Proteomic-based approaches to identify urine proteins associated with acute renal allograft rejection

by

Dr. Stefan Schaub, MD

A Thesis submitted to
The Faculty of Graduate Studies
In Partial Fulfilment of the Requirements for the Degree of:

Master's of Science

Department of Immunology
University of Manitoba
Winnipeg, Manitoba, Canada
June 2004

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Proteomic-based approaches to identify urine proteins associated with acute renal allograft rejection

BY

Dr. Stefan Schaub, MD

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

Contents

i) Acknowledgements

Everything starts with a dream…

When my wife and I decided to live two years in a foreign country, we agreed on Canada or Australia. Fortunately, my mentor in Switzerland (Jürg Steiger) knew Peter Nickerson from their collaboration in Boston. When I met Peter for the first time in Switzerland, I has captured by his research project about non-invasive diagnostics for renal allograft rejection. However, I did not really understand everything he told me, but it seemed to be very interesting. In addition, the research was conducted in Canada, which was one of our favourite countries.

Some months later my wife, our son Tim and I started our trip to Winnipeg, kindly supported by grants from the Swiss National Foundation and the Novartis Stiftung. The night before our departure I was dreaming about the goals, I would like to achieve within the two years in Winnipeg. First of all, I wanted to see Canada and learn proper English. Second, I wanted to make two publications in good journals. Third, I wanted to bring my body back into good shape. And forth, my wife and I wanted to have a Canadian child. But these were all just dreams…

With a delay of one and a half day we finally arrived in Winnipeg, happy but exhausted. The friendly and warm welcome we received from family Nickerson (Vivienne, Laura, Emily, Graeme and Peter) helped us getting started in our new life in Canada. Soon the research project caught my attention. The first part – reviewing patient charts, setting up a database – was not something new. But then I encountered for the first time pipettes and a mass spectrometer in the laboratory. All these new things were really exiting, and I tried to learn as much as possible from all the people I met. John Wilkins,

Tracey Weiler, Kevin Sangster, Mihaela Antunovici, Sandra Sousa, Patty Sauder, Kumar Dasuri and Oleg Krokhin were all very helpful answering my endless questions and they instructed me how to use all the proteomic tools. It didn't take long to feel at home in the research group, because the two α-women (Tracey Weiler and Sheryl Hagenstein) created an environment that was familiar to me from living with my wife and working with nurses ('women rule'). Numerous discussions with Peter Nickerson and David Rush gave me a much more sophisticated view of transplant immunology than I had before. I understand now how a small transplant centre like Winnipeg could become a leading group in the world: you just need the right people with critical thinking and a vision. Peter Nickerson not only introduced me to science, but also to medical politics, economics and laboratory management ('the hedgehog concept'). In addition, through the connections of the Winnipeg Transplant group I had the pleasure to meet many famous researchers, which I only knew by name before. After 16 months of work, I could harvest the fruits of the research with two publications in Kidney International and the Journal of the American Society of Nephrology. The first dream became true.

The most exciting thing in Canada is definitively its landscapes and its nature. We could enjoy the beauty of Canada on our holidays to Quebec, la Gaspesie, Montreal, the Algonquin National Park, Calgary, the Banff National Park, Lake Louise and Mont Tremblant. We hope that Canadians take good care of their nature, because it is of priceless value!! Due to travelling and several presentations my English also improved steadily. I am sure that I will never ask Sandra Sousa again to help me capping and uncapping storage tubes the way I did ('do you like to screw?'). Another dream turned into reality.

The first 16 months, I focused basically on research, which let my body become weak. With the help of my wife and the new MP3-player I started to exercise again and reduced my food intake. After five months my body weight and muscles were almost back at the level I had in my early twenties, when I was a dedicated soccer player. The third dream became true.

The most exciting event was the birth of Noé on December 26th, 2004. He is the only member of our family with a 'Canadian passport', which fulfilled my last dream.

All the mentioned people made our stay here in Winnipeg an exciting memory. In addition, special thanks go to Iga and Tom Dembinski for giving me rides from home to the Health Science Centre. But more importantly for the numerous serious and not-so-serious conversations we had while finding the fastest way through the traffic. In the end, I noticed that dreams can become true if you really try to realize them. In August 2004 the four of us will return to Switzerland...

And I am already dreaming about the things to come...

ii) Abbreviations

ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
ATN	Acute tubular necrosis
BK-NP	Polyomavirus type BK-nephropathy
CAN	Chronic allograft nephropathy
CHCA	α-cyano-4-hydroxycinnamic acid
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
CTL	Cytotoxic T-lymphocyte
DGF	Delayed graft function
GN	Glomerulonephritis
ICAT	Isotope coded affinity tag
IP-10	Interferon gamma induced protein of 10kDa
I-TAC	Interferon gamma inducible T-cell chemotactant
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
MAC	Membrane attack complex
MALDI-TOF-MS	Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry
MHC	Major histocompatibility complex
Mig	Monokine induced by interferon gamma

MS/MS	Tandem mass spectrometry
PN	Pyelonephritis
PSA	Prostate specific antigen
RP-HPLC	Reverse-phase high-pressure liquid chromatography
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass-spectrometry
SPA	Sinapinic acid
TFA	Trifluoroacetic acid
UTI	Urinary tract infection

Chapter 1

Abstract

Statement of the problem: Although short-term graft survival in renal transplantation has improved substantially and the incidence of acute clinical rejection episodes decreased significantly, the long-term graft survival has not. At present, the diagnosis of acute rejection can only be made by a renal allograft biopsy, which is expensive, inconvenient, has some associated morbidity and can therefore not be performed on a regular basis (e.g. weekly). Non-invasive immune monitoring of the rejection process would benefit patients by allowing frequent measurements to adjust immunosuppressive drugs to the needs of individual patients. The aim of this project is to test the hypothesis whether proteomic technology can identify candidate urine proteins associated with renal allograft rejection.

Methods: For this purpose we used a surface-enhanced laser desorption/ionization time-of-flight mass spectrometer (SELDI-TOF-MS) to profile urine samples from rigidly defined patient groups based on allograft function, clinical course, and allograft biopsy results.

Results: We found that the SELDI-TOF-MS is a high throughput technology with a good reproducibility, however, standardization of analysis conditions is essential, and both extrinsic (e.g. urine storage) and intrinsic factors (e.g. blood in urine, first-void vs. mid-stream urine, urine dilution) must be taken into account for accurate data interpretation. Urine protein profiling of clinical samples showed three prominent peak clusters in 94% of patients with acute rejection episodes, but only in 18% of patients without clinical and histological evidence for rejection and in none of the normal controls. In addition, the presence or absence of these peak clusters correlated with the clinico-pathological course in most patients. Identification by mass spectrometry revealed that all peaks could be assigned to a non-tryptic cleaved form of β2-microglobulin with very high confidence.

Conclusions: The presence of cleaved β2-microglobulin in urine is most likely to be associated with tubular epithelial cell stress/injury induced by allograft rejection. Further research efforts will concentrate on the development of an ELISA to reliably measure cleaved β2-microglobulin as well as the determination and enzymatic measurement of the involved proteinase(s). After validation, such assays may prove to be useful for non-invasive monitoring of tubulointerstitial renal allograft rejection.

Chapter 2

Introduction

2.1) Significance of rejection episodes and over-immunosuppression for long-term allograft and patient survival

2.2) Immunobiology of renal allograft rejection

2.3) Diagnosis of renal allograft rejection

- 2.3.1) Current clinical practice
- 2.3.2) Non-invasive immune monitoring post-transplant

2.4) Development of new non-invasive biomarkers for allograft rejection

- 2.4.1) Definition and general purpose of biomarkers
- 2.4.2) Proteomics versus genomics: advantages and disadvantages
- 2.4.3) Properties and limitations of proteomic technology

2.5) Pathophysiology of proteinuria

2.1) Significance of rejection episodes and over-immunosuppression for long-term allograft and patient survival

Although short and long-term kidney allograft survival has improved substantially from 1988-1996 (1), this trend did not continue from 1995-2000 (2). Specifically, despite a continuous decrease in reported acute clinical rejection rates within the 1st year post-transplant in the latter period, death-censored allograft survival even diminished (2). This was attributed to "a higher proportion of acute rejection episodes which have not resolved with full functional recovery in recent years" (2), but it may also be due to undetected - and therefore not treated - rejection episodes (i.e. subclinical rejection) which harm the allograft over time.

Both immunological and non-immunological (e.g. calcineurin-inhibitor (CNI)-toxicity, hypertension, recurrent disease) factors contribute to a continuous deterioration of allograft function, which is referred to as chronic allograft nephropathy (CAN) (3). Acute allograft rejection is the major immunological risk factor for developing CAN (4,5). However, as stated above there remains a consistent rate of late graft loss due to CAN with or without previous acute clinical rejection episodes suggesting the existence of subtle and 'subclinical' degrees of graft inflammation that are capable of progressing to CAN. Indeed while non-immunological factors may play a role, a recent analysis found that immunological factors were strong correlates of declining graft function beyond 6 months (6).

Currently about 50% of renal allografts are lost due to patient death with a functioning graft. These patients died mainly from cardiovascular diseases and malignancies (7,8). Indeed, the incidence of many cancers is increased compared to age-

matched general population, ranging from 2-fold for colon cancer to 20-fold for non-Hodgkin's lymphoma and 90-fold for non-melanoma skin cancers (9). Furthermore, the risk for developing a fatal or non-fatal cardiovascular event is 2-fold increased compared to the age- and cardiovascular risk factors-matched general population (10). These findings may be explained by more potent immunosuppressive regimens used to date, which increase cardiovascular risk factors (e.g. hypertension, hypercholesterolemia) and malignancy development. In addition, new emerging opportunistic viral infections such as polyomavirus BK-type nephropathy (11,12) underscore the observation that over-immunosuppression may have increased in current years. With this concern in mind, there has been a recent interest in the implementation of strategies that reduce the net immunosuppression delivered to the patient by avoidance, minimization, withdrawal or substitution drug protocols (13). The problem with such strategies, however, is that there has been to date no way other than a renal allograft biopsy of ascertaining whether the graft is free of rejection, and several attempts at reducing immunosuppression have been followed by acute rejection episodes.

Therefore, the individualisation of the immunosuppressive therapy tailored to the needs of every patient at every time point is a major goal. To achieve this, tools to monitor the rejection process in the allograft are mandatory. However, this is complicated by the complex and often redundant biology of allograft rejection.

2.2) Immunobiology of renal allograft rejection

Rejection of an allograft is mainly driven by differences in the major histocompatibility complex (MHC), but also by differences in 'minor' antigens (e.g. male H-Y antigen, endothelial antigens) (14). Therefore only identical twins share all antigens, whereas MHC-identical persons may still have differences in minor antigens.

From numerous experiments and *in vivo* observations the immune response to the kidney allograft can be divided into five phases (**Figure 1**). The first phase consists of immediate activation of the innate immune system upon reperfusion of blood through ischemic tissue. This leads to infiltration of inflammatory cells (e.g. neutrophils, macrophages), activation and deposition of complement and platelets, upregulation of adhesion-, MHC- and costimulatory molecules on allograft cells and particularly tissue residing donor antigen-presenting cells, as well as cytokine/chemokine release from various cells (15). Subsequently, these changes may trigger activation of the adaptive immune system. In cadaveric donors, brain death with its associated disturbances in the circulatory, endocrine and immune system precedes these events and cause injury to the graft even earlier (16). This may explain the increased incidence of delayed graft function (DGF), early acute rejection rates and inferior graft survival rates of allografts from cadaveric donors compared to living donors (1).

The second phase consists of antigen encounter and recognition, where recipient T-cells recognize allo-antigens presented either by donor-APC (direct allorecognition) or by recipient-APC (indirect allorecognition) (14). It has been shown that about 10% of T-cells are reactive to allo-MHC-molecules, which underscores that the donor-MHC molecule itself rather than the presented peptide on the donor-APC is responsible for the

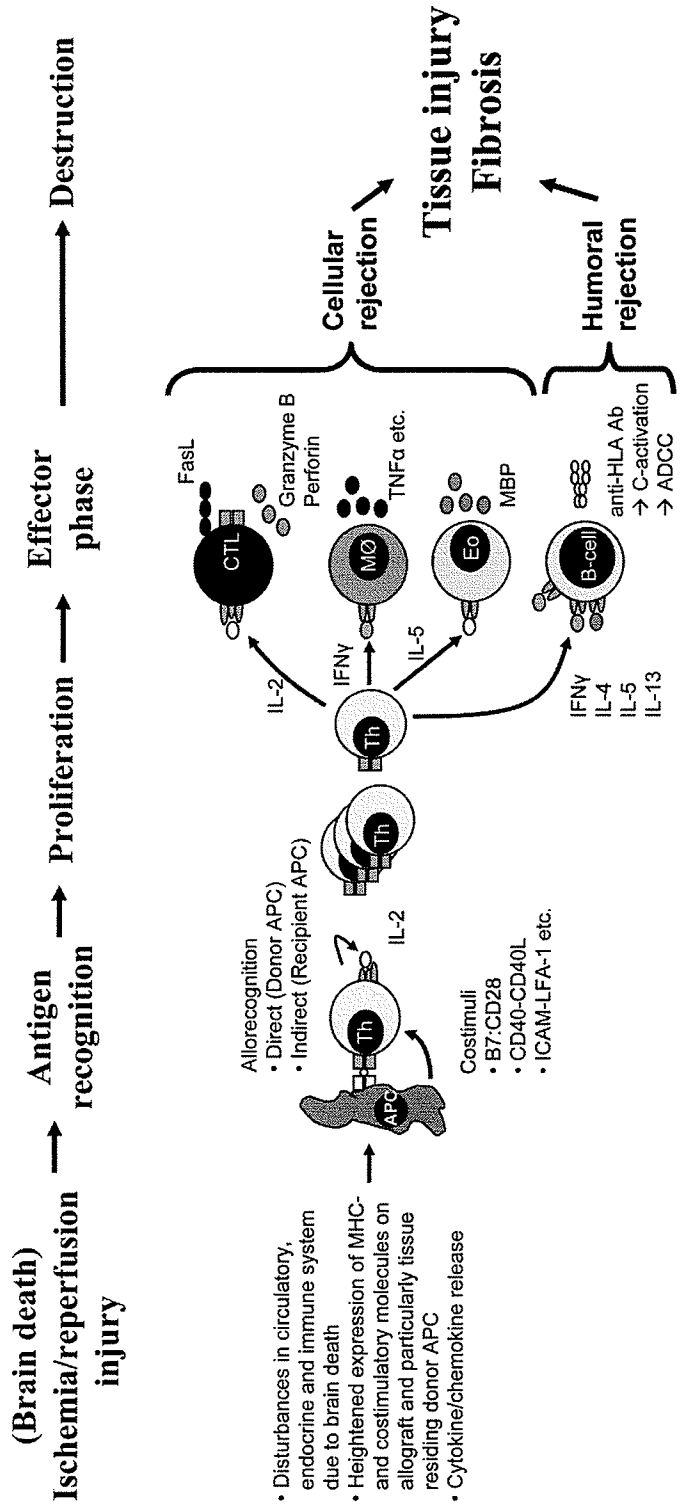


Figure 1. Immunobiology of allograft rejection. The process can be divided into five phases, [I] brain death and ischemia/reperfusion injury, [II] antigen recognition, [III] proliferation of activated T-cells, [IV] effector phase with the different pathways of humoral and cellular rejection, and [V] tissue injury.

activation of corresponding recipient T-cells. Direct allorecognition is thought to be mainly responsible for early rejection episodes, but later on donor-APC may become rare or even all undergo apoptosis. In contrast, indirect allorecognition requires uptake, processing and presentation of donor-derived antigens by recipient-APC, which needs a longer period to start up (14). Therefore, indirect allorecognition may largely be accountable to later rejection episodes and continues to happen over the lifetime of an allograft.

The third phase involves the differentiation and proliferation of antigen-specific $CD4^+$ and $CD8^+$ T-cells. The local microenvironment, the APC and their presented antigens can influence the differentiation into Th1- and Th2-cells. Some earlier studies suggested that a Th1-response is associated with rejection, whereas a Th2-response is protective and favours tolerance (17,18). But even 'pure' Th2-responses with eosinophils as their major effector cell can lead to allograft rejection (19,20,21,22,23,24,25,26). Most often there is a mixture of Th1 and Th2 cells and their relative amount may determine through a complex and redundant signalling process (e.g. INF-γ, IL-4, IL-5, IL-13), which effector cells are stimulated and activated. Both antigen encounter/recognition and differentiation/proliferation of T-cells happen in secondary lymphoid organs (i.e. lymph nodes, spleen) and are necessary for the adaptive immune response. This is supported by an experiment in mice, where vascularized transplants were accepted indefinitely in the absence of secondary lymphoid organs (27). The responsible mechanism was shown to be immunologic ignorance and not tolerance.

