipient and originate from either living or deceased donors. Despite the disadvantages of clinical studies, it should be possible to develop diagnostic tests from their results. Fast application, high throughput and minimal sample preparation must be possible for a routine diagnostic test. This is given for urine to a greater extent than for plasma.

5.3 2-DE MALDI-TOF MS

In recent years, many authors presented new and promising rejection markers (see section 1.5). However, none of them has made its way from bench to bedside routine diagnostics, as most methods are too expensive and/or too time-consuming. None of the markers has been evaluated in multicenter studies yet. Furthermore, most presented tests are not standardized with regard to sample preparation, reagents, methods, and interpretation of the results.

High-throughput proteomics is a novel technology to study highly complex mixtures of proteins in biological fluids and tissues. The field of transplantation stands to benefit from the application of proteomics in the discovery of new biomarkers and therapeutic targets. Several proteomic techniques are promising tools to better understand disease pathogenesis and as the basis for new diagnostic tests. Most studies in transplantation proteomics used MS-based technologies, and –to a minor extent– protein arrays have also been used. In the present study, the technique used was 2-dimensional electrophoresis combined with matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy (2-DE MALDI-TOF MS).

Because of the sheer number of proteins in urine samples and in order to identify individual proteins, the complexity of the mixture has to be decreased. 2-D polyacrylamide gel electrophoresis is a powerful tool to segregate complex protein mixtures. 2D-PAGE is useful for the analysis of protein phenotypes, monitoring disease process and discovering new disease markers in body fluids or tissue biopsies; it is particularly suited for the high-resolution separation of proteins and the simultaneous detection of diverse changes in protein expression. 2D-PAGE is therefore frequently used in a clinical research environment.

Previous studies tried to establish 2D urine proteome maps of healthy patients [86, 88, 96, 87], to monitor potential physiologic changes in disease states, and finally take advantage of this approach in kidney disease diagnosis. Unfortunately, all maps differ from each other, and protein detection varies with sample preparation and electrophoretic procedures. Some of the studies have been limited by the low protein content of urine or by the presence of compounds like glycosaminoglycans that can interfere with protein migration in SDS-PAGE. Various methods were investigated to improve electrophoretic migration [128]. Under the auspices of the World Human Proteome Organisation (www.hupo.org), the Human Kidney and Urine Proteome Project (www.hkupp.org) is currently concerned with those problems and publishes standardized methods for urine proteomic studies.

The preparation of the urine samples for 2-DE analysis is a critical point to achieve reproducible results. Many investigators used dialysis and concentration by lyophilization to remove interfering molecules, salts and albumin and described positive effects on reproducibility of the 2-DE gels [86, 129, 88]. Technologies exist to remove high-abundance proteins, such as albumin, IgG, IgA, transferrin and haptoglobin. However, those procedures carry the risk of losing interesting proteins, as many low molecular weight proteins are bound to albumin. As we neither filtrated nor centrifuged the samples before precipitation (see details in section 3.2), the protein pellet was probably contaminated with cell debris and whole cells. Streaking patterns, as they were rarely present in some of our gels, generally come from contaminations with salts. However, we aimed to resolve as many molecules as possible that could represent the process of rejection in the kidney. As all patients were equipped with double pigtail stents in the transplantation, we obtained nearly all urine samples directly from the renal calices without the risk of cellular contamination from the urogenital tract. Our method represents an easy and cheap way to obtain reproducible and high-resolving protein gels.

Low-abundance proteins can be missed in 2D-PAGE, as only those proteins are analyzed that form visible spots on a gel. Another disadvantage of 2D-PAGE is the limited resolution capacity, particularly for small proteins and peptides. High-performance liquid chromatography (HPLC) is an alternative method for fractionation and can be used to overcome

some limitations of 2D-PAGE. In HPLC, mixtures of molecules can be separated by several physico-chemical properties, and small proteins and peptides are easier handled than in 2D-PAGE. The weight cut-off for the visualisation of small molecules in 2-DE gels is about 5 kDa. In most of the studies cited, commercial gradient gels (9-16%) were used for the separation in the second dimension. In the present study, we prepared homogeneous polyacrylamide gels by ourselves from the chemical components. That enabled us to optimize the resolution by varying the gel concentration. A gel concentration of 18% polyacrylamide suited our demands best.

HPLC methods have also been adopted for proteomic analysis of urine and plasma because of the high-throughput capabilities; however, on 2-DE gels, relative variations in protein expression level can be demonstrated with molecular weights and pI. Separation of proteins in 2-DE gels provides not only the entities of the proteins in a sample but also a two-dimensional separation pattern, such as spot intensities and relative distances between neighboring proteins. It is easier to present post-translationally modified proteins in 2-DE gels than with the HPLC-MS/MS methods, and it may be possible to discover biomarkers by comparing 2-D patterns even if there is no newly expressed or suppressed protein in the sample.

For MS-analysis, samples of peptides or entire proteins are ionized by an ion source and analyte ions of similar mass-to-charge (m/z) ratio are resolved by a mass analyzer. The intensities of ions at a given m/z are then measured by an ion detector. The spectra obtained are fed into coupled databases for subsequent protein identification. Basically, two techniques are used to ionize peptides or proteins: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), or analogously surface-enhanced laser desorption ionization (SELDI). In contrast to MALDI, it is possible to analyze whole proteins with SELDI. Fractionation and digestion of biological samples can therefore be omitted, which makes SELDI less labor intensive and less expensive than MALDI or ESI. Previous studies showed that it is possible to generate informative predictive profiles of conditions related to transplantation [76, 77, 79]. However, protein identification is currently not possible with SELDI.

5.4 Transplanted patients selection criteria

The clear and distinct definition of control groups is essential for all large-scale genomic and proteomic investigations that aim to distinguish disease states from normal. Especially in proteomics, it is simply not possible to define a “normal” proteome because protein expression is far too variable even in healthy individuals. In comparison to plasma, where many proteins are kept in well-known, narrow ranges, it is more difficult to define a normal pattern

of proteins for urine as their expression reflects the kidney's function to maintain plasma homeostasis. Urinary protein excretion depends on blood pressure, volume status, gender, nutrition, body mass index, physical activity, and posture (upright or recumbent). Since a "normal" urine proteome cannot be defined, it is hardly possible to distinguish normal from disease states, and in particular, it is difficult to differentiate between rejecting and non-rejecting patients. Allograft rejection, as it is known so far, cannot be regarded as a sudden event, but as a continuous process between the two extremes of a fully functioning and a failing organ. Allograft rejection is diagnosed and treated as soon as immunologic defense mechanisms have started to attack the allograft to a certain degree. Treatment of the rejection is therefore nothing else than an augmentation of the transplant patients' immunosuppressive medication. Early processes of rejection might not be reflected in clinical signs or serum creatinine levels. Even allograft biopsy does not rule out rejections with certainty, as focal processes might be missed. Nevertheless, allograft biopsy remains the strongest proof for presence or absence of rejection. Schaub et al. therefore used protocol biopsies to distinguish between rejecting and control patients [79]. As protocol biopsies are not performed at our center, we had to set the most stringent criteria possible to define the control group of our study. The criteria included clinical appearance, serum creatinine and urea levels, 6-month-GFR, presence of urinary tract infections, need for dialysis, and the time of sampling (see section 3.1). From 288 urine samples collected from 66 patients, only some were selected for analysis according to those criteria.