The fourth or effector phase can be divided into cellular and humoral rejection responses (28). The cellular rejection involves mainly cytotoxic $CD8^+$ T-cells (CTL),

macrophages and $CD4^+$ T-cells. CTL can kill target cells through Fas-FasL interaction or through secretion of perforin and granzyme B, macrophages through secretion TNF-α and $CD4^+$ T-cells through Fas-FasL interaction. Even eosinophils are able to injure and kill graft cells through release of toxic molecules (e.g. major basic protein). There is some evidence that the presence of many eosinophils in the graft is associated with a poor outcome (22,23,29,30). Whether the increased presence of eosinophils in the graft itself is responsible for the poor outcome or whether they are only an indicator of a stronger activation of the whole effector phase is unknown. Cellular rejection (i.e. CTL, macrophages, $CD4^+$ T-cells) is histologically characterized by an interstitial infiltrate with tubulitis, but it can also lead to arteritis and glomerulitis, which both have worse prognosis than tubulointerstitial rejection (31,32) (**Figure 2**). Humoral rejection consists of plasma cell-derived donor-specific antibodies against MHC- or other antigens (e.g. endothelial antigens). The antibodies bind almost exclusively to antigens on endothelial cells of glomeruli and peritubular capillaries. After binding their targets, the antibodies can either activate the complement system or they can bind FcγR-bearing cells (e.g. neutrophils and macrophages) (14). Several mechanisms exist to protect the endothelial cells from being damaged after binding of the antibodies. The antibody-antigen complex can be removed from the surface by shedding or internalising. In addition, bound complement can be inactivated by CD59, CD55, protein I and other complement regulatory proteins (33). Once activated, complement leads to necrosis of endothelial cells through the membrane-attack complex (MAC). Most complement proteins are rapidly cleared from the cell surface after completing their task, but covalently bound C4d is a remnant that is readily detectable by immunohistochemistry on allograft biopsies and is widely used to determine

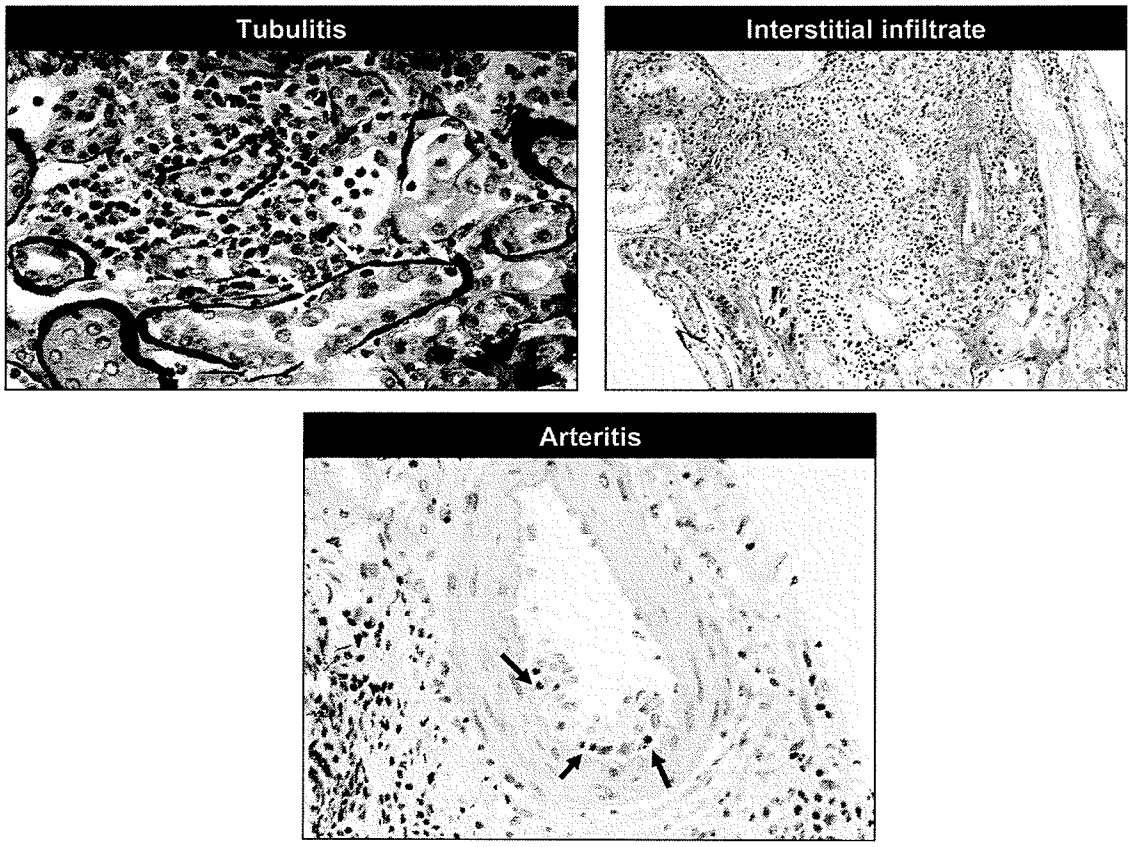


Figure 2. Histology of cellular renal allograft rejection. Cellular rejection is characterized by tubulitis, interstitial infiltrates and arteritis. T-cells pass through the basement membrane of tubuli and attack the tubular epithelial cells (white arrows in top left graphic). The interstitial compartment shows diffuse infiltrate consisting mainly of T-cells and macrophages (top right graphic). T-cells can also target arteries. After passing underneath the endothelial cells T-cells lead to inflammation of the arterial wall (black arrows in bottom graphic).

complement activation *in vivo*. Finally, necrosis of endothelial cells exposes the basement membrane, which activates the coagulation system and the platelets leading to intravascular microthrombi. Pure humoral rejection is almost exclusively seen in patients with circulating donor-specific antibodies at the time of transplantation and is mostly detected within the first 1-10 days after transplantation (personal observation by P.Nickerson, (34)). It is histologically characterized by the presence of neutrophils, macrophages and microthrombi in peritubular capillaries, glomeruli and rarely arteries, C4d deposits in peritubular capillaries (33,35,36) and circulating donor-specific antibodies (28) (**Figure 3**). Plasma cells are normally not abundant or absent in allograft biopsies, but their presence is associated with a poor outcome (37,38,39). However, whether the increased presence of plasma cells in the graft itself is responsible for the poor outcome or whether they are only an indicator of a stronger activation of the whole effector phase is unknown.

The fifth and last phase consist of tissue injury and destruction, which is histologically characterised by tubular atrophy, interstitial fibrosis, fibrous intimal thickening of arteries and glomerulosclerosis. Robertson et. al. demonstrated that intratubular T-cells (i.e. tubulitis) can directly induce adjacent tubular epithelial cells to transform into proliferating fibroblasts that migrate across the tubular basement membrane, producing fibrotic lesions within the renal interstitium (40). In analogy, similar processes lead to fibrous intimal thickening of arteries, which then can decrease perfusion of downstream tissue resulting in tubular atrophy and interstitial fibrosis (41). Finally, glomerulosclerosis follows as a consequence of extensive and permanent damage to the corresponding tubuli. Whereas the above described immunological processes may be responsible for most of the tissue damage post transplant, non-immunological processes

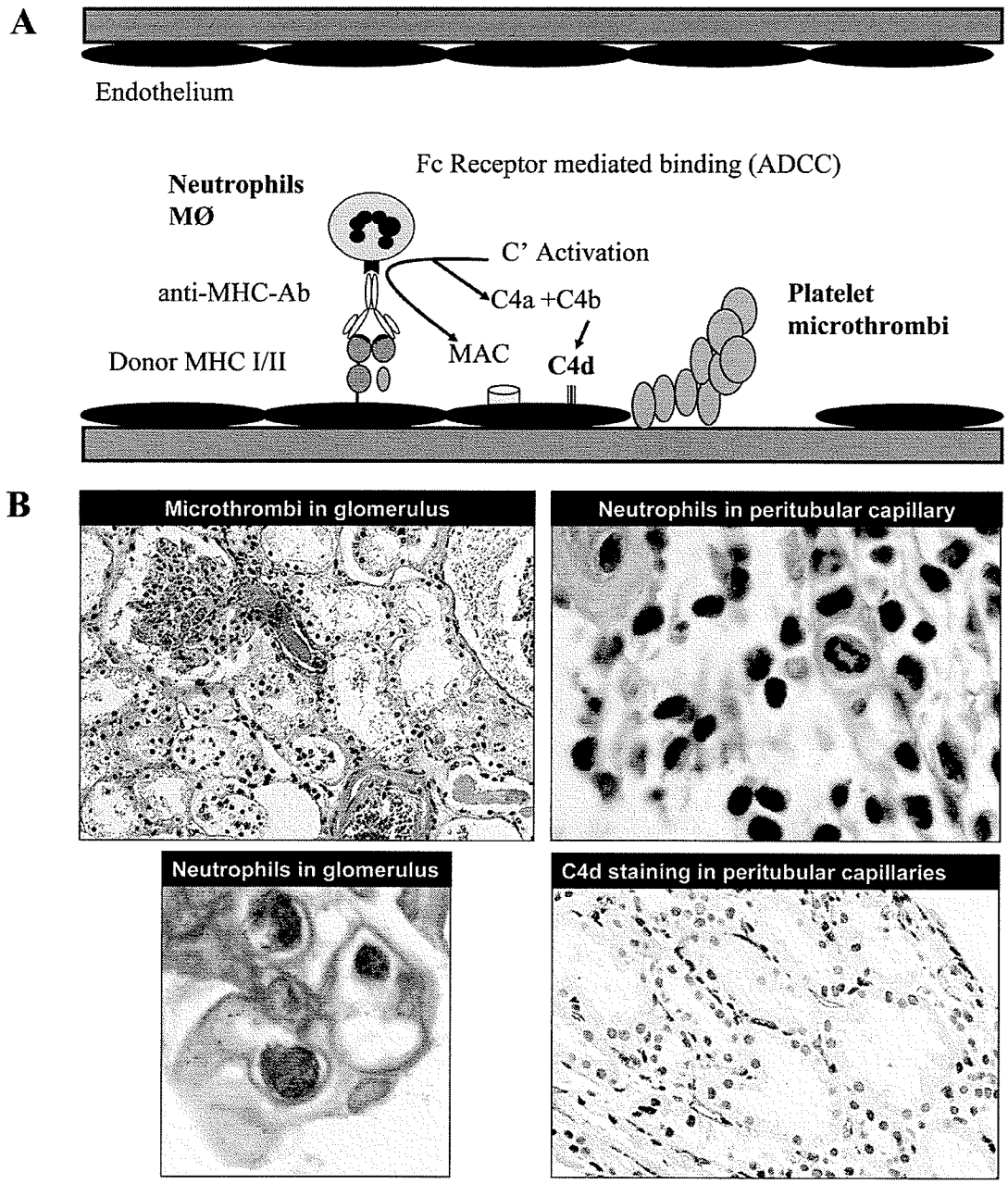


Figure 3. Pathogenesis and histology of humoral renal allograft rejection. A, Donor-directed antibodies bind their target antigens on endothelial cells. Subsequently, either complement is activated and/or neutrophils/macrophages bind through their FcγR. Both pathways lead to endothelial cell necrosis/apoptosis, exposing the basement membrane to the blood stream, which activates the coagulation cascade and causes platelet microthrombi formation. B, Histologically, humoral rejection is characterized by microthrombi and neutrophils/macrophages in small vessels (i.e. glomeruli and peritubular capillaries). In addition, immunohistochemistry shows C4d in small peritubular capillaries as proof of complement activation *in vivo*.

(e.g. hypertension, hypercholesterolemia, CNI-toxicity) lead to the same non-specific histological features, which are currently referred to as 'chronic allograft nephropathy' (3,28,31) (**Figure 4**). Therefore, the distinction of immunological from non-immunological damage is often not possible unless there are concomitant active cell infiltrates (i.e. tubulitis, arteritis) (42). A prospective protocol biopsy study found that by 10 years, severe chronic allograft nephropathy was present in 58% of patients, with sclerosis in 37% of glomeruli (43). Unfortunately, established tubulointerstitial and glomerular damage is irreversible, resulting in declining renal function and allograft failure. This underscores the need to prevent early tissue damage from both immunological and non-immunological processes.

2.3) Diagnosis of renal allograft rejection

2.3.1) Current clinical practice

At present, the diagnosis of acute rejection can only be made by renal allograft biopsy, which provides information about the type (humoral vs. cellular) and the severity of rejection (tubulointerstitial vs. vascular) that can be used to select the appropriate anti-rejection therapy. Sometimes it is 'practical' in a clinical setting to assume rejection by excluding other possibilities for graft dysfunction and to treat rejection. Nevertheless, most kidney transplant centres perform an allograft biopsy when rejection is a concern and allograft function (measured by serum creatinine) has deteriorated by more than 20-30% from baseline.

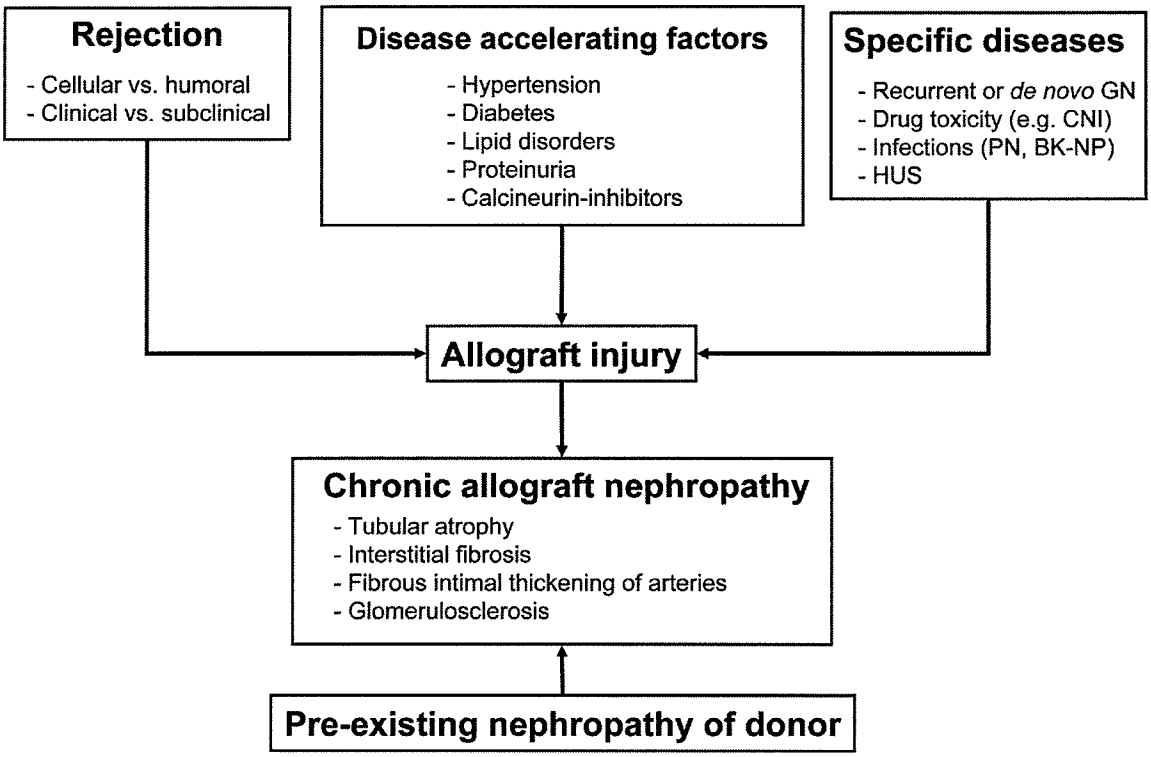


Figure 4. Pathogenesis of chronic allograft nephropathy. Immunologic factors (i.e. rejection) and non-immunological factors (i.e. disease accelerating factors and specific diseases) result in allograft injury. The response to injury is always the same which leads to non-specific changes histologically characterized by tubular atrophy, interstitial fibrosis, fibrous intimal thickening of arteries and glomerulosclerosis. Any pre-existing donor kidney pathology may show the same lesions. GN=glomerulonephritis, CNI=calcineurin-inhibitor, PN=pyelonephritis, BK-NP=polyoma BK-type nephropathy. Adapted from Halloran PF: Call for revolution: a new approach to describing allograft deterioration. *Am J Transplant* 2:195-200, 2002.

However, studies by the Winnipeg Transplant Group have demonstrated that the serum creatinine is an insensitive method for the early detection of renal allograft pathology. Indeed, the histologic criteria for acute rejection are present in 3-45% of protocol biopsies of renal allografts with stable function ('subclinical rejection') (43,44,45,46,47). The pathogenic potential of subclinical rejection was demonstrated in a randomized study in which the treatment of early subclinical rejection with corticosteroids improved both early and late outcomes (44). Specifically, there was a decrease in early (months 2-3) as well as late (months 7-12) clinical rejection episodes, a decrease in the chronic tubulointerstitial pathological score at 6 months, and a lower serum creatinine at 24 months in those patients randomized to treatment. Finally, similar to acute pathology, the Winnipeg Transplant Group reported that early chronic allograft pathology, detectable only by a 6-month protocol biopsy (i.e. graft function was stable), is predictive of both a subsequent decline in allograft function and time to graft failure (48,49). These data suggest that early detection and treatment of subclinical inflammation may be required to decrease the incidence of CAN.

2.3.2) Non-invasive immune monitoring post-transplant

With the advent of new immunosuppressive agents it is becoming apparent that a limitation to the 'gold standard' (i.e. renal biopsy) is the extent of heterogeneity of inflammation within the allograft resulting in sampling error (**Figure 5**) (50). To overcome this obstacle one could take additional cores, use larger biopsy needles or perform more frequent protocol biopsies. However, clearly this is restricted by patient risk for complications that limits the frequency with which they can be performed, not to

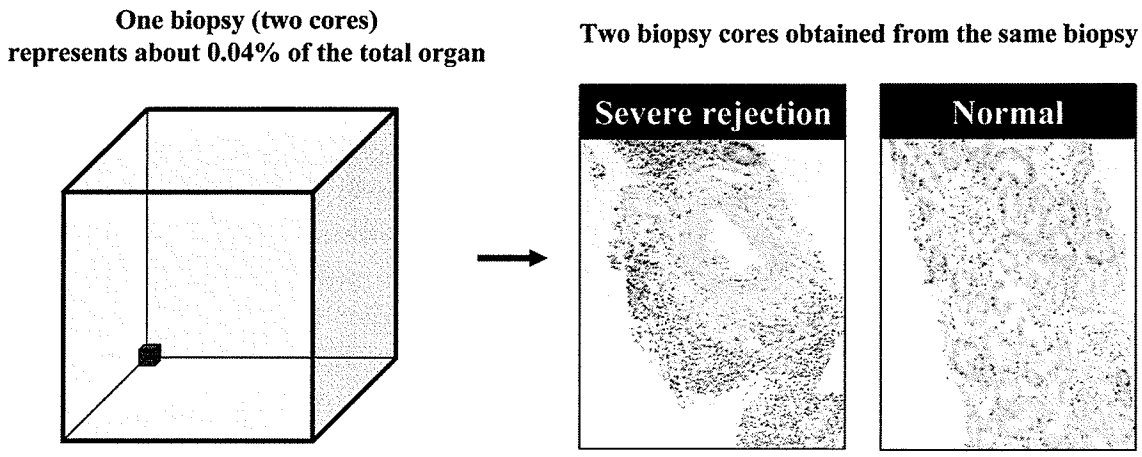


Figure 5. Sampling error of renal allograft biopsies. As one biopsy (two cores) represents only 0.04% of the whole allograft, sampling error can occur. In addition, early rejection may start as a patchy, focal process that spreads over the whole graft as severity increases. This may lead to a missed diagnosis of early rejection processes.

mention the associated cost. An alternative is to further increase the baseline immunosuppression for all patients, but this carries the known risks of infection in the short-term, and of drug toxicity and malignancy in the long-term. Therefore, in order to detect and eventually prevent these early pathogenic lesions, it is important to develop non-invasive approaches that sample the entire graft and can be performed repeatedly.

Non-invasive monitoring of the immune response directed at the kidney allograft is constrained to examine cells or proteins from the peripheral blood or urine. Strategies have broadly taken one of two approaches (51). The first takes advantage of donor-recipient MHC disparity, the central target of the alloimmune response, to design donor antigen specific assays. The second strategy is to assess global changes in immune system components of the recipient. As will be discussed below each approach offers distinct advantages and disadvantages. Independent of the strategy however, clinical utility will require that an assay be conducted easily with small volumes of blood or urine and be able to be repeated frequently.

Antigen specific assays: These approaches have largely employed donor cells as targets for either recipient T-cells or sera containing antibody targeting donor-MHC. To date the most successful by far has been the 'cross-match' assay examining pre-transplant sera for donor specific alloantibodies that target MHC molecules on the surface of donor T- or B-cells (52). In contrast to antibody assays, donor-specific T-cell assays have not proven to be as predictive (51). Tests have included limiting dilution assays (LDA), trans-vivo delayed type hypersensitivity (DTH) assays, enzyme-linked immunospot (ELISPOT) assay, flow cytometry based detection of cytokines, and tetramer staining. Like the antibody 'cross-match' assay, the LDA and ELISPOT assays have been successful in detecting pre-transplant donor-specific T-cell memory that predict risk for early acute

rejection (53). However, their utility to monitor for acute rejection post-transplant has been rather limited (54). While highly specific for donor antigens, the main disadvantages of these assays are [I] need for repository of donor cells (limits frequency of testing possible), [II] need for cell expansion (time consuming and labour intensive), [III] difficult reproducibility, [IV] complex interpretation, [V] low sensitivity, [VI] in the case of tetramers requires availability of a diverse panel with the number of potential donor-recipient disparities, and [VII] in the case of trans-vivo DTH the need for a large number of animals (51).

Antigen non-specific assays: To date antigen non-specific assay development (via immuno-phenotyping for immune cell activation markers, cytokine excretion, or mRNA analysis) has largely been limited to known inflammatory programs that are associated with clinical rejection (51,55,56,57,58,59,60,61). However, it is unclear whether these assays will reliably detect the more subtle (subclinical) forms of acute and/or chronic rejection. The Winnipeg Transplant group attempted to develop non-invasive markers correlating clinical and subclinical rejection with flow based detection of CD69 up-regulation on circulating T-cells (i.e. an early T-cell activation marker that we found in the biopsy infiltrate of acute clinical and subclinical rejection). In this study, CD69 expression tended to correlate with acute allograft inflammation, however, it was also up-regulated when even asymptomatic cytomegalovirus (CMV) viremia was present in the blood (62). This study highlighted the difficulty in using antigen non-specific biomarkers; specificity is difficult to ensure given that immune markers in blood can reflect inflammation generated through multiple pathways (i.e. rejection versus infection) occurring at multiple sites within the patient. In addition, T-cells in the circulation may not necessarily be representative of their abundance within the graft (63). The same problems apply also to

studies measuring serum proteins secreted by immune cells (e.g. IL-2, IL-6, INF-γ). Although statistically significant differences have been found in patients with or without acute rejection, the overlap of the two populations was often substantial (59,60) resulting in either many 'false positives' or many 'false negatives' for a selected cut-off (see also 2.4.1).

Urine as a specimen for immune monitoring offers some potential advantages compared to serum, because [I] it is in direct contact with the main target of rejection (tubular epithelial cells), [II] it may represent the whole kidney allograft, and [III] it may be less confounded by systemic inflammation. However, urine can be very heterogeneous concerning the amount of cells, the concentration of proteins and the pH. One group used mRNA measurement of granzyme B, perforin and CD103 in urinary lymphocytes to predict acute renal allograft rejection (57,58); others measured cytokines (60) or chemokines (61). Yet again, the major problem in these studies was the insufficient sensitivity and specificity, which limits the clinical usefulness of such assays. The unsatisfactory performances could partially be explained by the rather loose definition of 'no-rejection' in these studies, which was mostly based on stable allograft function without further support by allograft histology.

2.4) Development of new non-invasive biomarkers for allograft rejection

2.4.1) Definition and general purpose of biomarkers

'A biomarker may have various uses. An early intervention (or diagnostic) biomarker is used for early detection of disease to facilitate intervention. A prognostic biomarker is used to identify patients who may benefit from an intervention' (64). Ideally, a biomarker has both, diagnostic and prognostic properties, but this is rarely the case.

A diagnostic biomarker is described by its sensitivity, specificity and its receiver operating characteristics (ROC) curve. ROC-analysis allows finding the best cut-off value to assign the test result to be 'positive' or 'negative'. For clinical decision-making, it is more important to know the positive (PPV; 'true positives') and negative predictive value (NPV; 'true negatives') than its sensitivity and specificity. This calculation then allows determination of how many 'false positive' and 'false negative' results the test produces. These numbers should be as low as possible, because they represent the patients that are wrongly assigned to have either a 'positive' or a 'negative' test. Besides the -given and constant- sensitivity and the specificity of a diagnostic test, the prevalence of the target disease in the screened population largely influences the PPV, the NPV, the number of 'false positives' and the number of 'false negatives'. Therefore, these values should always be calculated based on the 'true prevalence' of the disease in the screened population rather than from a selected population, which may over- or underestimate the 'true prevalence' and consequently lead to wrongly calculated PPV and NPV (65).

The meaning of a prognostic biomarker can be confusing. Sometimes prognostic biomarkers just 'correlate' with a specific outcome, whereas ideally they should 'predict'

the outcome. Correlations denote a statistically significant relationship between two values (i.e. biomarker and outcome). Prediction requires the further criterion of showing that changes in the value have consequential changes in the outcome. Many prognostic biomarkers used to date only 'correlate' with an outcome (e.g. C-reactive protein and risk of acute myocardial infarction), fewer 'predict' (e.g. smoking and risk of lung cancer or acute myocardial infarction).

2.4.2) Proteomics versus genomics: advantages and disadvantages

The decoding of the human genome, developments in microtechnology, bioinformatics and mass spectrometry made it possible to investigate complex biological processes on a broad gene and protein level. Gene-microarrays (39,66) and MS-based proteomics (67,68) have gained widespread applications in biomedical research, including identification of candidate genes/proteins for diagnostic, prognostic and therapeutic purposes. However, both approaches have their limitations, which are mainly related to the technology itself (**Table 1**).

2.4.3) Properties and limitations of proteomic technology

At present, there are several techniques to identify and compare the expression of proteins, each with advantages and disadvantages. The most established method is protein separation by two-dimensional gel-electrophoresis (2-DE) followed by in-gel digestion and peptide mass fingerprinting by mass spectrometry. This method allows for the comparison of the relative abundance of proteins. However, there are several limitations

	Gene-microarrays	MS-based proteomics
Advantages	- Capturing of known mRNA molecules (identification is not a problem) - DNA and RNA are conservative - High sensitivity even in complex mixtures - Amplification possible	- Proteins are the final effector molecules - Proteins are the main target for therapeutics - Applicable to biological fluids, e.g. ascites and urine
Disadvantages	- Provides no information regarding protein expression levels - Reproducibility, especially for genes with low expression levels - Dependant on cells (difficult to apply to biological fluids with low cells counts, e.g. ascites and urine) - Larger sample amounts needed for analysis	- No amplification - Not quantitative - Comparison and identification is complicated by high dynamic range of proteins (mmol to fmol) and ion suppression - Reproducibility of high-throughput technologies (SELDI-TOF-MS, ICAT) - Proteins are not conservative (e.g. phosphorylation, glycosylation)
	Most human tissues are a mixture of different cells types. Changes in gene and protein expression patterns may just be related to different cell type composition of the tissue sample, rather then a 'real' up- or down- regulation. This bias can be overcome with cell type sampling by laser-capture-microdissection	

Table 1. Comparison of gene-mircoarrays and MS-based proteomics.

of 2-DE as a separation method for proteomic studies. The resolvable range of molecular weights is limited at both ends, with a bias toward high abundance proteins. In addition, the technique requires relatively large amount of sample, is labour-intensive, and good gel-to-gel reproducibility can be hard to achieve (69,70). Thus, this approach is not optimal for high-throughput profiling. An alternative approach uses one- or two-dimensional liquid chromatography as the separation step upstream from the mass spectrometer (LC-MS). While this technique provides information about the protein content of the samples, little information about their relative abundance can be obtained, unless the proteins/peptides are labelled first by isotope-coded affinity tags (71,72) or other protein/peptide labelling techniques (e.g. digestion with $H_2{}^{16}O$ and $H_2{}^{18}O$ mixture (73,74,75)). Furthermore, this method is still labour-intensive and has limited throughput. Surface-enhanced laser desorption/ionization time-of-flight mass-spectrometry (SELDI-TOF-MS) addresses some of the limitations of both 2-DE and LC-MS. It combines matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to surface chromatography. Specifically, a sample is applied to a chip surface carrying a functional group (e.g. hydrophobic, anion-exchange, cation-exchange, normal phase and metal-affinity). After incubation, proteins that do not bind to the surface are removed by a simple wash step, and bound proteins are analysed by mass spectrometry (**Figure 6**). This approach, in contrast to the others described, allows for high-throughput profiling of many clinical samples, but has limited sensitivity, resolution and mass accuracy (**Table 2**). Advantages and disadvantages of SELDI-TOF-MS for urine protein profiling are described in more details in the *results* and *discussion* section.

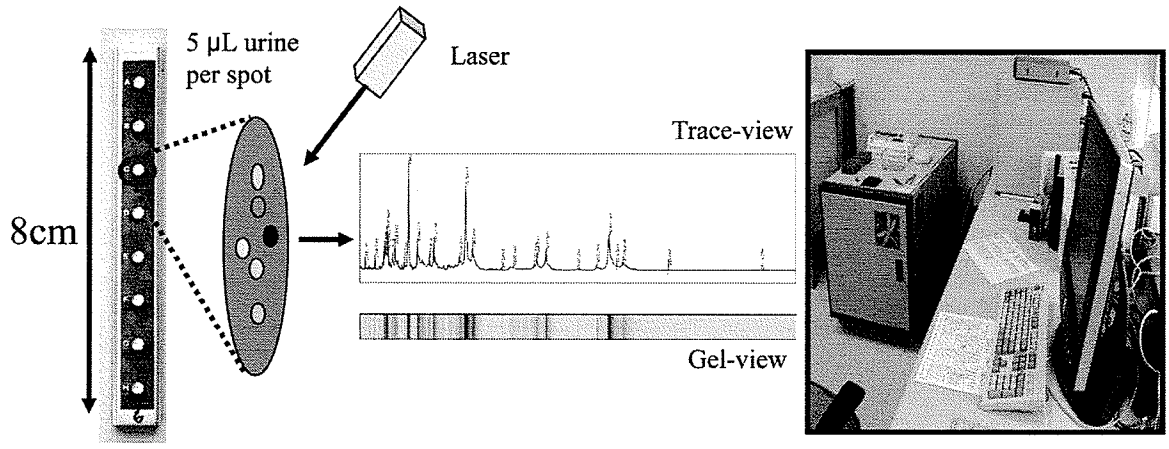


Figure 6. SELDI-TOF-MS. This technology couples surface chromatography to mass spectrometry. Specifically, 5µL of a sample is applied to a chromatographic surface, which binds a certain subset of proteins in the samples. Unbound proteins are then washed away and matrix is added, which allows for ionization of the proteins when energy is added in form of laser shots. Ionized proteins are then analyzed by mass spectrometry, which separates the proteins based on their molecular weight. The results can be displayed in a so-called trace view (x-axis=peak intensity; y-axis=molecular weight) or in a so-called gel view, where the darkness of the bands corresponds to the peak intensity in the trace view.

	2-DE	LC-MS	SELDI-TOF-MS	ELISA
Use for biomarker discovery	Yes	Yes	Yes	Limited *
Direct identification of detected biomarkers	Yes	Yes	No	No
Sensitivity	Medium	High	Medium	Highest
Throughput	Low	Low	Highest	High

Table 2. Comparison of different proteomic technologies for their use as biomarker discovery platforms. * Modified assays are used to detect allo- or autoantibodies which could yield biomarkers for allo- or autoimmunity.

Protein microarrays, consisting of thousands of protein-specific capturing molecules (e.g. antibodies) in analogy to gene-microarrays, may revolutionize protein expression profiling. However, the few currently available antibodies largely limit this technology.

2.5) Pathophysiology of proteinuria

Healthy people secrete less than 150mg of protein in urine each day. Depending on the kidney or urinary tract system disease proteinuria can reach more than 10g per day. Basically, there are four different pathophysiological pathways that influence the protein content and composition of urine (**Figure 7**).

[I] Filtration from serum: The major part of urine proteins is derived from serum by filtration through the glomerular barrier. The glomerular barrier consists of the fenestrated endothelial cells, the glomerular basement membrane and the slit-diaphragm of the podocytes. The latter is considered to be predominantly responsible for the characteristics of the barrier. Proteins are thought to be retained from filtration into the urine based on their molecular weight, size, shape and net charge (76). Normally, proteins below 20kDa are completely filtrated into urine, whereas larger proteins are more and more retained in the serum. Albumin (66kDa), for instance, would still pass the glomerular barrier based on its size, but it is speculated that its negative charge prevents filtration of large amounts. However, not everybody agrees to the hypothesis of charge selectivity (77).

[II] Tubular reabsorption and regurgitation: Many filtrated proteins bind to more or less specific receptors mainly on proximal tubular epithelial cells (e.g. megalin and

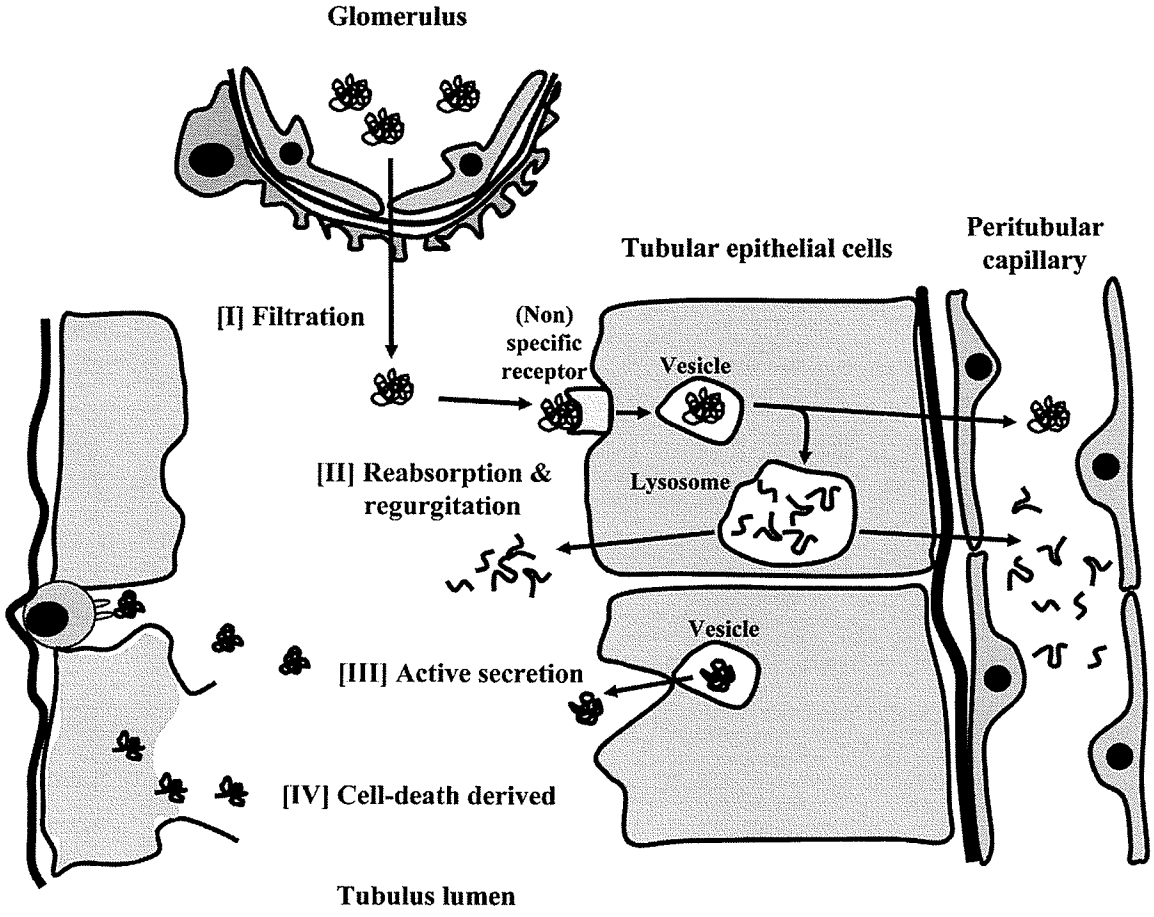


Figure 7. Pathophysiology of proteinuria. Urine proteins can be derived from [I] filtration of serum proteins through the glomerular barrier, [II] incomplete reabsorption or regurgitation by tubular epithelial cells, [III] active secretion by tubular epithelial cells or other cells with access to the urine, and [IV] lysis of urinary cells.

cubilin). After binding, ligands are trafficked to lysosomes for degradation or endocytic vesicles for transcytosis back to the blood stream (78). Lysosomal degraded proteins may be directed back to the blood stream, but they are also regurgitated into the tubular lumen and ultimately excreted. The latter pathway was not recognized until recently and may have been underestimated (77,79). It is critical to take this pathway into account for proteomic analysis in urine, because not only intact proteins but also fragments of the same protein may be detectable.

[III] Active secretion: Some proteins are produced and secreted from tubular cells into the urine by an active process (e.g. Tamm-Horsfall protein) (80). Even whole vesicles can be release. Furthermore, cells with access to the urinary tract system can secrete proteins into it (e.g. neutrophils secrete α-defensins).