5.5 The urine proteome map of renal transplant patients

Our aim was to establish a 2-DE urine proteome map of renal transplant patients. It was meant to serve as a reference map for protein spot positions and identifications. We therefore fused all (that is from rejecting and non-rejecting patients) sample gel images into one synthetic image. All protein spots from the single sample gels are collected in the proteome map (shown in section 4.2).

Several groups have presented 2-DE based urine proteome maps of healthy subjects [96, 87, 86] and of patients with renal [130] and urogenital diseases like bladder cancer [131], Bence-Jones proteinuria [132], kidney stones [133], IgA nephropathy [134], or even chronic exposure to cadmium [135]. 2D maps have also been applied for characterizing apolipoproteins in urine and for monitoring the adaptive changes of unilateral nephrectomy [136].

Some groups have attempted to characterize the urine proteome of healthy individuals. Spahr et al. used direct liquid chromatography-tandem mass spectrometry (LC-MS/MS) and identified 124 proteins being present in "normal male urine from a commercial pooled source"

[137]. In 2002, Thongboonkerd el al. published their 2-DE proteome map of urine from 5 healthy individuals. They identified 47 distinct proteins, most of them being reaffirmed by our study. Of the 17 areas of high-abundance proteins defined in our study, 9 were also seen and identified in this publication [96]. A Korean group presented their urine proteome map in 2004, using urine samples from 20 healthy males and 20 healthy females [86]. In 157 spots they identified 113 different proteins. They realized that even proteomic maps on 2-D gels are difficult to compare due to different gel patterns. Different preparation methods and either genetic or environmental variations in the sampled patient group might cause these differences. In fact, of the 17 high-abundance proteins identified in our study, only three were also found by the Korean group: Serum albumin, serotransferrin and AMBP protein. Also in 2004, Pieper et al. used a combined approach of LC and 2-DE followed by MALDI-MS and ion trap LC-MS/MS to present the most extensive effort in characterizing the human urine proteome [87]. They observed approximately 1400 distinct protein spots in their 2-DE gels, 420 of them were identified, representing 150 unique gene products. The protein identified in our and their study overlapped by nearly 30%, but all 17 high-abundance proteins that we defined were observed and identified by Pieper et al. as well.

We present one of the most extensive efforts published to date characterizing the human urine proteome. A reproducible and high-resolution proteome map was created by TCA precipitation for protein preparation. Although our preparation method (described in 3.2) was easier and cheaper than those used in previous studies, we obtained well-separated and reproducible 2-DE gels. More than 1400 discrete protein spots were detected in our 2-DE gels, and approximately 400 of those were identified by our high-throughput mass spectrometry analysis. 178 unique proteins were identified that achieved an overlap of nearly 30% to the most extensive human urine proteome study [87]. Nearly half of them were plasma proteins, the rest had different subcellular, mostly intracellular, origins. Further subgrouping of their annotated functions revealed the presence of transport proteins, proteases and protein inhibitors, glycoproteins, growth factors, extracellular proteins, complement factors and immunoglobulins. Approximately 50 of the 178 proteins were, to our knowledge, not previously reported as human urinary proteins. Yet nearly 1000 protein spots, mostly small spots in the gels, remained unidentified. Previous reports stated that differences in environment, genetic background and diet of the urine donors as well as different preparation methods make comparisons difficult. However, comparing our proteome map with those of other proteomic studies, we could define a typical pattern of 17 high-abundance protein areas (see section 4.2.1) that had been identified before and that were constitutively present in all urinary samples.

5.6 Rejection-associated proteins

As shown in section 4.2.3, we identified distinct sets of proteins that were differentially abundant in individuals undergoing episodes of rejection compared to those without rejection. Twenty-five urine proteins exhibited high relative abundance in rejection samples compared to samples from stable patients, and seven urine proteins were of low relative abundance compared to stable patients. All differentially expressed proteins were subjected to an extensive literature search to evaluate their possible role in the rejection process. The sets of proteins identified as differentially expressed might include some accidental findings, but it is likely that further studies of these potential markers will facilitate better understanding of the mechanisms of rejection and provide new avenues for diagnostic tests and therapy.

Heat shock cognate 71 kDa protein

Based on the idea of a "positivity" of a test, research for rejection markers has mainly focused on single molecules that are over-expressed during the rejection process. However, it is probable that only a set of distinct markers will show sufficient specificity and sensitivity for a routine clinical test, and in particular, there is no reason to exclude under-expressed markers from such a test.

Among the under-expressed urinary proteins found in our study, the most interesting was heat shock cognate 71 kDa protein. Heat shock proteins are found in nearly all living organisms, from bacteria to humans. Hsps are named according to their molecular weight and are a class of functionally related proteins. The heat shock response is a state of increased expression of those proteins when cells are exposed to elevated temperatures and other stresses. Although exact mechanisms of activation remain unclear, it is discussed that an increase in damaged and abnormal proteins induces expression of Hsps. Extracellular and membrane-bound Hsps, especially Hsp70 is involved in binding antigens and delivering them to antigen presenting cells. It is recognized by signaling receptors on APCs, such as CD40 and Toll-like receptor (TLR) 2 and TLR4. On the one hand, it upregulates the expression of MHC class II and costimulatory molecules as well as the secretion of cytokines and chemokines via activation of the nuclear factor κB (NF-κB) pathway. On the other hand, it is recognized by Hsp receptors such as CD91 and LOX-1, internalized and processed together with the attached antigens for presentation on MHC class I molecules.

It has been proposed that expression of Hsps in allograft tissue promotes acute and chronic graft rejection [101, 102, 103]. Rizzo et al. found increased expression of Hsp40 and Hsp70 during rejection in transbronchial biopsy specimens from lung transplant recipients [103]. Trieb et al. tested the responsiveness of T cell obtained from rejected human kidney allografts to recombinant human heat shock protein 72. They observed a proliferative response

in both CD4+ and CD8+ T cell lines upon stimulation with Hsp72 and renal epithelial cells. It was deduced that released Hsps might play a role as target molecules and, by inducing an immune response, aggravate the rejection process [101]. The same Austrian group demonstrated the expression of Hsps in renal tissue in healthy, minimal change nephropathy and rejecting patients by immunohistochemical staining. Besides the constitutive presence of all Hsp70 and Hsp60 subtypes in all tissues, they found an enhanced expression in rejection samples. It was proposed that Hsps might protect cells and increase transplant survival during ischemia, reperfusion and surgery itself. Later on, this effect might change to activation and aggravation of rejection [102]. Mycobacterial Hsp65-induced growth of graft infiltrating lymphocytes from endomyocardial biopsies was shown to correlate with cardiac graft rejection [138].