[IV] Cell-death derived proteins: Tubular cells undergo constant renewal and 'old'/apoptotic cells are shed into the urine. Prescott estimated that, under physiological conditions, almost 2,000,000 tubular epithelial cells are sloughed into the urine each day (81). In addition, red and white blood cells as well as urothelial cells can be present in urine in significant amounts. All cells may stay intact, but their membranes could also be disrupted and the intracellular proteins released into the urine.

Chapter 3

Statement of the rationale

3.1) General hypothesis

3.2) Specific aims

3.1) General hypothesis

Post-transplant immune monitoring of renal transplant recipient is currently based on the integrated information gathered from the allograft function (i.e. serum creatinine), the risk profile of a patient (e.g. number of MHC-mismatches, presensitization), the clinical course (e.g. prior rejections) and ultimately the allograft biopsy results. While these tools have proved to be invaluable for adjusting the immunosuppressive therapy, they still have major shortcomings as described in the introduction. Immune monitoring with non-invasive markers, which allows for frequent measurement, may further improve the clinical outcome of kidney allograft recipient by better individualisation of immunosuppressive therapy. Specifically, this includes reduction of immunosuppressive therapy for patients inferred to be free of rejection by the non-invasive test, as well as increasing immunosuppressive therapy before tissue damage occurs and the rejection process becomes obvious (i.e. worsening allograft function). Non-invasive, antigen-specific tests are mostly labour intensive, expensive and required donor cells (with the exception of tetramer-staining), which does not lend themselves to high-throughput analysis in a busy clinical setting. Non-antigen specific tests are cheaper and have high-throughput capabilities, but they often lack sensitivity and specificity for allograft rejection. As urine is [I] in direct contact with the main target of rejection (tubular epithelial cells), [II] may represent the whole kidney allograft, and [III] may also be less confounded by systemic inflammatory processes, we anticipate that non-invasive biomarker in urine may have a higher sensitivity and specificity than serum biomarker. Finally, proteins, as the effector molecules, may be more informative and specific for the rejection process than gene transcription products (i.e. mRNA). **Therefore, we**

hypothesized that proteomic technology will identify urine proteins associated with renal allograft rejection.

3.2) Specific aims

3.2.1) To establish a high-throughput urine protein profiling system

In order to be able to compare the proteome of many samples, a high-throughput platform is mandatory. We eventually choose the SELDI-TOF-MS system for this purpose because it is currently the only high-throughput platform available. However, before clinical samples can reliably be profiled, the reproducibility and the limitations of the SELDI-TOF-MS platform have to be determined. In addition, several intrinsic (e.g. urine concentration, cellular components) and extrinsic (e.g. stability of urine proteins, storage) factors of urine have to be studied first to confidently contribute differences in protein composition in various disease states to the disease process itself and not to confounding factors.

3.2.2) To define and characterise the 'normal' post-transplant urine proteome by SELDI-TOF-MS

Definition of the 'normal' post-transplant urine proteome is critical for further comparison with urines from patients with allograft rejection or other diseases affecting the allograft. Therefore, we speculated that the definition of 'normal' post-transplant urine has to be

very stringent and based on the clinical course, the allograft function and the histology of an allograft biopsy. In addition, we will compare these 'normal' post-transplant urines to urines from healthy persons to determine whether they express the same kind and amount of proteins.

3.2.3) To detect and identify urine proteins associated with acute renal allograft rejection

We hypothesized that by choosing more severe rejections (i.e. acute clinical rejections), chances would be higher to detect differences in comparison to the 'normal' post-transplant proteome. Once urine proteins associated with acute clinical allograft rejection are detected, urines from patients with less severe forms of rejection (i.e. acute subclinical rejections) can be screened for the presence or absence of these proteins. As control groups urines from transplanted patients with other pathologies than allograft rejection such as recurrent glomerulopathies, acute tubular necrosis, CNI-toxicity and infections of the transplant (e.g. polyomavirus BK-type nephropathy) will be included in the analysis. Once specific proteins associated with acute renal allograft rejection are found, they will be identified using peptide mass fingerprinting and/or peptide sequencing.

Chapter 4

Methods

4.1) Transplanted patients and non-transplanted control groups

4.2) Urine collection, preparation and microscopic analysis

4.3) Surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

4.4) Protein purification and identification methods

4.5) Determination of cytomegalovirus (CMV) viremia

4.6) Statistical analysis

4.1) Transplanted patients and non-transplanted control groups

4.1.1) Transplanted patients

All patient (e.g. allograft function measured by serum creatinine, biopsies) and urine data were stored and managed in a central Access database. From July 1997 to March 2003, 2400 serial mid-stream urine samples from 212 renal transplant patients were collected. These 212 patients underwent a total of 693 protocol or clinically indicated core needle allograft biopsies. All patient charts were review and additional information extracted as needed. Biopsies were analysed by experienced renal pathologists, and scored according to the Banff 1997 classification (**Table 3**) (23). The acute Banff score determines acute interstitial (ai 0-3), tubular (at 0-3), vascular (av 0-3) and glomerular (ag 0-3) changes, whereas the chronic Banff score assesses chronic interstitial (ci 0-3), tubular (ct 0-3), vascular (cv 0-3) and glomerular (cg 0-3) changes. The individual scores are added to a total acute (a 0-12) and total chronic (c 0-12) score. A biopsy specimen was judged adequate, when $\geq$7 glomeruli and $\geq$1 vessel were available for analysis. All patients were treated with a triple immunosuppressive regimen consisting of calcineurin-inhibitor (cyclosporine or tacrolimus), prednisone and mycophenolate-mofetil or azathioprine.

4.1.2) Non-transplanted control groups

[1] Normal control group: Consists of 28 healthy individuals (14 female and 14 male, age 20-50 years).

Acute Banff Score	Chronic Banff Score
i0 - No or trivial interstitial inflammation (<10% of unscarred parenchyma) i1 - 10 to 25% of parenchyma inflamed i2 - 26 to 50% of parenchyma inflamed i3 - more than 50% of parenchyma inflamed	ci0 - Interstitial fibrosis in up to 5% of cortical area ci1 - Mild - interstitial fibrosis in 6 to 25% of cortical area ci2 - Moderate - interstitial fibrosis in 26 to 50% of cortical area ci3 - Severe - interstitial fibrosis in >50% of cortical area
Indicate the presence of remarkable numbers of eosinophils, PMNL, or plasma cells (specify which) with an asterisk (*).	
t0 - No mononuclear cells in tubules t1 - Foci with 1 to 4 cells/tubular cross section (or 10 tubular cells) t2 - Foci with 5 to 10 cells/tubular cross section t3 - Foci with >10 cells/tubular cross section, or the presence of at least two areas of tubular basement membrane destruction accompanied by i2/i3 inflammation and t2 tubulitis elsewhere in the biopsy	ct0 - No tubular atrophy ct1 - Tubular atrophy in up to 25% of the area of cortical tubules ct2 - Tubular atrophy involving 26 to 50% of the area of cortical tubules ct3 - Tubular atrophy in >50% of the area of cortical tubules
[a] Applies to tubules no more than mildly atrophic	
g0 - No glomerulitis g1 - Glomerulitis in less than 25% of glomeruli g2 - Segmental or global glomerulitis in 25 to 75% of glomeruli g3 - Glomerulitis (mostly global) in more than 75% of glomeruli	cg0 - No glomerulopathy - double contours in <10% of peripheral capillary loops in most severely affected glomerulus cg1 - Double contours affecting up to 25% of peripheral capillary loops in the most affected of nonsclerotic glomeruli cg2 - Double contours affecting 26 to 50% of peripheral capillary loops in the most affected of nonsclerotic glomeruli cg3 - Double contours affecting more than 50% of peripheral capillary loops in the most affected of nonsclerotic glomeruli
	Note the number of glomeruli and percentage sclerotic.
v0 - No arteritis v1 - Mild-to-moderate intimal arteritis in at least one arterial cross section v2 - Severe intimal arteritis with at least 25% luminal area lost in at least one arterial cross section v3 - Transmural arteritis and/or arterial fibrinoid change and medial smooth muscle necrosis with lymphocytic infiltrate in vessel	cv0 - No chronic vascular changes cv1 - Vascular narrowing of up to 25% luminal area by fibrointimal thickening of arteries ± breach of internal elastic lamina or presence of foam cells or occasional mononuclear cells[a] cv2 - Increased severity of changes described above with 26 to 50% narrowing of vascular luminal area[a] cv3 - Severe vascular changes with >50% narrowing of vascular luminal area[a]
Note number of arteries present and number affected. Indicate infarction and/or interstitial hemorrhage by an asterisk (with any level v score).	[a] In the most severely affected vessel. Note if lesions characteristic of chronic rejection (breaks in the elastica, inflammatory cells in fibrosis, formation of neointima) are seen.

Table 3. Banff 1997 working classification of renal allograft pathology (revised 2003). The acute and the chronic Banff Score describe changes in the four structural compartments of the allograft (interstitial, tubular, glomerular, vascular). Scores from 0 to 3 are given for each compartment according to the severity of the changes. Individual scores may be added to give one total acute and chronic Banff Score.

[2] Urinary tract infection (UTI) group: Consists of 5 females with an episode of a lower UTI, which was defined as requiring the clinical symptoms of a UTI, a leukocyte count in the urine sediment >40/high power field and a positive bacterial culture (>10^8 colony forming units).

4.2) Urine collection, preparation and microscopic analysis

4.2.1) Urine collection and storage for evaluation of SELDI-TOF-MS platform

Second-morning urine from healthy men and women were collected into two different containers. The first 10-20 mL of urine was considered as first-void urine, the following 50-80 mL as mid-stream urine. Urines were centrifuged in a fixed angle centrifuge for 10 minutes at 2000 rpm (900 g), the supernatants were transferred into 2 mL cryo-tubes (Gordon Technologies Inc., Mississauga, ON) and stored at –80°C until further analysis. All samples were obtained with informed consent and ethics approval of the University of Manitoba Institutional Review Board.

For urine sediment analysis 10 mL of freshly collected urine was centrifuged for 10 minutes at 2000 rpm. The pellet was analyzed with a phase-contrast microscope at 400x magnification and is reported as cells per high power field (hpf).

4.2.2) Urine collection in transplanted patients and non-transplanted control groups for biomarker discovery

All urine samples were stored non-centrifuged at -80°C until further analysis. All transplanted patient and control group urine samples were obtained with informed consent and ethics approval by the University of Manitoba institutional review board.

4.3) SELDI-TOF-MS

Urine samples were thawed on ice, shortly vortexed and centrifuged for 5 minutes at 10000 rpm (to remove remaining cell particles). Two different ProteinChips were used for the analysis. They were prepared as follows:

[1] Normal phase chips (ProteinChip NP20; Ciphergen, Freemont, CA): Five µL of urine supernatant were applied in duplicate to the chip and incubated for 20 minutes in a humidity chamber. Spots were then washed three times with 5 µL HPLC-grade water and air-dried for 10 minutes.

[2] Hydrophobic chips (ProteinChip H4): Five µL of 50% acetonitrile in HPLC-grade water were applied to the spots for 5 minutes to activate the surface. This solution was removed and 5 µL urine supernatant were applied in duplicate to the chip and incubated for 20 minutes in a humidity chamber. Spots were washed twice with 5 µL 10% acetonitrile in HPLC-grade water and then once with 5 µL HPLC-grade water. Chips were air-dried for 10 minutes.

As matrices saturated α-cyano-4-hydroxycinnamic acid (CHCA: Ciphergen) and sinapinic acid (SPA: Ciphergen) were prepared in 50% acetonitrile / 0.5% trifluoro-acetic acid according to the manufacturer's instructions and 1μL of matrix solution (35% CHCA unless otherwise specified) was applied to each spot and air-dried. Unless stated otherwise, chips were read with the following SELDI-TOF-MS instrument (ProteinChip Reader II: Ciphergen) settings in the positive ion mode: Laser intensity 230; detector sensitivity 6; detector voltage 1800 V; positions 20 to 80 were read with an increment of 5 (resulting in 13 different sampling positions); sixteen laser shots were collected on each position (total shots collected and averaged: 208/sample); eight warming shots were fired at each position, which were not included in the collection; the acquired mass range was from a mass-over-charge (m/z) ratio of 0 to 80000; lag time focus of 900 ns. Calibration was done externally with a mixture of 4 proteins with masses ranging from 2 to 16 kD. After baseline subtraction, peak labelling was performed by the ProteinChip Software (Version 3.1) for peaks with a signal-to-noise (S/N) ratio of ≥3 in the m/z range from 2000-80000. For some comparisons and presentations spectra were normalized according to the total ion current.

4.4) Protein purification and identification methods

4.4.1) Determination of point of iso-electricity (pI) of rejection pattern proteins

A urine sample with the rejection pattern proteins was dialysed with 7kD cut-off dialysis cassettes (Slide-A-Lyzer, Pierce, Rockford, IL) against 50mmol/L MES pH 6 and 50mmol/L Tris pH 8, respectively. Cation-exchange (CM HyperD, Ciphergen) and anion-exchange (Q HyperD, Ciphergen) beads were washed three times for 20 minutes with 1 mL 50mmol/L MES pH 6 or 50mmol/L Tris pH 8, respectively. The pH 6 fraction was incubated on CM-beads for 2h in a ratio of 5µL beads per 1 mL urine. The supernatant was transferred in a separate tube. After washing the CM-beads twice with two bead-volumes 50mmol/L MES pH 6 for 15 minutes, proteins were eluted with increasing concentrations of KCl in 50mmol/L MES pH 6 (two bead-volumes for 30 minutes each). The supernatant and the eluted fractions were checked for the presence or absence of the rejection pattern proteins by SELDI-TOF-MS. The pH 8 fraction was incubated on Q-beads for 2h in a ratio of 5µL beads per 1 mL urine. The supernatant was transferred to a separate tube. After washing the Q-beads twice with two bead-volumes 50mmol/L Tris pH 8 for 15 minutes, proteins were eluted with increasing concentrations of NaCl in 50mmol/L Tris pH 8 (two bead-volumes for 30 minutes each). The supernatant and the elution fractions were checked for the presence or absence of the rejection pattern proteins by SELDI-TOF-MS.

4.4.2) Purification of rejection pattern proteins with cation exchange (CM) beads and reverse-phase high-pressure liquid chromatography (RP-HPLC)

Fifteen mL of urine sample with the rejection pattern proteins was dialysed with 6-8kD cut-off dialysis tube membrane (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA) against 50mmol/L MES pH 6.2. Dialysed urine was transferred into 1.5 mL siliconized tubes (Fisherbrand) and previously washed CM-beads (see above) were added in a ratio of 5µL beads per 1 mL urine. After 2h incubation the supernatant was transferred to a separate tube and the CM-beads were washed twice with two bead-volumes 50mmol/L MES pH 6.2 for 15 minutes. Proteins were eluted with two bead-volumes 200mmol/L KCl in 50mmol/L MES pH 6.2. Those fractions containing the rejection pattern proteins were lyophilized and resuspended in a 5 times smaller volume of HPLC-grade water.

Further purification was done by RP-HPLC using an Agilent 1100 Series with a C4 column (Zorbax SB-C4, 5µm, 0.5x150mm; Agilent Technologies, Paulo Alto, CA). Five µL of concentrated sample was applied and eluted using a 1.6% acetonitrile increment per minute in 0.1% TFA during the first 17 minutes, followed by a 0.3% increment per minute for 24 minutes and a 16% increment per minute for the last 4 minutes at a flow rate of 20µL/minute. Peak fractions containing the rejection pattern proteins were pooled, lyophilised and resuspended in 50mmol/L ammonium bicarbonate for in solution digestion. The purification process was monitored with SELDI-TOF-MS using H4 chips.

4.4.3) Identification of rejection pattern proteins by liquid-chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS)

Concentrated and purified protein (from about 10 mL starting material) was reduced with 10mM DDT for 30 minutes at 57.5°C, alkylated with 50mM iodoacetamide for 30 minutes in the dark, then dialysed against 50mmol/L ammonium bicarbonate, and finally digested with 100ng trypsin (sequencing-grade modified trypsin, Promega) over night at 37°C. Peptides were lyophilised, resuspended in 5μL 0.1% TFA, and subjected to RP-HPLC separation using an Agilent 1100 Series system with a C18 column (Vydac 218 TP C18, 5μm, 0.15x150mm). Peptides were eluted with a linear gradient of 1.3% acetonitrile increment per minute in 0.1%TFA during 35 minutes and a 10% increment for the last 5 minutes. The column effluent (4μl/min) was mixed online with 2,5-dihydroxybenzoic acid (0.16g/ml, Sigma-Aldrich) matrix solution (0.5μl/min) and deposited by a small computer-controlled robot onto a movable MALDI target at one-minute intervals. Forty such fractions were collected over a total period of 40 minutes. The spots on the target were analyzed individually, both by single mass spectrometry (MS) and by tandem mass spectrometry (MS/MS) in the Manitoba/Sciex prototype quadrupole/time-of-flight mass spectrometer (QqTOF) (82). In this instrument, ions are produced by irradiation of the target with photon pulses from a 20-Hz nitrogen laser (Laser Science) with 300 mJ energy per pulse. Orthogonal injection of ions from the quadrupole into the TOF section normally produce a mass resolving power 10,000 FWHM and accuracy within a few mDa in the TOF spectra in both MS and MS/MS modes, as long as the ion peak is reasonably intense. MS and MS/MS peak list were submitted to Profound and searched against the non-

redundant NCBI human database using a mass accuracy of 20ppm of monoisotopic peaks. Partial methionine oxidation and one trypsin miscleavage was allowed.

4.5) Determination of cytomegalovirus (CMV) viremia

CMV-viremia was measured on peripheral blood buffy coat specimens using a semi-quantitative PCR assay developed at the Manitoba Cadham Provincial Laboratory that is accredited by the College of American Pathologists.

4.6) Statistical analysis

We used JMP IN software version 4.0.4 (SAS Institute Inc., Cary, NC) for statistical analysis. For categorical data, Fisher's exact test or Pearson's chi-square test was used. Parametric continuous data was analyzed by Student t-tests or one-way analysis of variance. For nonparametric continuous data, Wilcoxon or Kruskal-Wallis rank sum tests were used. A P-value < 0.05 (two-sided test) was considered to indicate statistical significance.