In contrast, we found enhanced urinary Hsp 71 expression to be related with stable graft function. In stable patients, the spot identified as heat shock cognate 71 kDa protein (spot 56) was found to be more intense by a factor of 7.2 in comparison to rejecting patients. It can be deduced that a lack of Hsp 71 is related to the development of acute rejection. Our finding suggests a protective role of Hsp 71 in the early post-transplant period. In fact, Hsps have cytoprotective attributes and enhanced expression during the early peri- and post-transplant period could reflect a protective response targeted towards the maintenance of cell and tissue integrity. Several authors reported that Hsp 70 expression appears to represent an endogenous protective mechanism against ischemic injury [139]. Evidence for this theory was published by Currie et al., who suggested that Hsp70 limits the extent of the myocardial infarct as it was found to be expressed preferentially at the border of the ischemic area [140]. Ischemic preconditioning was shown to provide protection against a subsequent myocardial infarction and is accompanied by an increase in Hsp 70 expression [141]. It has also been shown that overexpression of Hsp 70 in transgenic mice reduces the susceptibility to ischemic injury [139]. Further evidence to support this comes from studies that show that Hsps attenuate preservation and ischemia/reperfusion injury [142], and protect endothelial cells from neutrophil-mediated necrosis. Tesar and Goldstein showed that Hsp 70 does not activate dendritic cells (DCs) *in vitro*. *In vivo* transplant studies demonstrate that Hsp 70 levels are not increased during acute allograft rejection in the HY-incompatible skin transplant model [106]. Additionally, Flohé et al. reported that lower levels of Hsp 70 in pre-liver transplant biopsies and organ perfusates are associated with early graft loss [143]. Mueller et al. examined the first urine of six pediatric allograft recipients for proteinuria and urinary Hsp 72 excretion. Their results suggest that urinary excretion of Hsp 72 is associated with an increased renal stress response and loss of tubular cell integrity after clinical and experimental renal ischemia [144]. They showed that measurements of urinary Hsp 72 are possible in practice.

Is there a relevance of our findings with regard to Hsp 71 as future therapeutic tool in transplant medicine? Previous studies reported, that upregulation of Hsp 70 raised the resistance to subsequent injury in several models of transplantation [145, 141]. Upregulation of Hsp 71 might therefore represent an option for new therapeutic interventions. For example, pretreatment of donor organs with transient hyperthermia might be part of future protocols to investigate whether overexpression of Hsp 71 will indeed result in cytoprotection in human transplant medicine similar to that observed in animal models. Different methods on how to stimulate Hsp 71 expression still need to be investigated.

Osteopontin

Osteopontin is a secreted multifunctional glycoprotein, which is over-expressed in several types of cancer and plays a principal role in urinary stone formation as it forms the stone matrix. Other functions are related to bone metabolism, wound healing, cell survival, and immune regulation. It is a potent chemoattractant for mononuclear cells that is up-regulated in various inflammatory states of the kidney. Osteopontin activity is mediated by different receptors including the hyaluronan receptor CD44, and many integrins. CD44 is involved in the activation, proliferation, adhesion, and extravasation of lymphocytes. In order to determine its role in the pathogenesis of acute renal allograft rejection, Rouschop et al. studied tissue expression and plasma levels of CD44 and its ligands, OPN and hyaluronic acid. They observed elevated levels of CD44, OPN, and hyaluronic acid in allograft tissue during rejection episodes by immunohistochemistry [117]. It is thought that some intrarenal proinflammatory cytokines act via autocrine/paracrine mechanisms to induce OPN gene transcription. Inflammatory mediators including TNF-α, IL-1β, NO and LPS stimulate OPN expression [112]. Cytokine mRNA expression revealed the presence of the first two mediators during acute allograft rejection [146]. Osteopontin is also well-known to mediate tubulointerstitial injury in glomerulonephritis.

Chemoattractants including osteopontin are supposed to initiate acute rejection episodes with concomitant monocyte accumulation and activation in the graft, acting directly or in concert with other parts of the immune system. OPN is a potent chemotactic molecule for macrophages *in vivo* [147], and its up-regulated expression by proximal tubular epithelial cells in association with monocyte/macrophage infiltrates has been described in a number of rodent models of renal injury [148], and in human renal diseases [119]. Previous studies have found tubular OPN expression to be induced by organ ischemia [149].

Hudkins et al. found strong OPN protein and mRNA expression by tubular epithelium in pretransplant biopsies and in biopsies with cyclosporine toxicity without an inflammatory cell infiltration, however, correlations could not be calculated due to the limited number of

donor biopsies [120]. Another study also suggested OPN as an indicator for cyclosporine toxicity [121].

However, we found over-expression of the osteopontin spot rather related to rejecting patients than to those suffering from cyclosporine toxicity or to stable patients. Comparing rejection vs. stable group, we found the averaged spot intensity to be 8.902 times stronger in samples from rejecting patients. This is supported by recent findings by Alchi et al., who suggest a potential role for osteopontin in acute renal allograft rejection. They examined intrarenal expression of osteopontin in allograft biopsies of rejecting and non-rejecting patients by immunohistochemistry and in situ hybridization. Osteopontin expression was enhanced in rejection biopsies, especially in tubules surrounded by inflammatory cells, whereas in non-rejection and perioperative donor biopsies expression was weak or nil [113]. As OPN works as a chemotactic molecule it is probably expressed already early in the rejection process of an allograft and could eventually be used as an early marker. We therefore tested the feasibility of urinary osteopontin measurements with an easy immunoassay kit (see sections 3.6 and 4.4) and found that reproducible results were possible. Furthermore, we found that urinary osteopontin concentration strongly correlated with the incidence of acute rejection, even in samples taken up to 6 days **before** overt rejection.

β_2-microglobulin

Serum and urinary levels of β_2-microglobulin have been studied in renal transplantation for decades. Already in 1978, a French group measured serum and urinary β_2-microglobulin concentrations daily after transplantation in 44 renal allograft recipients by radioimmunoassay. They observed peaks of urinary β_2-microglobulin levels either before (in 22/30 cases) or concomitant (in 16/30 cases) with episodes of acute rejection. Urinary β_2-microglobulin excretion was related to proximal tubular dysfunction. However, they also found massive urinary β_2-microglobulin excretion in some stable patients without signs of rejection [122]. Roberts et al. found increased urinary β_2-microglobulin concentrations in both acute and chronic transplant rejection. In particular, for acute allograft rejection, they observed an elevation in serum β_2-microglobulin levels preceding the rise in serum creatinine [123]. In 1981, Roxe et al. stated that measurement of urinary and serum β_2-microglobulin could be used as an adjunct to classical methods for detecting acute rejection, in particular subclinical rejections. They monitored 31 renal allograft recipients in the first 21 days after transplantation, and showed that 29 of them met the differentiation criteria they had set for observations on 15 cases [124]. By monitoring serum β_2-microglobulin concentrations in 83 renal transplant recipients, Bäckmann et al. found significantly elevated levels during pretransplant uremia, rejection, cyclosporine-induced nephrotoxicity, and infections, in particular cytomegalovirus (CMV) infections. Patients treated with cyclosporine showed higher

serum β_2-microglobulin and creatinine levels than azathioprine-treated patients. They also observed significantly elevated β_2-microglobulin levels in patients with irreversible rejections compared to patients responding to rejection treatment [115]. It was concluded that serum β_2-microglobulin concentrations cannot distinguish between rejection, cyclosporine nephrotoxicity, or infection. Pacheco-Silva et al. came to the same conclusion after they had studied serum β_2-microglobulin levels in 20 renal transplant patients and had evaluated its use for detecting acute rejections. The sensitivity for the test was 87.5%, but specificity was poor [125]. For many years, it seemed that β_2-microglobulin as a biomarker for renal allograft rejection had fallen into oblivion. However, recent studies supported the assumption that urinary β_2-microglobulin excretion is closely associated with acute rejection episodes. In 2004, Schaub et al. found a 'rejection pattern' in the urine proteome using SELDI-TOF MS [79], and identified the peaks as cleavage products of β_2-microglobulin in their follow-up study [80]. Another urine proteomic study found a protein peak at 11.7 kDa that strongly correlated with acute rejection, and also identified this peak as β_2-microglobulin [81]. Most recently, Schaub et al. evaluated urinary β_2-microglobulin concentrations for detection of subclinical rejection episodes in a rigid clinical setting. Although they found slightly elevated levels in patients with subclinical tubulitis vs. stable patients with normal histology, the comparison between patients with clinical rejection and the subclinical tubulitis group did not reach significance [82]. As shown in section 4.4, our findings strongly support the suggestion that β_2-microglobulin is an interesting biomarker of acute rejection.