Chapter 5

Results

5.1) Urine protein profiling with SELDI-TOF-MS

5.2) Detection of urine proteins associated with acute renal allograft rejection

5.3) Identification of proteins associated with acute renal allograft rejection

5.1) Urine protein profiling with SELDI-TOF-MS

5.1.1) Evaluation of reproducibility

It is critical to evaluate the reproducibility of urine protein profiling using the SELDI-TOF-MS approach before establishing whether the urine protein profiles differ in various clinical states. Reproducibility was evaluated by applying one urine sample to 14 spots and reading the spots using the protocol described above. The total number of detected peaks with an S/N-ratio ≥3 was 25peaks/spectrum (range 23-29). Fourteen peaks common to all spectra were selected and compared with regard to their peak intensity by calculating the coefficient of variation. They ranged from 8 to 30%, with the lowest variation seen in the high intensity peaks and the higher variation seen in lower intensity peaks (**Figure 8A**). This is expected, as small differences in low intensity peaks (e.g. 1.0 vs. 0.5) have a large influence on the calculated coefficient of variation. Independent of the software assignment of protein peaks, it is important to conduct manual inspection of the spectra, to determine whether a specific peak is present. Low intensity peaks with a S/N-ratio near the selected detection threshold (i.e. ≥3) can be unlabelled and undetected by the software (**Figure 8B**).

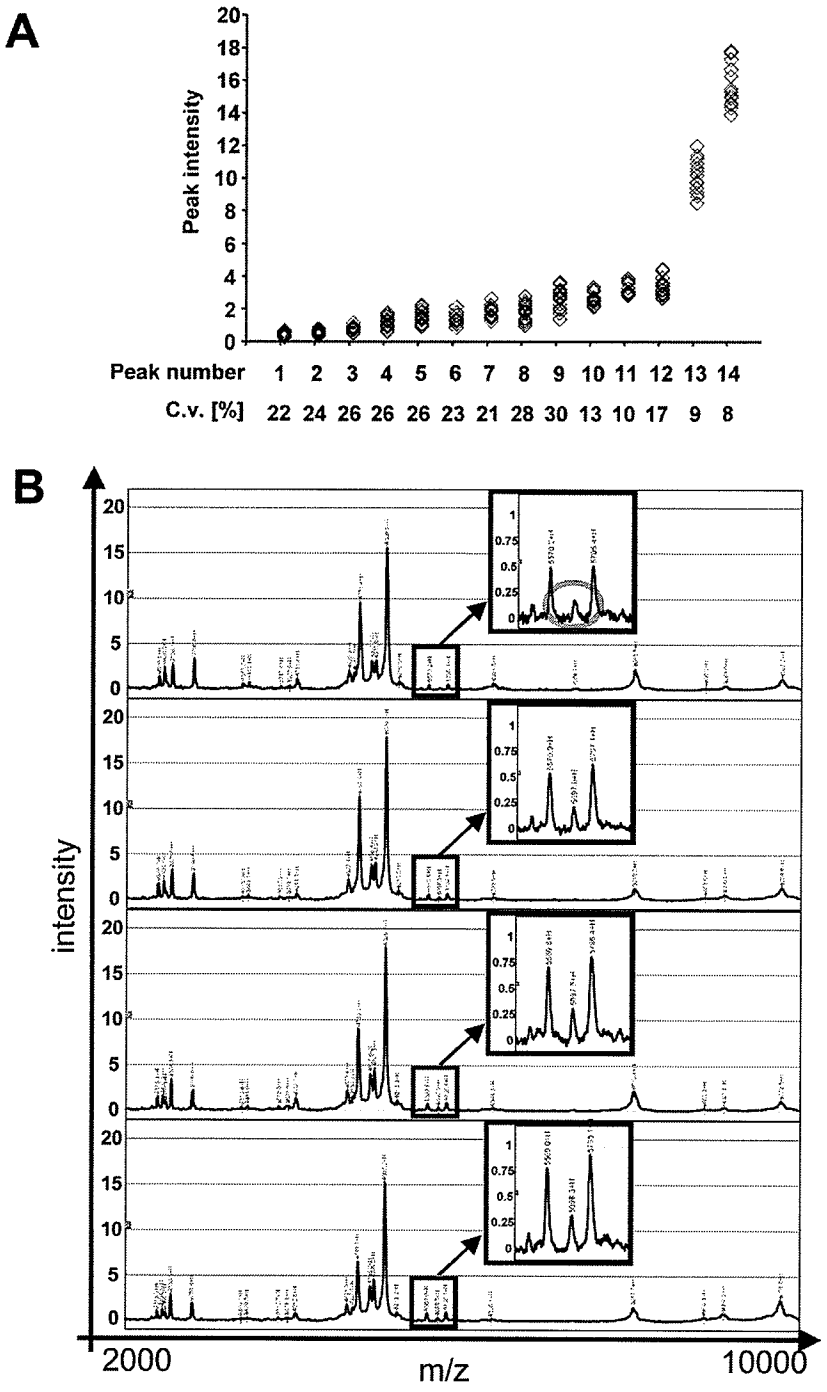


Figure 8. Reproducibility of urine protein profiles. One urine sample was applied to 14 spots and analysed. **A**: Fourteen peaks common to all spectra were selected and compared with regard to their peak intensity by calculating the coefficients of variation. They ranged from 8 to 30%, with the lowest variation seen in the high intensity peaks and the higher variation seen in lower intensity peaks. **B**: Parts of 4 randomly selected spectra from the obtained 14. Manual inspection of the spectra showed the presence of an unlabelled peak (circle in insert), although the spectra look the same by "eyeball".

5.1.2) Impact of extrinsic factors on reproducibility and peak detection of urine protein profiles

The impact of matrix on the urine protein profile was determined by comparing different dilutions of CHCA and SPA (20%, 35%, 50% and 100%) with the otherwise unchanged protocol stated above. In the range from 2-25 kD, 22, 26, 19 and 16 peaks were detected using 20%, 35%, 50% and 100% CHCA, respectively. In contrast, 13, 19, 11 and 10 peaks were detected using 20%, 35%, 50% and 100% SPA. Peak intensity below 8-10 kD was higher with CHCA, whereas SPA yielded higher peak intensities above 8-10 kD (urine protein profiles not shown).

The impact of spot sampling protocols was determined by comparing three different spot sampling protocols with respect to peak detection in undiluted and diluted urine: protocol 1 (standard protocol; see above); protocol 2 (standard protocol modified to sample on only 5 different positions for a total of 80 shots/sample); protocol 3 (standard protocol modified to use a higher detector sensitivity (10 instead of 6)). Protocol 1 detected 34 peaks in undiluted urine, whereas protocols 2 and 3 detected only 21 and 26 peaks, respectively. In diluted urine (urine creatinine 3.75 mmol/l) the peak counts were 20, 11 and 13, respectively (urine protein profiles not shown).

If the SELDI-TOF-MS approach is to be used in the assessment of clinical samples, it is important to assess the stability of the urine proteins prior to analysis. First-void and mid-stream urine samples from three females and three males were analyzed within 2 hours from the time of collection, after storage for three days at room temperature and after three days at 4°C. In all six samples, only minor differences in the mid-stream urine protein profiles could be detected after storage for three days at 4°C. However, in

three first-void urines (two female, one male), storage for three days at room temperature or at 4°C changed the spectra considerably. A series of new peaks in the low molecular weight range were detected (**Figure 9**). Storage of the urine samples at -70°C did not change the spectra compared to those obtained before freezing. Furthermore, almost the same spectra could be generated after four freeze-thaw cycles, however, a loss of peaks was observed after the 5th freeze-thaw cycle (**Figure 10**).

5.1.3) Impact of intrinsic factors on normal urine protein profiling

A potential confounding variable in the clinical setting is whether a urine sample is first-void or mid-stream. In all three urine samples from males, there are almost no differences between the protein profile of first-void and mid-stream urine (**Figure 11A**). However, in all three urine samples from females, there are prominent peaks between 3.3 and 3.5 kD in the first-void urine fraction. These peaks are greatly diminished in the mid-stream urine sample, together with other changes in peak intensities (**Figure 11B**). Three of these peaks with average masses of 3370.3, 3441.2 and 3484.3 Da are consistent with the masses of the α-defensins 2, 1, and 3, respectively.

Another confounding variable in urine proteomic analysis is the presence of blood in urine. It can be present in urine under normal conditions (e.g. menstruation) or in association with urogenital tract pathologies. To investigate the impact of blood on the normal urine profile, we spiked 500 μL urine with 10 μL of blood, which resulted in a red colouring of the sample (sediment analysis showed >100 red blood cells (RBC)/hpf).

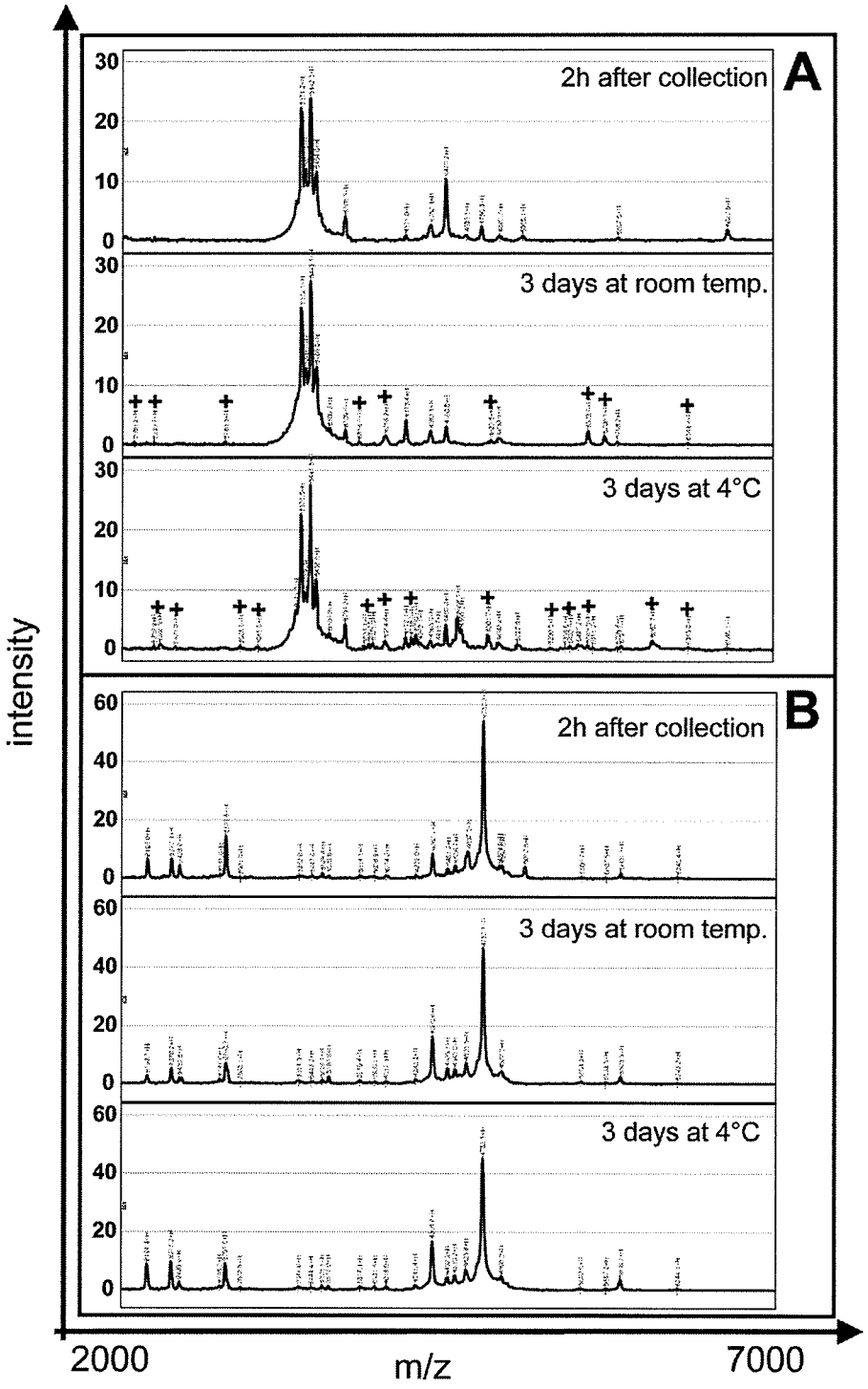


Figure 9. Impact of storage on urine protein profiles. A: Representative female first-void urine showing the appearance of new peaks (+) in the 2-6kD range after storage for 3 days at room temperature or at 4°C. **B**: Representative male mid-stream urine protein profile, which showed only minor changes, whether it was analysed 2 hours after collection or after storage for 3 days at room temperature or at 4°C, respectively.

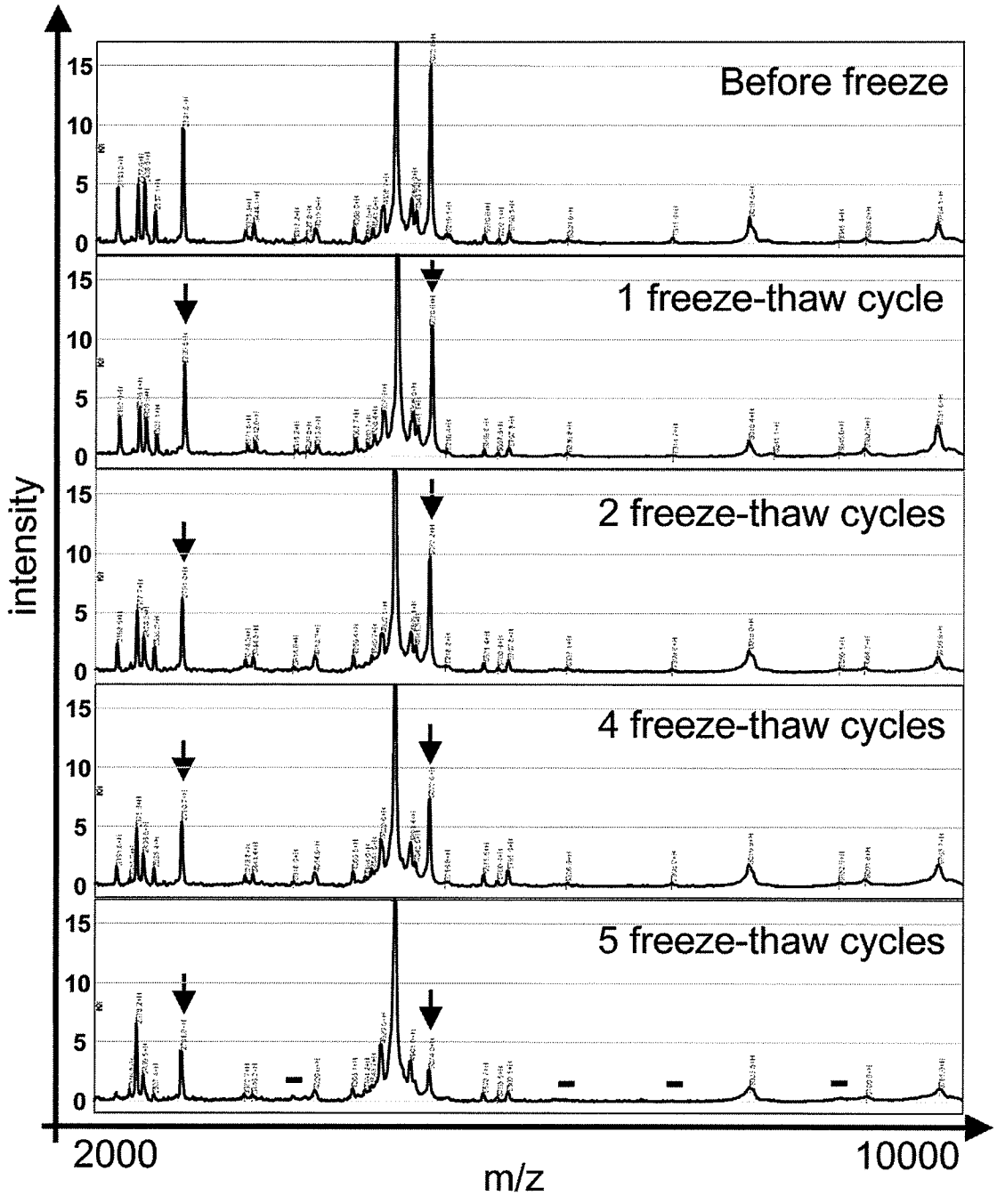


Figure 10. Impact of freeze-thaw cycles on urine protein profiles. Urine protein profiles obtained before freeze and after 1 to 4 freeze-thaw cycles were unchanged, but an increasing loss of intensity in some peaks was detected (↓). After the 5th freeze-thaw cycle some weak intensity peaks were not detected (-).

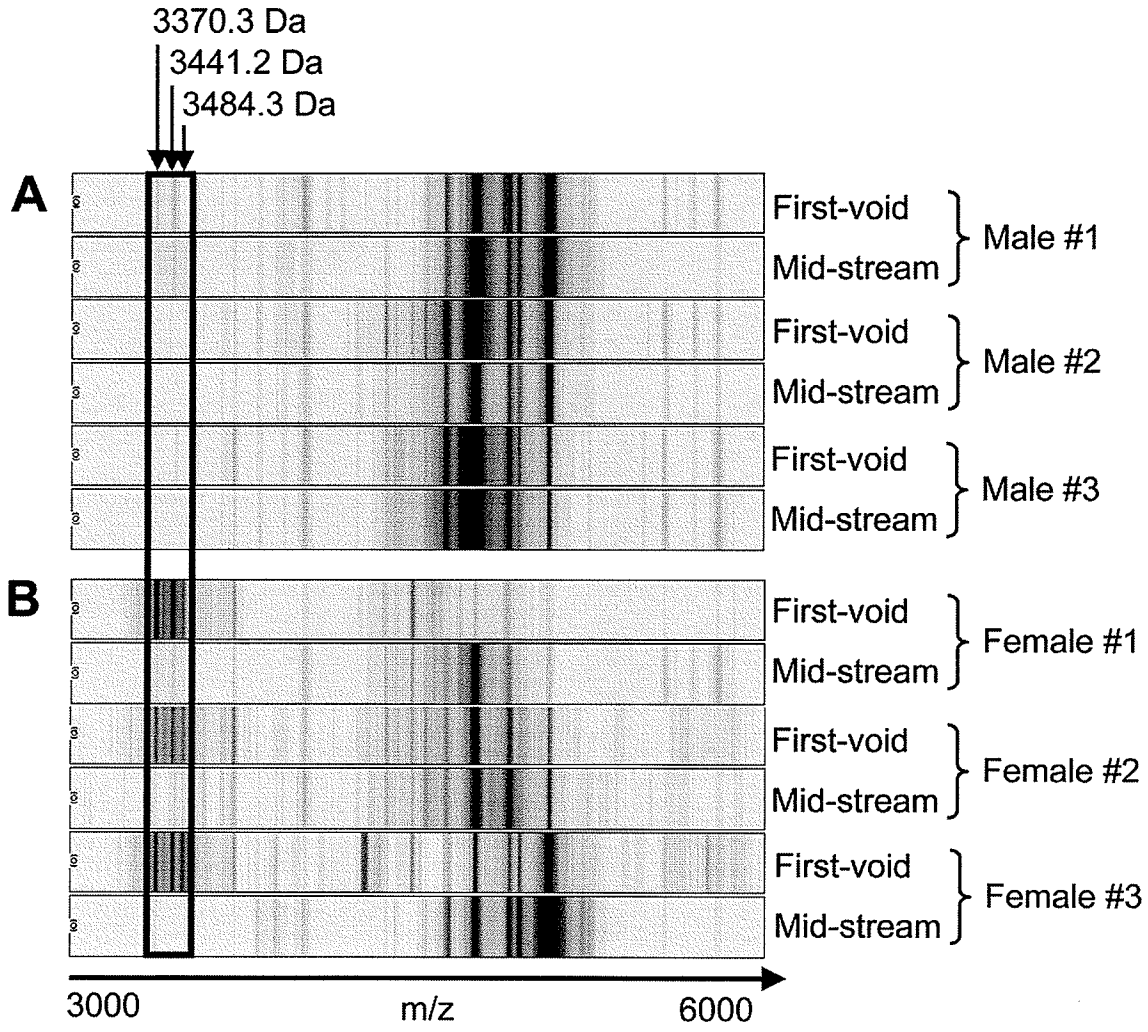


Figure 11. Comparison between first-void and mid-stream urine protein profiles (gel-view). First-void and mid-stream urine protein profiles obtained from three females and three males. In males both urine samples had similar protein profiles, whereas in females there are significant differences. The most prominent difference in female first-void urine are three peaks at 3370.3, 3441.2 and 3484.3 Da (↓), which are consistent with the masses of the α-defensins 2, 1, and 3, respectively (Swiss-Prot P59665+P59666; 3371.9, 3442.5, 3486.5 Da)). The calculated mass accuracy of the SELDI-TOF-MS in this example is < 0.07%, which is within the limits given by the manufacturer (<0.1%).

In the subsequent analysis by SELDI-TOF-MS, four major peaks were detected (**Figure 12B**), which are consistent with the masses of the hemoglobin α- and β-chains and their doubly charged ions. Based on the virtual disappearance of these peaks after sample centrifugation prior to SELDI-TOF-MS analysis, it is likely that these peaks represent hemoglobin. They were easily detectable as the most intense peaks up to a 1:128 dilution of this sample, corresponding to 10 μL blood in 64 ml diluted urine (urine protein profiles not shown). However, even when the RBC were removed by centrifugation, the urine was still contaminated with serum proteins. This is suggested by the presence of peaks with masses consistent with albumin in the urine protein profile (**Figure 12C**).

A dilute urine sample may limit the ability to detect the normal urine protein profile. To address the issue of urine concentration, urine was sampled from a healthy male person with a body weight of 75 kg after 20 hrs of no fluid intake. The measured urine creatinine was 15 mmol/L and the total protein was 0.11 g/L. At another time point, the same individual was challenged with 4 L of fluid over 2 hrs, leading to dilute urine with a creatinine of 0.9 mmol/L and a total protein of 0.03 g/L. While the concentrated urine showed the normal peak profile (**Figure 13A**), the dilute urine sample showed only three peaks in the range from 2-25 kD (**Figure 13E**). To determine the detection threshold of the normal urine profile, the concentrated urine sample was serially diluted. At a 1:2 dilution, which corresponds to a urine output of 2L/day (calculated in our test person by: creatinine production/day [0.2 mmol/kg/day * 75 kg] divided by urine creatinine [7.5 mmol/L] = 2 L/day) the profile remained unchanged (**Figure 13B**). A progressive loss of urine profile peaks started with a 1:4 dilution. The 1:16 dilution showed a spectrum similar to the urine profile obtained after the fluid challenge (**Figure 13E**).

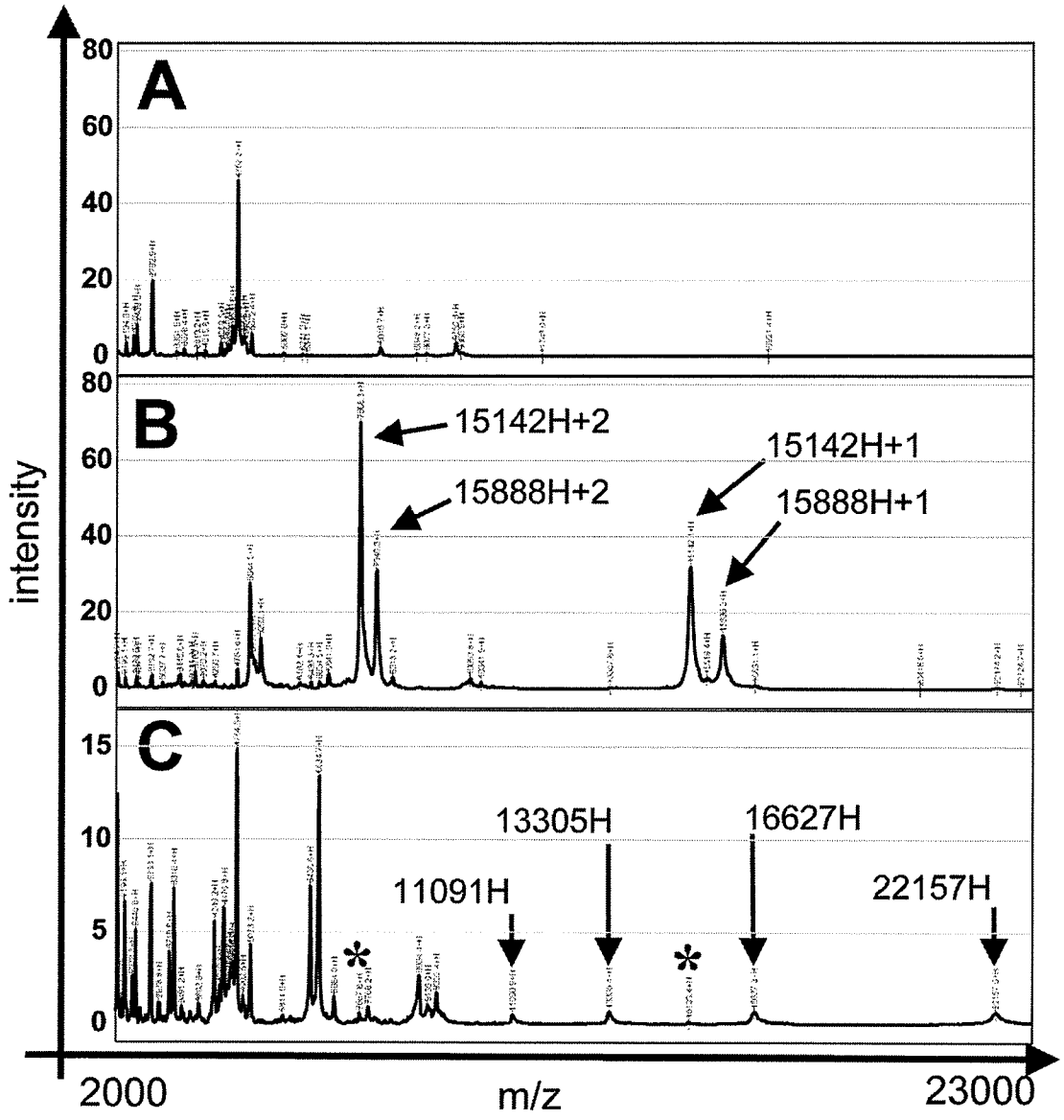


Figure 12. Impact of blood in urine on urine protein profiles. **A**: Protein profile of urine sample from a healthy male. **B**: Protein profile after spiking the same sample from A with blood (10 µL blood in 500 µL urine). Four peaks appear which are consistent with the masses of singly- and doubly-charged hemoglobin α- and β-chains (Swiss-Prot P01922: 15126 Da; P02023: 15867 Da). The calculated mass accuracy of the SELDI-TOF-MS in this example is < 0.13%, which is slightly above the limits given by the manufacturer (<0.1%). **C**: Protein profile after centrifugation of the same blood-spiked urine sample from B. Only trace amounts of two of these peaks were detectable (*), however contamination with serum proteins was obvious (e.g. peaks consistent with masses of serum albumin were detected). Albumin has a molecular weight of 66472 Da with its multiply-charged ions at an m/z of 33236 (double-charged), 22157 (triple-charged), 16618 (quadruple-charged), 13294 (quintuple-charged) and 11079 (sextuple-charged).

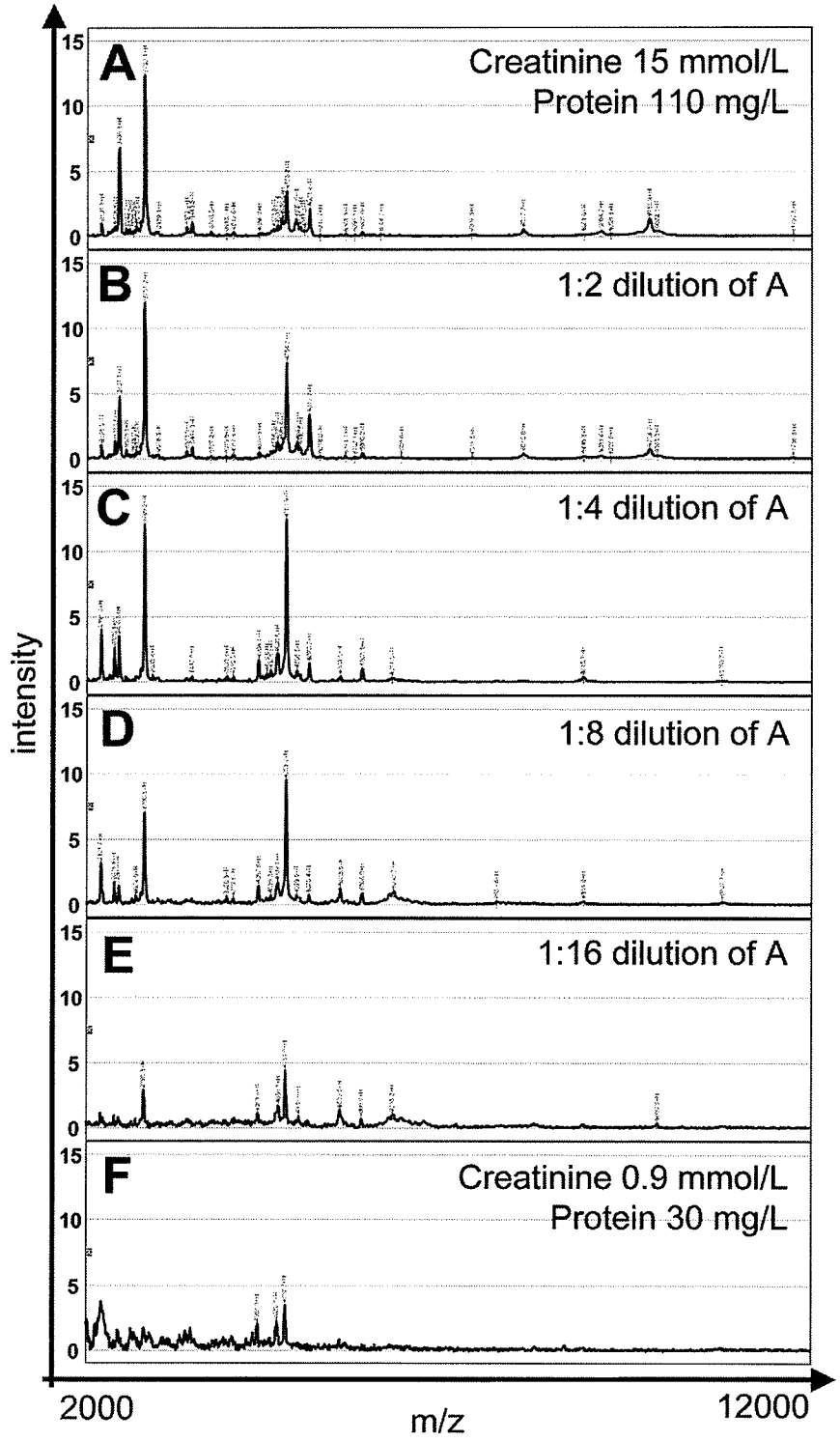


Figure 13. Impact of dilution on urine protein profiles. Protein profile obtained from **A:** Urine collected after a 20 h period of no fluid intake; **B-E:** Serial dilution of urine sample A; **F:** Urine collected after a 4 L fluid challenge. Starting with a 1:4 dilution, a continuous loss of peaks was observed.

5.1.4) Protein quantification and detection limits with SELDI-TOF-MS

To determine if either the spectral peak intensity or area provides a means for reliable protein quantification, serial dilution of a single protein (ubiquitin, 8565 Da) was performed. There was an excellent correlation between the amount of protein in the sample and peak intensity (r^2=0.95) or the area under the peak (r^2=0.98) in non-normalized spectra (**Figure 14A**). Even in a mixture containing four other proteins, the correlation was maintained (r^2=0.99 for peak intensity and for the area under the peak), but the peak intensities were 10 times lower with the same amount of ubiquitin (**Figure 14B**). When a complex protein mixture (i.e. normal urine with a protein concentration of 110 mg/L) was spiked with 1.0, 0.1 and 0.01 pmol/μL ubiquitin, only the first two concentrations of ubiquitin were detectable (**Figure 14C**). The peak intensity dropped from 0.32 (1.0 pmol/μL) to 0.09 (0.1 pmol/μL), which is only a 3.5 times decrease instead of the expected 10 times. Because only two measurements of peak intensity were obtainable, no correlation was calculated.

This experiment also showed that the detection limit for Ubiquitin, spiked in urine, is 0.01pmol/μL or 10000pmol/L, respectively (**Figure 15B, red box**). Other experimental evidence for the detection limit of SELDI-TOF-MS can be extracted from dilution experiments. Several urine proteins (i.e. hepcidin at 2191, 2436 and 2789Da; β-defensins at 4636, 4750 and 5069Da; β2-microglobulin at 11730Da; albumin at 66500Da) are readily detectable in urine from a healthy individual by SELDI-TOF-MS. Serial dilution of this sample has demonstrated that all these proteins are no longer detectable starting at a 1:4 and ending at 1:16 dilution. Because the concentration of these proteins is known, the

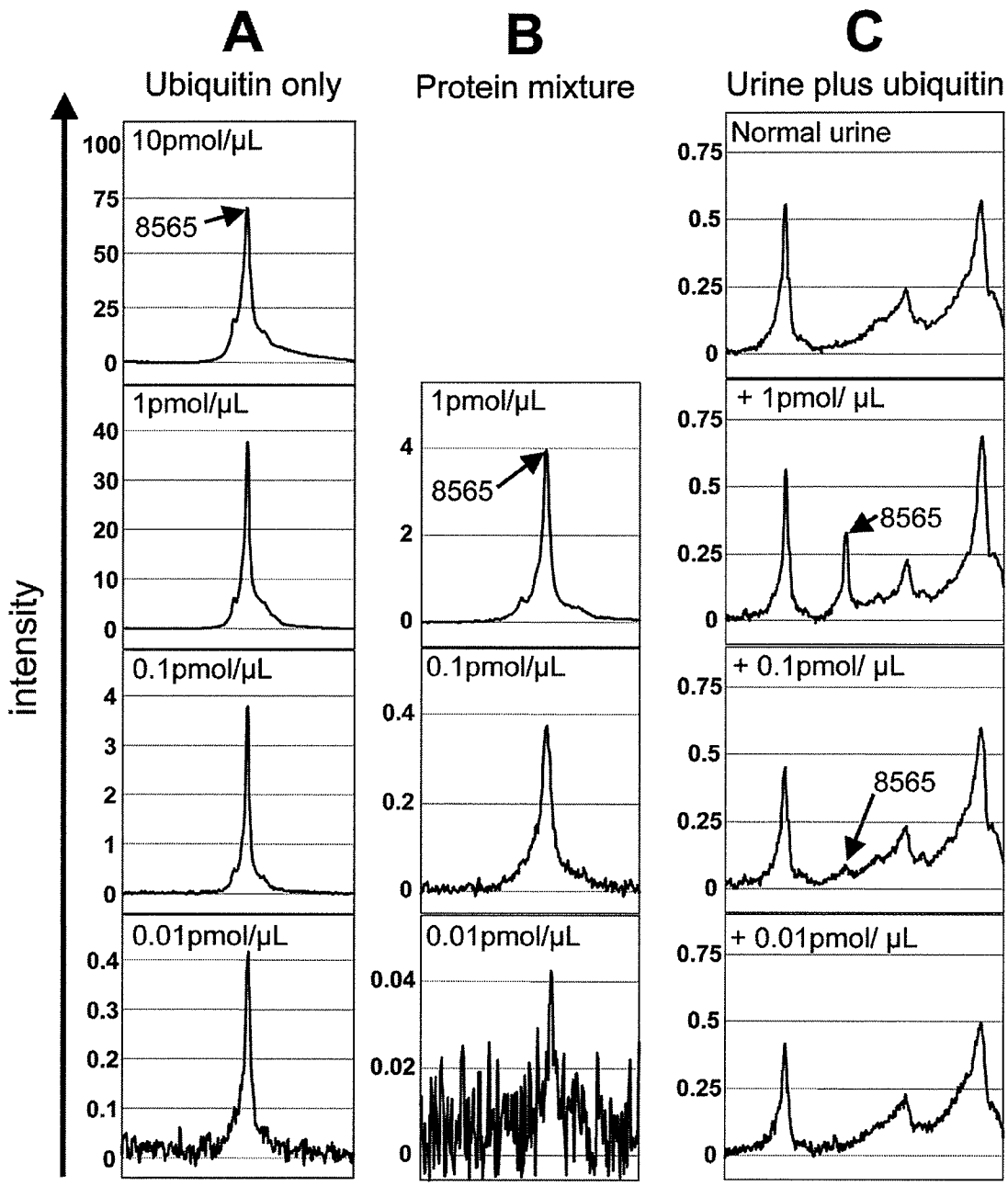


Figure 14. Impact of protein concentration on peak intensity. **A**: Dilution series of a single protein (ubiquitin, 8565 Da) from 10 pmol/µL to 0.01 pmol/µL (equals 85.6, 8.56, 0.85 and 0.08 ng/µL, respectively). **B**: Dilution series of ubiquitin from 1 pmol/µL to 0.01 pmol/µL in a mixture of four other proteins with constant concentrations (1.5 pmol/µL dynorphin A, 1 pmol/µL insulin, 0.3 pmol/µL cytochrome C and 0.3 pmol/µL superoxide dismutase). **C**: Dilution series of ubiquitin from 1 pmol/µL to 0.01 pmol/µL spiked into normal male urine with a protein concentration of 110 mg/L.