5.7 Concluding remarks

Despite the development of new immunosuppressive agents which has led to an improved short-term survival, acute rejection remains a major risk factor for graft loss in the long-term. Strong immunosuppression also mitigates classical signs for allograft rejection like fatigue, decrease in diuresis, fever, increased blood pressure, or transplant pain. Strategies currently used for monitoring and diagnosing acute rejections including serum urea and creatinine levels are not capable of detecting acute rejections at an early stage, which means that the allograft is already damaged at the time of diagnosis. Identification of new rejection markers is therefore essential to identify patients at risk for a rejection episode. New markers could also reveal insights into the pathogenesis of the rejection process. In this study, we present our proteomic technique for the identification of non-invasive rejection markers. However, we do not propose the 2-D electrophoretic gels as a diagnostic test, but rather as a tool to detect proteins that are specific for the pathogenesis of rejection. Furthermore, the patient selection criteria set in this study reflect the extremes of the rejection spectrum (stable transplant *versus* acute clinical rejection) rather than the whole spectrum seen in

regular clinics. Therefore, and also because of the semi-quantitative approach and the small patient number, we have avoided calculating parameters that characterize a clinical test (e.g., sensitivity, specificity, positive and negative predictive value) and tried to validate potential markers with simple, specific ELISAs.

However, it is unlikely that one marker alone will fulfil all expectations. A combination of markers could reflect the heterogeneous process of rejection better, i.e. combining the individual sensitivities and specificities should increase the overall sensitivity and specificity. We have examined several promising markers for such a marker set and suggest that regular monitoring of serum creatinine should be paralleled by non-invasive examination of rejection markers. On the one hand, measurements of urinary β_2-microglobulin excretion is not specific but helpful, whereas detection of osteopontin and IP-10 which are involved in immune activation and of the cytotoxic molecules granzyme B and perforin suggests acute rejection. An allograft biopsy should then be performed to clarify if rejection is present. This approach could make it possible to diagnose acute rejection at an early stage, for the benefit of the transplant patients.

6 Summary

Kidney transplantation is nowadays the established therapy of end-stage renal disease. Due to tissue incompatibilities between donor organ and recipient, a lifelong immunosuppression with a broad range of side-effects is inevitable after transplantation. However, immunosuppressive agents are the only option to control humorally and cellularly mediated rejections. Despite matching of histocompatibility and the application of immunosuppressants, the incidence of acute rejection episodes is still high. Infiltration of the donor organ with alloreactive leucocytes leads to the destruction of transplant tissue and compromises long-term graft survival. For those reasons, accurate and timely diagnosis of acute rejection, even if subclinical, is essential. Gold standard for diagnosis of acute rejections is the histopathological examination of allograft tissue, which requires a needle biopsy of the organ. The invasive procedure of needle biopsies implies the risk of complications and is not suitable for routine examinations. Measuring of serum creatinine levels as an indicator of renal function allows a close monitoring, but is neither sensitive nor specific for rejections. Serum creatinine levels will not rise until the allograft is already damaged, and incipient rejections and the adequate therapy might be missed.

Urine is an obvious source of material for the identification of specific and sensitive rejection markers and the development of new diagnostic tests. Its non-invasive obtainability in sufficient amounts, and anatomical proximity to the kidneys raise the probability that alterations in its composition can be detected during episodes of rejection. New proteomic techniques allow the high-resolution separation of proteins and the simultaneous detection of diverse changes in protein expressions. In this study, we used 2-dimensional electrophoresis combined with mass spectrometry to define the urine proteome of renal transplant patients and to identify differentially expressed proteins during episodes of acute rejection. A total of 178 unique proteins was identified and 17 of them were defined as high-abundance. Among the differentially expressed proteins in rejecting patients we found osteopontin, β_2-microglobulin and Hsp71. Osteopontin was evaluated quantitatively in free urine by immunoassay.

7 Zusammenfassung

Die Nierentransplantation ist mittlerweile Methode der Wahl zur Therapie der terminalen Niereninsuffizienz. Aufgrund der Gewebeunverträglichkeit von Spenderorgan und Empfänger ist postoperativ eine lebenslange Immunsuppression unumgänglich, die jedoch ein breites Spektrum an Nebenwirkungen hervorrufen kann. Trotzdem bietet nur die Gabe von Immunsuppressiva die Möglichkeit, humoral und zellulär vermittelte Abstoßungsreaktionen zu kontrollieren. Trotz der Anpassung von Histokompatibilitätskomplex-Spezifitäten von Spender und Empfänger und der Gabe von immunsuppressiven Therapeutika kommt es immer noch häufig zum Auftreten von akuten Abstoßungen. Die Infiltration des Organs durch immunkompetente Zellen des Empfängers führt zur Schädigung des Transplantatgewebes und zu einer eingeschränkten Langzeitfunktion. Deshalb ist die rechtzeitige und sichere Diagnose der Abstoßungsreaktion notwendig, auch wenn diese sich noch nicht klinisch zeigt. Der Goldstandart zur Diagnose einer akuten Abstoßung nach Nierentransplantation ist die histopathologische Untersuchung von Transplantatgewebe, welches durch eine Nadelbiopsie gewonnen wird. Dieser invasive Eingriff ist mit gewissen Risiken für den Patienten verbunden und kann nicht mehrfach innerhalb kurzer Zeit durchgeführt werden. Der Serumkreatininspiegel als Indikator der Nierenfunktion erlaubt ein tägliches Monitoring, ist aber weder sensitiv noch spezifisch für Abstoßungsreaktionen - subklinische Abstoßungen können dadurch lange übersehen werden und unbehandelt bleiben. Der Serumkreatininspiegel steigt zudem erst bei fortgeschrittener Schädigung des Transplantats, sodass eine beginnende Abstoßung nicht erkannt und deren adäquate Therapie verzögert werden kann.

In der Entwicklung diagnostischer Tests bietet sich Urin als Materialquelle zur Identifikation von sensitiven und spezifischen Abstoßungsmarkern an. Urin kann in ausreichenden Mengen und nicht-invasiv gewonnen werden. Die anatomische Nähe zu den Nieren erhöht die Wahrscheinlichkeit, dass während einer Abstoßungsreaktion Veränderungen in seiner Zusammensetzung gefunden werden können. Unsere Arbeitsgruppe konnte bereits zeigen, dass die Hochregulation der mRNA von Granulysin und IP-10 im Urinsediment prädiktiv für akute Abstoßungen ist. Fortschritte im Bereich der Proteomics erlauben heute die hochauflösende Auftrennung hochkomplexer Proteingemische und die Erfassung von dynamischen Veränderungen der Proteinexpression. In der vorliegenden Studie nutzten wir 2-D Gelelektrophorese in Kombination mit Massenspektrometrie zur Identifikation der im Urin gelösten Proteine und erfassten die Veränderung ihrer Expression während einer Abstoßungsreaktion gegen das Transplantat. Dabei wurden 178 einzelne Proteine identifiziert, 17 von ihnen wurden als "Hauptproteine" definiert. Es zeigte sich, dass u.a. Osteopontin, β_2-Mikroglobulin und Hsp71 während einer Abstoßungsepisode unterschiedlich zu stabilen Verläufen exprimiert wurden. Auch im freien Urin konnte mittels ELISA eine erhöhte Osteopontin Pro-

teinkonzentration bei Patienten mit Abstoßungsreaktionen festgestellt werden.