	A				B	C
	SELDI-TOF-MS detected proteins in normal urine				Spiked into urine	Chemokine in urine
Protein	Hepcidin	β-defensins	β2-micro-globulin	Albumin	Ubiquitin	IP-10
MWt [Da]	2191 2436 2789	4636 4750 5069	11730	66500	8565	10881
Protein concentration	pmol/L	pmol/L	pmol/L	pmol/L	pmol/L	pmol/L
100mg/L	40453074.4	20755500.2	8525149.2	1503759.4	11675423.2	
10mg/L	4045307.4	2075550.0	852514.9	150375.9	1167542.3	919033.2
1mg/L	404530.7	207555.0	85251.5		116754.2	91903.3
100µg/L	40453.1	20755.5		1503.8		
10µg/L	4045.3	2075.6	852.5	150.4	1167.5	919.0
1µg/L	404.5		85.3	15.0	116.8	91.9
100ng/L		20.8	8.5	1.5	11.7	9.2
10ng/L	4.0	2.1	0.9	0.2	1.2	0.9
1ng/L	0.4	0.2	0.1	0.0	0.1	0.1

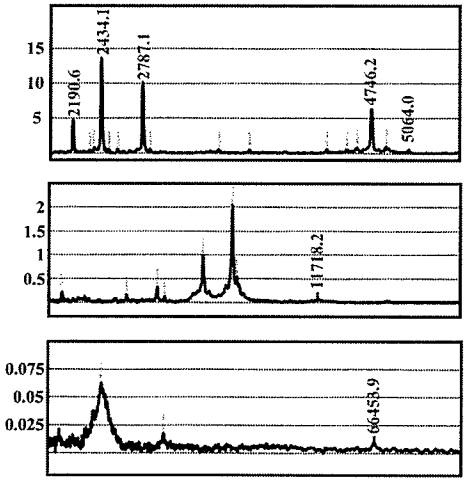


Figure 15. Estimation of detection threshold for urine proteins detected by SELDI-TOF-MS. **A**, Selected urine proteins with different molecular weights detected by SELDI-TOF-MS (H4-chip) in urine from a healthy person with total urine protein 150mg/L and urine creatinine 18mmol/L. The light grey boxes indicate the normal concentration range in healthy individuals. The spectra below show the detection of these proteins by SELDI-TOF-MS. In a 1:4 to 1:16 dilution these proteins are not detectable anymore. Therefore the detection threshold is approximately 10 times below the normal concentration of these proteins (grey boxes). **B**, In the previous experiment (**Figure 14C**) ubiquitin spiked in urine from the same person was detectable down to 0.1pmol/µL or 100000pmol/L, respectively, but not anymore at 0.01pmol/µL or 10000pmol/L, respectively. The detection threshold (grey box) lies is the same range than the one for β2-microglobulin. **C**, As an example for chemokine concentration in urine, IP-10 is shown, which was measured by ELISA technology. The bottom box indicates the normal range in healthy individuals, middle boxes represent values measured during allograft rejection. The anticipated detection threshold for IP-10 by SELDI-TOF-MS (grey box) is 100-1000 times higher than the measured values during rejection and 10000-100000 times higher than normal values.

detection threshold can be approximated (**Figure 15A, red boxes**). To illustrate the importance of knowing the detection threshold, an example with the chemokine IP-10 in urine is described. Normal values measured by ELISA are 1-20ng/L; during allograft rejection concentrations up to 1μg/L have been reported (61). Even the later concentrations are 100-1000 times below the anticipated detection threshold of 100μg-1mg/L based on the experimental evidence from proteins in the same molecular weight range (Ubiquitin and β2-microglobulin).

5.2) Detection of urine proteins associated with acute renal allograft rejection

5.2.1) Patient characteristics

From July 1997 to March 2003, 2400 serial mid-stream urine samples from 212 renal transplant patients were collected. These 212 patients underwent a total of 693 protocol or clinically indicated core needle allograft biopsies. Based on allograft function, the clinical course and the allograft biopsy result, four rigidly defined patient groups were extracted from the whole patient population (n=212) as follows:

[1] Stable transplant group: Consists of 22 mid-stream urine samples (from 22 patients) obtained immediately before a protocol renal allograft biopsy performed within the first 12 months post-transplant. None of these patients had experienced DGF. All had stable allograft function (i.e. serum creatinine within 110% of baseline value at the time of biopsy), and none experienced a clinical or protocol biopsy-proven rejection prior to the

date of examination. All biopsies met the criteria for adequacy and all were required to have an acute and chronic Banff score of zero (i.e. ai0t0v0g0 and ci0t0v0g0).

[2] Acute clinical rejection group: Consists of 18 mid-stream urine samples (from 18 patients) obtained immediately before a renal allograft biopsy performed within the first 12 months post-transplant. All experienced an elevation in creatinine >110% from baseline and the diagnosis of acute rejection required an acute Banff score ≥ai2t2v0g0. Patients with a chronic Banff score >ci1t1v0g0 were excluded in order to avoid chronic allograft nephropathy as a confounding variable in the analysis.

[3] Acute tubular necrosis (ATN) group: Consists of 5 mid-stream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed within the first 6 days post-transplant to diagnose the cause of delayed graft function (DGF), which was defined as the need for hemodialysis within the first week or a drop of serum creatinine <50% from pre-transplant levels by day 5 post-transplant. Antibody mediated rejection was excluded based on a negative flow-crossmatch, and histological changes on the biopsy consistent with ATN. In all biopsies, the acute Banff score was ai0t0v0g0 and significant donor pathology was excluded by requiring a chronic Banff score of ≤ci1t1v0g0.

[4] Recurrent (or de novo) glomerulopathy group: Consists of 5 mid-stream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed to diagnose the cause of proteinuria (≥1.5g/day). The patients had diagnoses of membranous glomerulonephritis, focal-segmental glomerulosclerosis or IgA-nephropathy and all had acute Banff scores ≤ai1t1v0g0.

The acute clinical rejection group had more HLA-mismatches and a higher mean serum creatinine level at the time of the renal allograft biopsy compared to the stable transplant group. Otherwise, there were no significant differences between these groups (**Table 4**).

5.2.2) Non-transplanted control group characteristics

Normal control group: Consists of 28 mid-stream urine samples from 28 healthy individuals (14 female and 14 male, age 20-50 years).

Urinary tract infection (UTI) group: Consists of 5 mid-stream urine samples from 5 females obtained during an episode of a lower UTI, which was defined as requiring the clinical symptoms of a UTI, a leukocyte count in the urine sediment >40/high power field and a positive bacterial culture (>10^8 colony forming units).

5.2.3) Characterisation of urine protein profiles associated with individual patient groups

In the m/z range from 5000 to 12000 we observed two distinct urine protein patterns when comparing the normal control group or stable transplant group to the acute clinical rejection group. One urine protein profile (rejection pattern) had prominent peak clusters in three regions corresponding to m/z values of 5270-5550 (Region I; 5 peaks), 7050-7360 (Region II; 3 peaks), and 10530-11100 (Region III; 5 peaks) that always occurred together, whereas the other urine protein profile (normal pattern) had no peak clusters in these m/z regions (**Figure 16**). All 28 urine samples (100%) from the normal control group, 18 of 22 urine samples (82%) from the stable transplant group, and 1 of 18 urine samples (6%) from the acute clinical rejection group showed the normal pattern.

Variable	Stable transplant (n=22)	Acute clinical rejection (n=18)	ATN (n=5)	Recurrent or *de novo* glomerulopathy (n=5)
Female Sex – no. (%)	12 (55)	6 (33)	2	2
Age – mean ± SD	45±13	43±10	40±18	47±9
Caucasian Race – no. (%)	14 (64)	15 (83)	3	5
Nephropathy				
- Diabetic – no. (%)	6 (27)	3 (17)	1	0
- Glomerulonephritis – no. (%)	6 (27)	6 (33)	3	4
- others – no. (%)	10 (46)	9 (50)	1	1
First transplant – no. (%)	21 (95)	16 (89)	5	4
Cadaveric donor – no. (%)	15 (68)	10 (56)	3	5
HLA-mismatches – median (range)	3 (1-5)	4 (2-5) [a]	3 (2-4)	3 (3-5)
Panel-reactive antibodies (PRA)				
- Peak PRA >10% – no. (%)	2 (9)	0	0	1
- Current PRA >10% – no. (%)	1 (5)	0	0	1
Cytomegalovirus serology	3 (14)	3 (17)	1	1
- Recipient neg. / Donor pos. – no. (%)	7 (32)	4 (22)	0	2
- Recipient neg. / Donor neg. – no. (%)	4 (18)	9 (50)	3	1
- Recipient pos. / Donor pos. – no. (%)	8 (36)	2 (11)	1	1
- Recipient pos. / Donor neg. – no. (%)				
Allograft biopsy				
- Week post transplant – median (range)	8 (3-51)	8 (1-18)	day 5 or 6 [d]	253 (7-442)
- Rejection type (Banff 1997)		7 (39)		
- IA (moderate tubulitis) – no. (%)		8 (44)		
- IB (severe tubulitis) – no. (%)		3 (17)		
- IIA (moderate arteritis) – no. (%)				
Creatinine at biopsy [μmol/L] – mean ± SD	91±26	180±59 [b]	942±80 [e]	122±29
- % above baseline – median (range)		25 (11-76)		
Proteinuria at biopsy [g/L] – median (range)	0.07 [c] (0.03-0.17)	0.09 (0.03-0.28)		3.20 (0.58-6.00)

[a] P=0.003 vs. stable transplant group
[b] P<0.001 vs. stable transplant group
[c] P=0.14 vs. acute clinical rejection group. P<0.001 vs. recurrent or *de novo* glomerulopathy group
[d] Not included for statistical analysis
[e] Not included for statistical comparison (3 of 5 patients were on hemodialysis)

Table 4. Patient characteristics.

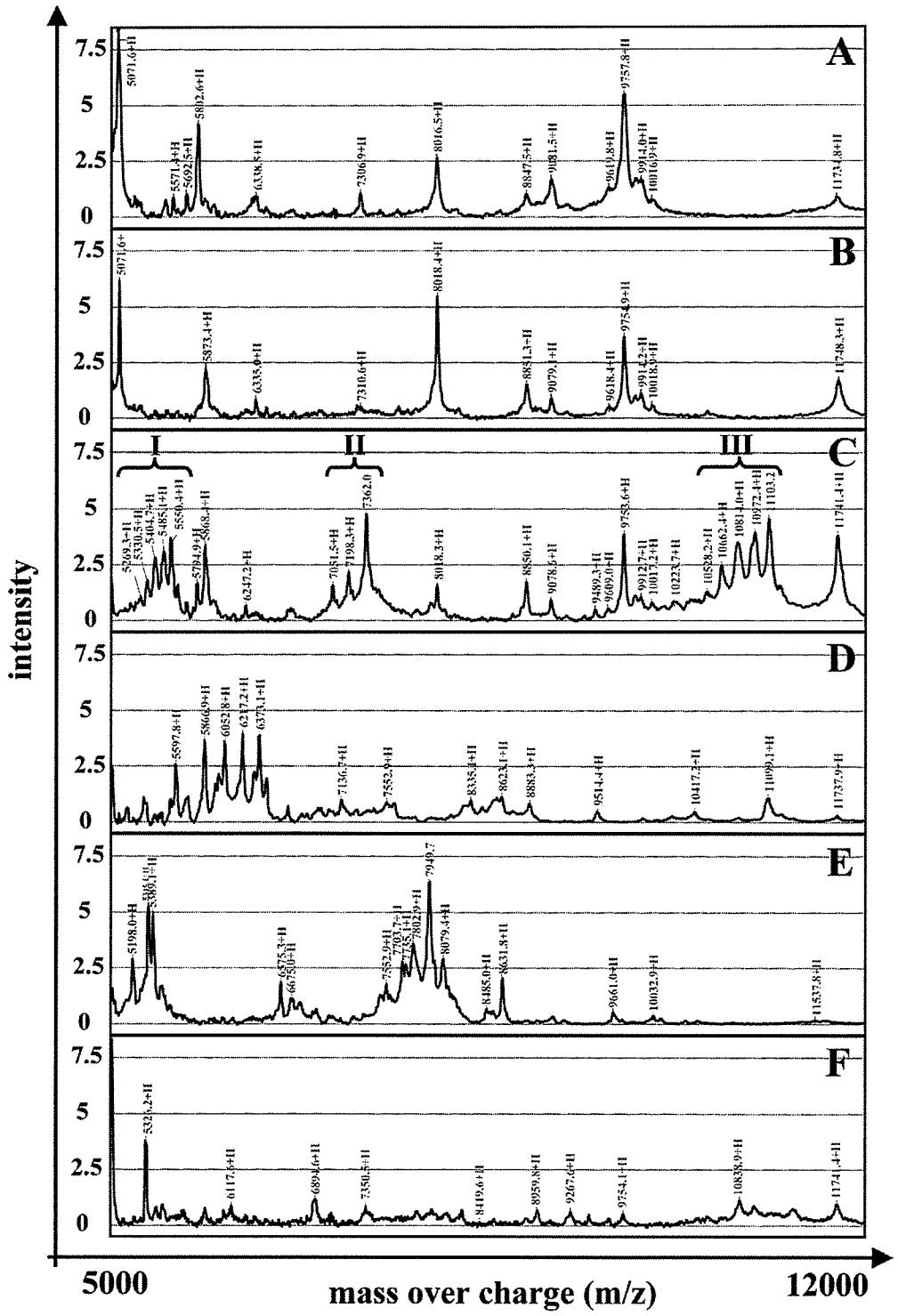


Figure 16. Representative urine protein profiles. **A**, Normal control with normal pattern. **B**, Stable transplant with normal pattern. **C**, Acute clinical rejection with rejection pattern. **D**, Glomerulopathy. **E**, ATN. **F**, UTI. The rejection pattern had prominent peak clusters in three regions corresponding to m/z values of 5270-5550 (Region I; 5 peaks), 7050-7360 (Region II; 3 peaks), and 10530-11100 (Region III; 5 peaks).

The rejection pattern was detected significantly more often in the acute clinical rejection group (17 of 18; 94%) than in the stable transplant group (4 of 22; 18%) ($p<0.0001$) (**Figure 17**). The ATN, the recurrent (or *de novo*) glomerulopathy and the UTI groups had urine protein profiles that were different from both the normal and the rejection pattern (**Figure 16 and Figure 17**).

5.2.4) Influence of CMV-viremia on urine protein profile pattern

Twenty-seven of 40 patients (68%) in the stable transplant and acute clinical rejection groups were tested for the presence of CMV-viremia at the time of renal allograft biopsy. Five patients tested positive; however none had or developed CMV-disease subsequently. CMV-viremia was found in 2 of 21 patients (10%) with the rejection pattern and in 3 of 19 patients (16%) with the normal pattern ($P=0.83$) (**Table 5**). We could not detect any additional peaks in the urine protein profiles from patients who had CMV-viremia.

5.2.5) Sequential urine protein profile analysis

To further determine the specificity of the normal and rejection pattern, we examined serial urine protein profiles in the stable transplant and acute clinical rejection groups and correlated them with the clinico-pathological course of the renal allograft. In particular we were interested in four specific outcomes: [1] the stable course persisted; [2] the stable transplant patient subsequently had an acute clinical rejection; [3] acute clinical rejection resolved to a stable course; [4] acute clinical rejection recurred.