A Protein lists

Table 10: *Spot identifications in the proteome maps*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
Areas					
A	Alpha-2-HS-glycoprotein precursor	P02765	Plasma	39299.71	5.43
B	Alpha-1-antichymotrypsin precursor	P01011	Plasma	47620.54	5.33
	Kininogen-1 precursor	P01042	Plasma	71912.15	6.34
C	Alpha-1-antitrypsin precursor	P01009	Plasma	46707.02	5.37
	Kininogen-1 precursor	P01042	Plasma	71912.15	6.34
D	Alpha-1B-glycoprotein precursor	P04217	Plasma	54238.58	5.58
E	Vitamin D-binding protein precursor	P02774	Plasma	52929.03	5.4
F	Serum albumin	P02768	Plasma	69321.49	5.92
G	Serotransferrin	P02787	Plasma	76999.61	6.81
H	Alpha-1-acid glycoprotein 1 precursor	P02763	Plasma	23496.76	4.93
I	Leucine-rich alpha-2-glycoprotein precursor	P02750	Plasma	38154.11	6.45
J	Zinc-alpha-2-glycoprotein precursor	P25311	Plasma	33850.89	5.57
K	Complement factor B precursor	P00751	Plasma	85478.52	6.67
L	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
M	Prostaglandin-H2 D-isomerase precursor	P41222	Various	21015.34	7.66
N	Haptoglobin precursor [Contains: Haptoglobin alpha chain; Haptoglobin beta chain]	P00738	Plasma	45176.56	6.13
O	Ig gamma-2 chain C region	P01859	Plasma	35861.76	7.66
	Ig gamma-4 chain C region	P01861	Plasma	35917.86	7.18
P	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]	P02675	Plasma	55892.26	8.54
	Fibrinogen gamma chain precursor	P02679	Plasma	51478.87	5.37
Q	Ig kappa chain C region	P01834	Plasma	11601.67	5.58
	Ig kappa chain V-I region AG	P01593	Plasma	11984.88	5.67
	Ig kappa chain V-I region Kue	P01604	Plasma	12119.04	8.96
	Ig kappa chain V-I region Ni	P01613	Plasma	12238.01	5.25

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
	Ig kappa chain V-II region TEW	P01617	Plasma	12308.15	5.69
	Ig kappa chain V-II region MIL	P01616	Plasma	12048.05	9.39
	Ig kappa chain V-III region GOL	P04206	Plasma	11822.95	9.34
	Ig kappa chain V-III region NG9 precursor	P01621	Plasma	10722.28	6.29
	Ig kappa chain V-III region HIC precursor	P18136	Plasma	14062.05	7.74
	Ig kappa chain V-III region B6	P01619	Plasma	11627.77	9.34
	Ig kappa chain V-III region SIE	P01620	Plasma	11767.84	8.7
	Ig kappa chain V-III region Ti	P01622	Plasma	11780.9	8.72
	Ig kappa chain V-III region WOL	P01623	Plasma	11738.9	9.07
	Ig lambda chain C regions	P01842	Plasma	11229.53	6.92
	Ig lambda chain V-IV region Hil	P01717	Plasma	11509.6	6.04
Spots in 12% proteome map					
1	Basement membrane-specific heparan sulfate proteoglycan core protein precursor	P98160	Extracellular	468527.5	6.06
2	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
3	Cadherin-11 precursor	P55287	Cell membrane	87910.8	4.75
4	Complement C3 precursor [Contains: Complement C3 beta chain; Complement C3 alpha chain; C3a anaphyl	P01024	Plasma	187029.9	6.02
5	Kininogen-1 precursor	P01042	Plasma	71912.15	6.34
6	Nebulin	P20929	Cytoplasm	772727.3	9.1
7	Lumican precursor	P51884	Extracellular	38404.79	6.16
8	Complement component C9 precursor [Contains: Complement component C9a; Complement component C9b] -	P02748	Plasma	63132.7	5.43
9	Ig mu heavy chain disease protein	P04220	Plasma	43030.3	5.13
	Ig mu chain C region	P01871	Plasma	49525.62	6.35
10	Alpha-2-macroglobulin precursor	P01023	Plasma	163174.9	6

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
11	Alpha-amylase 1 precursor	P04745	Plasma	57730.91	6.47
	Alpha-amylase 2B precursor	P19961	Plasma	57673.01	6.64
	Pancreatic alpha-amylase precursor	P04746	Plasma	57670.02	6.6
12	Beta-2-glycoprotein 1 precursor	P02749	Plasma	38272.66	8.34
13	Lysozyme C precursor	P61626	Lysosome	16526.28	9.38
14	Arylsulfatase A precursor	P15289	Lysosome	53553.67	5.65
15	Phosphoglucomutase-1	P36871	Cytoplasm	61410.53	6.3
16	Antithrombin-III precursor	P01008	Plasma	52568.86	6.32
17	Angiotensinogen precursor	P01019	Plasma	53120.51	5.87
18	Fibrinogen gamma chain precursor	P02679	Plasma	51478.87	5.37
19	Monocyte differentiation antigen CD14 precursor	P08571	Cell membrane	40050.74	5.84
20	Nesprin-1	Q8NF91	Nucleus	1010412	5.38
21	Alpha-1-antitrypsin precursor	P01009	Plasma	46707.02	5.37
	Anthrax toxin receptor 1 precursor	Q9H6X2	Cell membrane	62749.04	7.53
22	Kelch-like ECH-associated protein 1	Q14145	Cytoplasm	69620.73	6
23	Plasma retinol-binding protein precursor	P02753	Plasma	22995.26	5.76
24	Alpha-1-acid glycoprotein 1 precursor	P02763	Secreted	23496.76	4.93
	DNA polymerase iota	Q9UNA4	Nucleus	80295.58	6.37
25	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Clusterin precursor	P10909	Plasma, extracellular	52461	5.89
	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Nesprin-2	Q8WXH0	Nucleus	795943.5	5.26
	Nidogen-1 precursor	P14543	Extracellular	136366.8	5.14
	Transformation/transcription domain-associated protein	Q9Y4A5	Nucleus	437318.2	8.49
26	Endonuclease domain-containing 1 protein precursor	O94919	Plasma	54981.26	5.55

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
27	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	Plasma	103261	6.51
28	Clusterin precursor	P10909	Plasma, extracellular	52461	5.89
29	Tropomyosin alpha-1 chain	P09493	Cytoplasm	32688.68	4.69
	Tropomyosin beta chain	P07951	Cytoplasm	32830.57	4.66
30	Clusterin precursor	P10909	Plasma, extracellular	52461	5.89
	Hemopexin precursor	P02790	Plasma	51643.27	6.55
	Zinc-alpha-2-glycoprotein precursor	P25311	Plasma	33850.89	5.57
31	Hemopexin precursor	P02790	Plasma	51643.27	6.55
32	Apolipoprotein A-IV precursor	P06727	Plasma	45371.47	5.28
33	Gelsolin precursor	P06396	Cytoplasm	85644.19	5.9
34	Myotubularin-related protein 7	Q9Y216	Cytoplasm	75758.35	5.94
35	Actin, alpha cardiac muscle 1	P68032	Cytoplasm	41991.88	5.23
	Actin, alpha skeletal muscle	P68133	Cytoplasm	42023.85	5.23
	Actin, aortic smooth muscle	P62736	Cytoplasm	41981.8	5.23
	Actin, cytoplasmic 1	P60709	Cytoplasm	41709.73	5.29
	Actin, cytoplasmic 2	P63261	Cytoplasm	41765.79	5.31
	Actin, gamma-enteric smooth muscle	P63267	Cytoplasm	41849.79	5.31
36	Spectrin beta chain, erythrocyte	P11277	Cytoplasm	246169.5	5.13
37	Clusterin precursor	P10909	Plasma, extracellular	52461	5.89
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
	Vesicular integral-membrane protein VIP36 precursor	Q12907	Endoplasmic reticulum	40203.1	6.46
38	Zinc-alpha-2-glycoprotein precursor	P25311	Plasma	33850.89	5.57
39	Cathepsin Z precursor	Q9UBR2	Lysosome	33846.22	6.7
40	Aminoacylase-1	Q03154	Cytoplasm	45855.99	5.77
41	Tripeptidyl-peptidase 1 precursor	O14773	Lysosome	61209.63	6.01

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
42	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
43	Septin-1	Q8WYJ6	Plasma	41944.24	5.56
44	Serum albumin precursor	P02768	Plasma	69321.49	5.92
45	Dual specificity protein phosphatase 3	P51452	Plasma	20465.31	7.66
46	Structural maintenance of chromosomes protein 1A	Q14683	Nucleus	143144.1	7.51
47	Protein disulfide-isomerase precursor	P07237	Endoplasmic reticulum	57080.67	4.76
48	Hemopexin precursor	P02790	Plasma	51643.27	6.55
49	N-acetylgalactosamine-6-sulfatase precursor	P34059	Lysosome	57989.08	6.25
50	Septin-1	Q8WYJ6	Plasma	41944.24	5.56
51	Endoplasmin precursor	P14625	Endoplasmic reticulum	92411.34	4.76
52	Angiopoietin-related protein 2 precursor	Q9UKU9	Plasma	57068.44	7.23
53	Lipocalin-1 precursor	P31025	Plasma	19237.81	5.39
54	Protein disulfide-isomerase precursor	P07237	Endoplasmic reticulum	57080.67	4.76
55	Carbonic anhydrase 1	P00915	Cytoplasm	28852.39	6.59
56	Heat shock cognate 71 kDa protein	P11142	Cytoplasm	70854.22	5.37
57	Carbonic anhydrase 1	P00915	Cytoplasm	28852.39	6.59
58	Hemopexin precursor	P02790	Plasma	51643.27	6.55
59	Carbonic anhydrase 1	P00915	Cytoplasm	28852.39	6.59
60	Neutrophil cytosol factor 1	P14598	Cytoplasm	44654.87	9.12
61	Vesicular integral-membrane protein VIP36 precursor	Q12907	Endoplasmic reticulum	40203.1	6.46
62	Complement factor B precursor	P00751	Plasma	85478.52	6.67
	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	Plasma	103261	6.51
63	Serum albumin precursor	P02768	Plasma	69321.49	5.92
64	Apolipoprotein A-IV precursor	P06727	Plasma	45371.47	5.28

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
65	Inter-alpha-trypsin inhibitor heavy chain H4 precursor	Q14624	Plasma	103261	6.51
66	Cathepsin D precursor	P07339	Lysosome	44523.62	6.1
67	Carbonic anhydrase 1	P00915	Cytoplasm	28852.39	6.59
68	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Immunoglobulin J chain	P01591	Plasma	15584.55	4.62
69	Apolipoprotein D precursor	P05090	Plasma	21261.76	5.06
	Lysosomal protective protein precursor	P10619	Lysosome	54431.12	6.16
70	Antithrombin-III precursor	P01008	Plasma	52568.86	6.32
71	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Connective tissue growth factor precursor	P29279	Plasma, extracellular	38064.85	8.43
	Lysozyme C precursor	P61626	Lysosome	16526.28	9.38
72	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
	Galectin-3-binding protein precursor	Q08380	Plasma, extracellular	65289.28	5.13
73	26S proteasome non-ATPase regulatory subunit 9	O00233	Cytoplasm	24638.47	6.46
74	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Lysozyme C precursor	P61626	Lysosome	16526.28	9.38
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
75	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
76	Programmed cell death 6-interacting protein	Q8WUM4	Cytoplasm	95963.12	6.13
77	Hemopexin precursor	P02790	Plasma	51643.27	6.55
	Vitelline membrane outer layer protein 1 homolog precursor	Q7Z5L0	Extracellular	21520.3	4.9
78	Dermatopontin precursor	Q07507	Extracellular	23988.8	4.7

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
79	Elongation factor 2	P13639	Cytoplasm	95276.95	6.41
80	Myosin light polypeptide 6	P60660	Cytoplasm	16919.13	4.56
	Zinc-alpha-2-glycoprotein precursor	P25311	Plasma	33850.89	5.57
81	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	P31930	Mitochondrion	52612.43	5.94
82	Elongation factor 1-gamma	P26641	Cytoplasm	50087.14	6.25
83	Transthyretin precursor	P02766	Plasma	15877.05	5.52
	Trem-like transcript 1 protein precursor	Q86YW5	Cell membrane	32657.9	5.7
84	Gamma-tubulin complex component 5	Q96RT8	Centrosome	118245.6	5.58
85	3-mercaptopyruvate sulfurtransferase	P25325	Cytoplasm	33157.64	6.13
86	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	Transthyretin precursor	P02766	Plasma	15877.05	5.52
87	Osteopontin precursor	P10451	Extracellular, plasma	35401.24	4.37
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
88	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
	Kininogen-1 precursor	P01042	Plasma	71912.15	6.34
	Osteopontin precursor	P10451	Extracellular, plasma	35401.24	4.37
89	Moesin	P26038	Cell membrane	67777.79	6.08
90	Ezrin	P15311	Cell membrane	69369.74	5.94
91	Ig kappa chain C region	P01834	Plasma	11601.67	5.58
	Immunoglobulin superfamily member 8 precursor	Q969P0	Cell membrane	64993.51	8.23
	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
92	Radixin	P35241	Cell membrane	68521.39	6.03

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
93	26S protease regulatory subunit 4	P62191	Cytoplasm	49153.69	5.87
94	Tryptophanyl-tRNA synthetase, cytoplasmic	P23381	Cytoplasm	53131.61	5.83
95	Ig kappa chain C region	P01834	Plasma	11601.67	5.58
	Kyphoscoliosis peptidase	Q8NBH2	Cytoplasm	63843.95	7.1
96	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
	Lysosomal alpha-glucosidase precursor	P10253	Lysosome	105270.8	5.62
97	Splicing factor U2AF 65 kDa subunit	P26368	Nucleus	53467.19	9.19
98	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
99	DNA replication licensing factor MCM4	P33991	Nucleus	96497.63	6.28
100	Plasma retinol-binding protein precursor	P02753	Plasma	22995.26	5.76
101	Crk-like protein	P46109	Cytoplasm	33755.92	6.26
102	Adenosylhomocysteinase	P23526	Cytoplasm	47685.2	5.92
103	Elongation factor 1-gamma	P26641	Cytoplasm	50087.14	6.25
104	Cystathionine beta-synthase	P35520	Cytoplasm	60548.16	6.2
105	AMBP protein precursor [Contains: Alpha-1-microglobulin]	P02760	Plasma	38973.98	5.95
106	WD repeat-containing protein 61	Q9GZS3		33559.77	5.16
107	Coagulation factor XII precursor	P00748	Plasma	67773.91	8.04
108	Sarcoma antigen 1	Q9NXZ1		99162.11	6.02
109	Asparagine synthetase [glutamine-hydrolyzing]	P08243	Plasma	64328.63	6.39
	Cystathionine beta-synthase	P35520	Cytoplasm	60548.16	6.2
110	Plasma retinol-binding protein precursor	P02753	Plasma	22995.26	5.76
111	Ig kappa chain C region	P01834	Plasma	11601.67	5.58
112	Kininogen-1 precursor	P01042	Plasma	71912.15	6.34
113	78 kDa glucose-regulated protein precursor	P11021	Endoplasmic reticulum	72288.43	5.07
114	Ubiquitin-conjugating enzyme E2 N	P61088	Cytoplasm	17126.97	6.13

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
115	Lithostathine 1 alpha precursor	P05451	Cytoplasm	18718.78	5.65
	Lithostathine 1 beta precursor	P48304	Cytoplasm	18652.67	5.67
	Secreted and transmembrane protein 1 precursor	Q8WVN6	Extracellular, cell membrane	27021.74	7
116	Glutathione S-transferase P	P09211	Plasma	23341.02	5.43
	Secreted and transmembrane protein 1 precursor	Q8WVN6	Extracellular, cell membrane	27021.74	7
117	Serine/threonine-protein kinase PAK 2	Q13177	Cytoplasm	58005.88	5.69
118	Histone H2A type 1-A	Q96QV6	Nucleus	14224.91	10.86
	Histone H2A type 1-E	P28001	Nucleus	14126.95	11.05
	Histone H2A type 1-H	Q96KK5	Nucleus	13897.83	10.88
	Histone H2A type 2-B	Q8IUE6	Nucleus	13986.84	10.88
	Histone H2A.x	P16104	Nucleus	15135.41	10.74
	Histone H2A.Z	P0C0S5	Nucleus	13544.55	10.58
119	Transthyretin precursor	P02766	Plasma	15877.05	5.52
120	Plasma retinol-binding protein precursor	P02753	Plasma	22995.26	5.76
121	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
	Uncharacterized protein C19orf10 precursor	Q969H8	Plasma	18783.31	6.2
122	Cytochrome b5	P00167	Endoplasmic reticulum	15320.51	4.88
123	Protein FAM3C precursor	Q92520	Plasma	24664.58	8.52
124	Dipeptidyl-peptidase 1 precursor	P53634	Lysosome	51808.25	6.54
125	78 kDa glucose-regulated protein precursor	P11021	Endoplasmic reticulum	72288.43	5.07
126	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
127	Serum amyloid P-component precursor	P02743	Plasma	25371.13	6.1
128	78 kDa glucose-regulated protein precursor	P11021	Endoplasmic reticulum	72288.43	5.07
129	Glutathione peroxidase 3 precursor	P22352	Plasma	25488.96	8.2

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
	Glutathione S-transferase P	P09211	Plasma	23341.02	5.43
130	Peroxiredoxin-2	P32119	Cytoplasm	21878.24	5.66
Spots in 18% proteome map					
131	Clusterin precursor	P10909	Plasma, extracellular	52461	5.89
	Hemopexin precursor	P02790	Plasma	51643.27	6.55
	Nidogen-1 precursor	P14543	Extracellular	136366.8	5.14
132	Hemopexin precursor	P02790	Plasma	51643.27	6.55
133	Apolipoprotein E precursor	P02649	Plasma	36131.75	5.65
134	Napsin-A precursor	O96009	Cytoplasm, extracellular	45357.78	6.15
135	Cathepsin D	P07339	Lysosome	44523.62	6.1
136	Keratin, type II cytoskeletal 1	P04264	Extracellular	65977.98	8.16
137	Serum albumin precursor	P02768	Plasma	69321.49	5.92
138	Serum albumin precursor	P02768	Plasma	69321.49	5.92
139	Serotransferrin	P02787	Plasma	76999.61	6.81
140	Ester hydrolase C11orf54	Q9H0W9	Nucleus	35094.61	6.23
141	Retinol-binding protein 4	P02753	Plasma	22995.26	5.76
142	Hemopexin	P02790	Plasma	51643.27	6.55
143	Alpha-1-antitrypsin	P01009	Plasma	46707.02	5.37
144	Complement factor B	P00751	Plasma	85478.52	6.67
145	Ig gamma-1 chain C region	P01857	Plasma	36083.17	8.46
	Ig gamma-2 chain C region	P01859	Plasma	35861.76	7.66
	Ig gamma-3 chain C region	P01860	Plasma	32309.82	7.89
146	Complement C4-A precursor	P0C0L4	Plasma	192650.5	6.65
	Complement C4-B precursor	P0C0L5	Plasma	192672.5	6.73
	T-complex protein 1 subunit theta	P50990	Cytoplasm	59582.5	5.42
147	Immunoglobulin J chain	P01591	Plasma	15584.55	4.62
148	Dermatopontin precursor	Q07507	Extracellular	23988.8	4.7
149	Dermatopontin precursor	Q07507	Extracellular	23988.8	4.7

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
	Mannan-binding lectin serine protease 2 precursor	O00187	Plasma	75684.64	5.47
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
150	Ganglioside GM2 activator	P17900	Lysosome	20824.73	5.17
151	CD59 glycoprotein	P13987	Cell membrane	14167.79	6.02
152	CD59 glycoprotein precursor	P13987	Cell membrane	14167.79	6.02
153	Lithostathine-1-alpha	P05451		18718.78	5.65
154	AMBP protein	P02760	Plasma	38973.98	5.95
	Secreted and transmembrane protein 1	Q8WVN6	Extracellular, cell membrane	27021.74	7
155	Retinol-binding protein 4	P02753	Plasma	22995.26	5.76
156	Mannan-binding lectin serine protease 2	O00187	Plasma	75684.64	5.47
157	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
	Lithostathine 1 beta precursor	P48304		18652.67	5.67
158	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
159	Lipocalin-1 precursor	P31025	Plasma	19237.81	5.39
160	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
	Lithostathine 1 beta precursor	P48304		18652.67	5.67
161	Transthyretin	P02766	Plasma	15877.05	5.52
162	Lithostathine 1 alpha precursor	P05451		18718.78	5.65
	Lithostathine 1 beta precursor	P48304		18652.67	5.67
163	Secreted and transmembrane protein 1 precursor	Q8WVN6	Extracellular, cell membrane	27021.74	7
164	Transthyretin	P02766	Plasma	15877.05	5.52
165	AMBP protein precursor	P02760	Plasma	38973.98	5.95
166	Secreted and transmembrane protein 1	Q8WVN6	Extracellular, cell membrane	27021.74	7
167	Alpha-1-antitrypsin precursor	P01009	Plasma	46707.02	5.37

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
168	Haptoglobin	P00738	Plasma	45176.56	6.13
	Secreted and transmembrane protein 1	Q8WVN6	Extracellular, cell membrane	27021.74	7
169	Mannan-binding lectin serine protease 2	O00187	Plasma	75684.64	5.47
170	Peroxiredoxin-2	P32119	Cytoplasm	21878.24	5.66
171	Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	Extracellular	468527.5	6.06
172	Ig kappa chain C region	P01834	Plasma	11601.67	5.58
173	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
174	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Multimerin-2 precursor	Q9H8L6	Extracellular	104351.9	5.5
175	Prostaglandin-H2 D-isomerase precursor	P41222	Various	21015.34	7.66
176	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
177	Haptoglobin	P00738	Plasma	45176.56	6.13
	Haptoglobin-related protein	P00739	Plasma	38982.65	6.42
178	Fatty acid-binding protein. adipocyte	P15090	Cytoplasm	14709.5	6.59
179	Serum albumin precursor	P02768	Plasma	69321.49	5.92
180	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
	Hemoglobin subunit delta	P02042	Cytoplasm	16045.29	7.85
181	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
	Hemoglobin subunit delta	P02042	Cytoplasm	16045.29	7.85
182	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
	Hemoglobin subunit delta	P02042	Cytoplasm	16045.29	7.85
183	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Lipocalin-1 precursor	P31025	Plasma	19237.81	5.39

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
	Putative lipocalin 1-like protein 1 precursor	Q5VSP4	Plasma, extracellular	17907	4.93
184	Keratin, type I cytoskeletal 10	P13645	Extracellular	59474.91	5.13
	Keratin, type II cytoskeletal 1	P04264	Extracellular	65977.98	8.16
185	Protein FAM3C precursor	Q92520	Plasma, extracellular	24664.58	8.52
	Serum albumin precursor	P02768	Plasma	69321.49	5.92
186	Serum albumin precursor	P02768	Plasma	69321.49	5.92
187	Serum albumin precursor	P02768	Plasma	69321.49	5.92
188	Cystatin-M	Q9H112	Extracellular, Plasma	16500.35	8.31
189	Cystatin-M	Q9H112	Extracellular, Plasma	16500.35	8.31
190	Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3]	P61769	Plasma	13705.91	6.06
191	Alpha-1-antitrypsin	P01009	Plasma	46707.02	5.37
192	Cystatin-M	Q9H112	Extracellular, Plasma	16500.35	8.31
193	Ubiquitin	P62988	Cytoplasm	8559.62	6.56
194	Keratin, type II cytoskeletal 1	P04264	Extracellular	65977.98	8.16
	Keratin, type II cytoskeletal 2 epidermal	P35908	Extracellular	65825.39	8.07
195	Ubiquitin	P62988	Cytoplasm	8559.62	6.56
196	Meprin A subunit alpha precursor	Q16819	Cell membrane	84314.31	5.42
197	Keratin, type II cytoskeletal 2 epidermal	P35908	Extracellular	65825.39	8.07
198	Hemoglobin subunit alpha	P69905	Cytoplasm	15247.93	8.72
	Hemoglobin subunit beta	P68871	Cytoplasm	15988.29	6.75
199	Ig gamma-3 chain C region	P01860	Plasma	32309.82	7.89
200	Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	P02671	Plasma	94914.41	5.7
	Ig gamma-1 chain C region	P01857	Plasma	36083.17	8.46
	Ig gamma-2 chain C region	P01859	Plasma	35861.76	7.66

Table 11: *Keratin types identified and taken out of the list*

Protein Name	Accession Number	Protein MW	Protein PI
Keratin type II cuticular Hb4	Q9NSB2	64854.55	8
Keratin, type I cytoskeletal 10	P13645	59474.91	5.13
Keratin, type I cytoskeletal 9	P35527	62091.91	5.19
Keratin, type II cytoskeletal 1	P04264	65977.98	8.16
Keratin, type II cytoskeletal 2 epidermal	P35908	65825.39	8.07
Keratin, type II cytoskeletal 2 oral	Q01546	65830.1	8.38
Keratin, type II cytoskeletal 6A	P02538	60008.27	8.09
Keratin, type II cytoskeletal 6B	P04259	59962.25	8.09
Keratin, type II cytoskeletal 6C	P48668	59988.3	8.09
Keratin, type II cytoskeletal 8	P05787	53671.13	5.52

Table 10: *(continued)*

Label	Protein Name	Accession Number	Cellular Designation	MW	pI
	Ig gamma-3 chain C region	P01860	Plasma	32309.82	7.89
201	SH3 domain-binding glutamic acid-rich-like protein	Q9H299	Cytoplasm	12766.38	5.22
202	Haptoglobin	P00738	Plasma	45176.56	6.13
203	Mannan-binding lectin serine protease 2	O00187	Plasma	75684.64	5.47
204	Mannan-binding lectin serine protease 2	O00187	Plasma	75684.64	5.47
205	Ig gamma-1 chain C region	P01857	Plasma	36083.17	8.46
	Ig gamma-2 chain C region	P01859	Plasma	35861.76	7.66
	Ig gamma-3 chain C region	P01860	Plasma	32309.82	7.89
206	Filamin-C	Q14315	Cytoplasm, cell membrane	290777.6	5.68
207	SH3 domain-binding glutamic acid-rich-like protein 3	Q9H300	Cytoplasm	10431.26	4.82
208	Guanylin	Q02747	Plasma	12440.24	4.56
209	Apolipoprotein C-III precursor	P02656	Plasma	10845.5	5.23

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Publikationen und Präsentationen

Stauch D, Solmaz M, Melloh GC, **Füldner A**, Yayahzadeh A, Schönemann C, Baron U, Olek S, Pratschke J, and Kotsch K
In vivo induction of CD4+CD25+FOXP3+ regulatory T cells following induction therapy with polyclonal antithymocyte globulin is reflected at the epigenetic level
Paper, eingereicht

Kotsch K, **Füldner A**, Melloh GC, Stauch D, Pascher A, Volk H, Neuhaus P and Pratschke J
In vivo expansion of CD4+CD25+ regulatory T cells after Thymoglobulin induction therapy
American Transplant Congress, Toronto, Canada, 2008

Kotsch K, **Füldner A**, Melloh GC, Stauch D, Pascher A, Volk HD, Neuhaus P and Pratschke J
In vivo expansion of CD4+CD25+FOXP3+ regulatory T cells after ATG induction therapy
Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie (DGTI), Düsseldorf, Germany, 2008

Kotsch K, Stauch D, **Füldner A**, Melloh GC, Yayahzadeh A, Volk HD, Pascher A, Neuhaus P and Pratschke J
In vivo expansion of CD4+CD25+FOXP3+ regulatory T cells after ATG induction therapy
Deutsche TransplantationsGesellschaft (DTG), Bochum, Germany, 2008

Erklärung

"Ich, Arne Füldner, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: *The urinary proteome of kidney transplant patients: A new approach for the identification of non-invasive rejection markers* 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."

Datum, Unterschrift

Danksagung

Ich möchte allen, die mir bei der Erstellung dieser Arbeit geholfen und den Rücken gestärkt haben, meinen tiefsten Dank aussprechen.

Arne