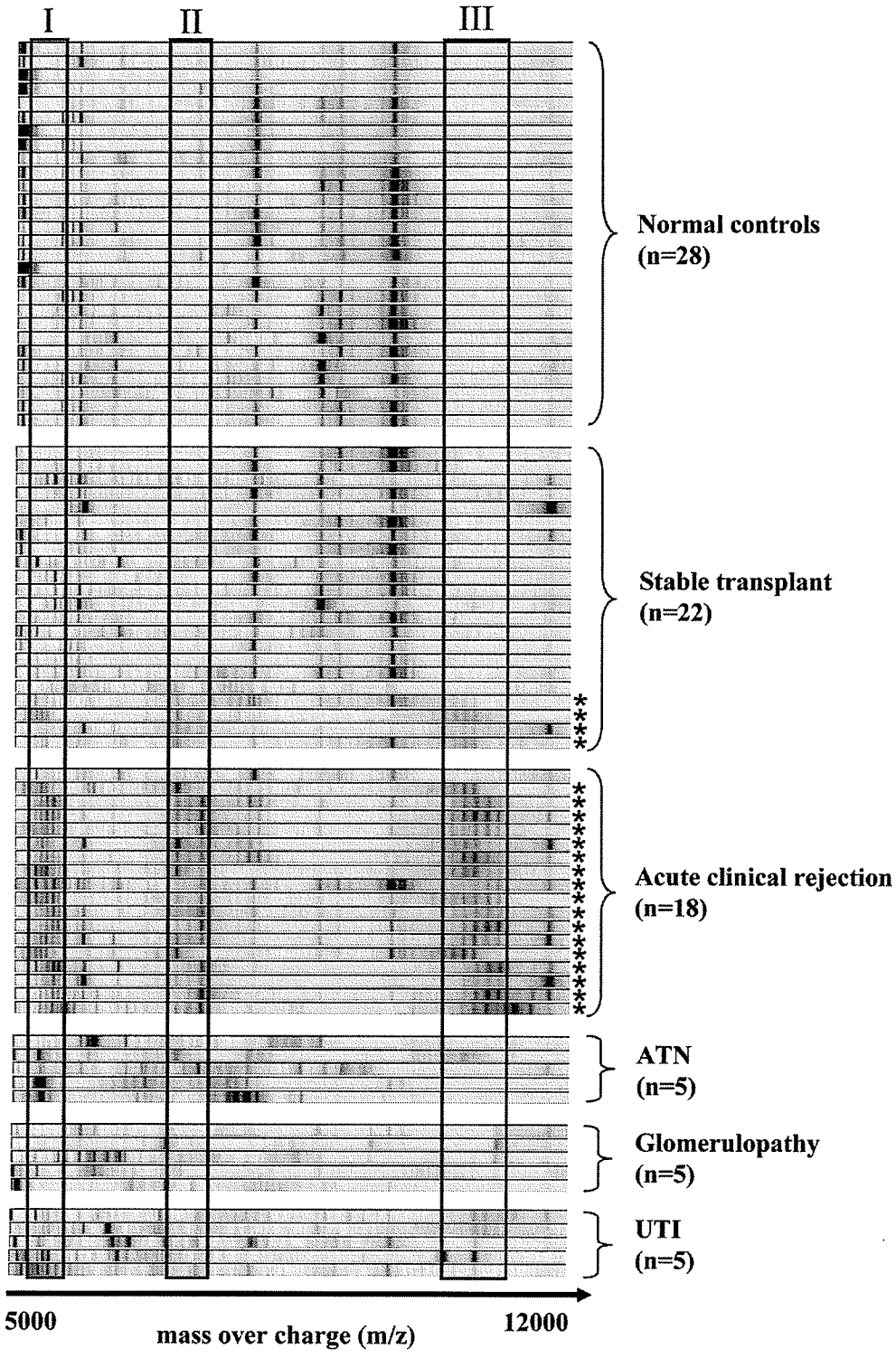


Figure 17. Software generated gel-view of urine protein profiles from all groups. Box frames represent the three regions corresponding to m/z values of 5270-5550 (Region I), 7050-7360 (Region II), and 10530-11100 (Region III). * indicates a urine sample with the rejection pattern.

CMV-viremia	Normal pattern (n=19) [a)]	Rejection pattern (n=21) [b)]	P-value
CMV-DNA positive – no.	3	2	
CMV-DNA negative – no.	10	12	P=0.83
No CMV-PCR available – no.	6 [c)]	7 [d)]	

[a)] Consists of 18 patients from the stable transplant group plus 1 patient from the acute clinical rejection group

[b)] Consists of 4 patients from the stable transplant group plus 17 patients from the acute clinical rejection group

[c)] CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (n=2); test was not ordered (n=3); or only CMV pp65-antigen was evaluated (n=1; patient tested negative)

[d)] CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (n=3); test was not ordered (n=3); or only CMV pp65-antigen was evaluated (n=1; patient tested negative)

Table 5. Correlation between CMV-viremia and urine protein pattern.

In the stable transplant group, we had sufficient urine and histology samples for sequential analysis to evaluate 12 of the 18 patients that originally had a normal pattern (**Figure 18**). One patient went on to have stable allograft function and two normal protocol biopsies, but the urine profile could not be classified. One patient developed acute clinical rejection (Banff type IA) and the urine protein profile changed from the normal to the rejection pattern. In 10 patients stable allograft function persisted and 20 subsequent protocol biopsies were interpreted as normal (n=18) or borderline rejection (n=2). Eight of these 10 patients showed the normal pattern throughout (**Figure 19A**), whereas two patients exhibited the rejection pattern in a single urine sample that subsequently reverted to the normal pattern.

In the acute clinical rejection group, we had sufficient urine and histology samples for sequential analysis to evaluate 12 of the 17 patients that originally had a rejection pattern (**Figure 18**). One patient had two subsequent normal protocol biopsies, but the creatinine remained elevated at the level seen during the acute rejection episode (20% above baseline) and the urine always showed the rejection pattern. In 6 patients the allograft function returned to baseline and subsequent protocol biopsies were interpreted as normal (n=3) or borderline rejection (n=3). All urine samples from these patients changed to the normal pattern (**Figure 19B**). Five patients had further episodes of acute clinical rejections and all of them kept the rejection pattern throughout (**Figure 19C**).

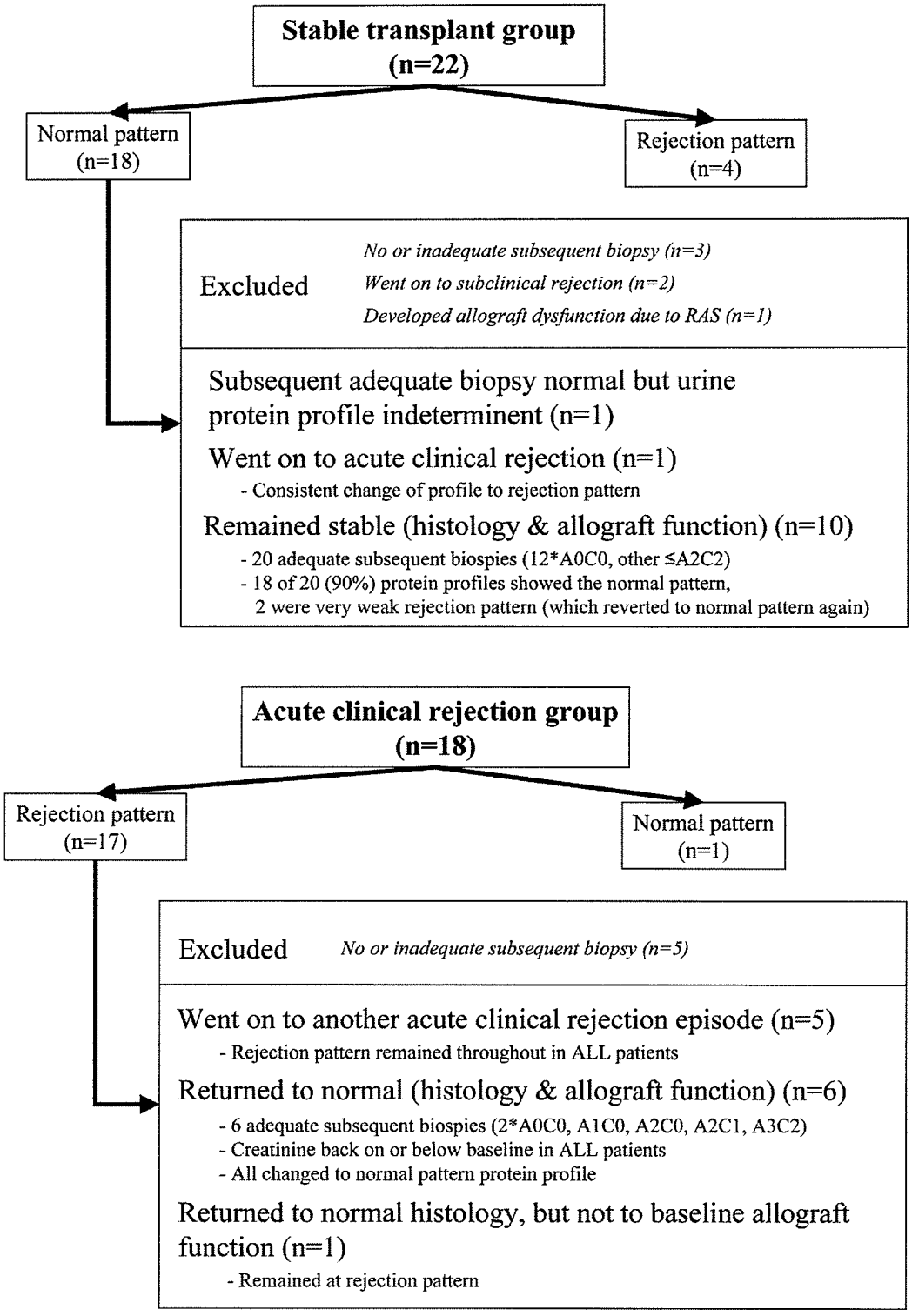


Figure 18. Patients eligible for sequential profiling analysis. Eleven of 22 (50%) in the stable transplant group, and 12 of 18 (66%) in the acute clinical rejection group were eligible for sequential analysis. Exclusion criteria were mainly missing subsequent urine or biopsy samples.

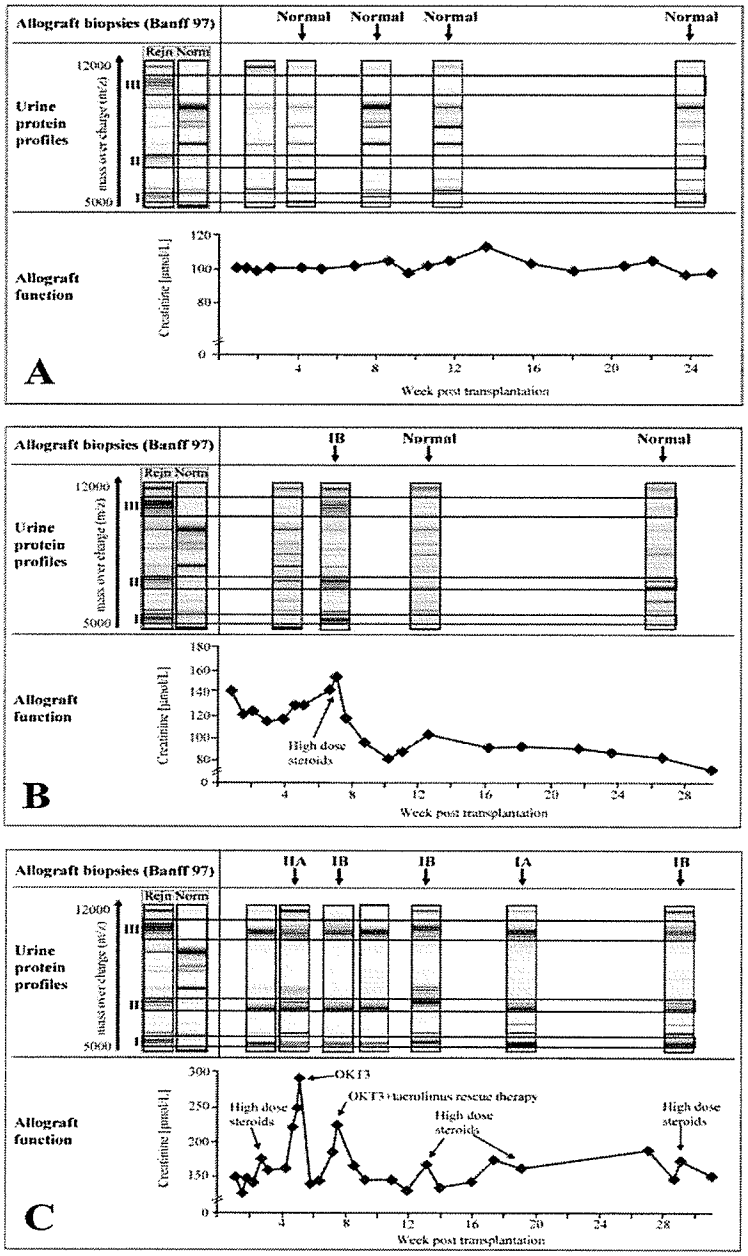


Figure 19. Sequential urine protein profiles in representative patients. Urine protein profiles highlighted in the grey box are examples of the rejection (Rejn) and the normal (Norm) pattern for comparison. Black box frames indicate the three regions corresponding to m/z values of 5270-5550 (Region I), 7050-7360 (Region II), and 10530-11100 (Region III). **A**, Patient with stable allograft function, normal protocol allograft biopsies, and normal pattern urine protein profiles throughout the post-transplant course. **B**, Patient with acute clinical rejection (Banff IB) on week 7 post-transplant. After treatment with high dose oral steroids the serum creatinine normalized and remained stable. Subsequent allograft biopsies were normal. The urine protein profile showed the normal pattern 3 week prior to the rejection episode, changed to the rejection pattern at the time of rejection, and returned to the normal pattern consistent with the subsequent allograft biopsies and the allograft function. **C**, Patient with recurrent acute clinical rejection episodes (Banff IA to IIA). Despite treatment with OKT3, high dose steroids and increased baseline immunosuppression, the patient always exhibited the rejection pattern.

5.2.6) Impact of intrinsic factors on the detection of the rejection pattern

As our urine samples from the transplanted patients were stored non-centrifuged at –80C, leading to subsequent cell lysis due to freeze-thawing, the analysed urine samples will contain intracellular proteins from cells present in the urine. To investigate whether the release of intracellular proteins of red blood cells (RBC), leucocytes and tubular epithelial cells due to freeze-thawing is responsible for generating the rejection pattern, we compared an 'acute clinical rejection' urine sample frozen with and without pre-centrifugation (**Figure 20**). In this case, the urine sample was collected from a clinically rejecting patient at day 6 post-transplant. The patient had persistent hematuria since the time of transplantation, which can be easily seen by the peaks consistent with hemoglobin in the non-centrifuged sample. In the pre-centrifuged sample these peaks almost disappeared and the rejection pattern could be clearly seen. Based on this documented case we suppose that the rejection pattern proteins are not intra-cytoplasmatic proteins that are released after disruption of the cell membrane due to freeze-thawing. In addition, this experiment is a good example of ion suppression due to high abundant proteins (i.e. hemoglobin in this sample).

5.3) Identification of proteins associated with acute renal allograft rejection

We first determined the pI of the rejection pattern proteins in order to subsequently use an extraction method (i.e. ion-exchange beads) as an initial step to concentrate the target proteins. With the used of cation- and anion-exchange beads we estimated the pI to be

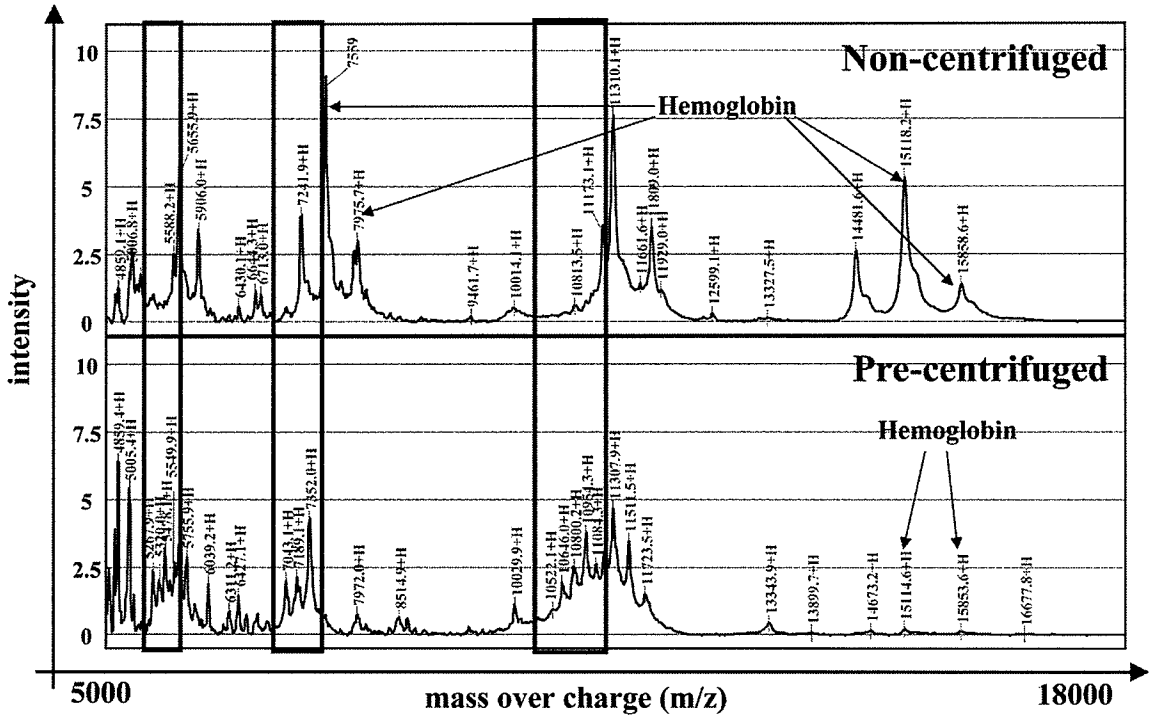


Figure 20. Impact of cell lysis due to freeze-thawing on the detection of the rejection pattern. These spectra show urine from a patient with hematuria and acute clinical allograft rejection. Peaks consistent with hemoglobin were detectable in the non-centrifuged sample. In the pre-centrifuged sample these peaks almost disappeared and the rejection pattern could be clearly seen. Based on this documented case we suppose that the rejection pattern proteins are not intra-cytoplasmatic proteins that are released after disruption of the cell membrane due to freeze-thawing. Box frames indicate the regions of interest with the rejection pattern.

around 7.0 (**Figure 21**). This allowed binding the target protein to anion-exchange beads at a pH 6.2, and subsequently eluting them with potassium as a counter-ion. This initial step resulted not only in a substantial concentration of the target proteins (100 fold) but also in a decrease of the complexity of the sample. Indeed, many major components of the urine proteome could be separated out, as their pI's are below that of the target proteins (e.g. albumin: pI 5.67; retinal-binding protein: pI 5.27) (**Figure 22**).

Additional and final purification was achieved by RP-HPLC, where the target proteins could be almost perfectly separated (**Figure 23**). After in-solution digestion, the target proteins were identified by LC-MS and LC-MS/MS as a cleaved form of β2-microglobulin. Every single peptide covering the whole sequence (except for the cleaved fragment F62YLLYYT68) was found and all amino acid sequences of the peptides were confirmed by MS/MS. In addition, eleven non-tryptic cleavage sites were observed (**Figure 24**). Based on the protein fragments detected by SELDI-TOF-MS with the highest intensities, the main non-tryptic cleavage sites may be S61, T68 and E69, resulting in two protein fragments with a predicted molecular weight of 10653.93Da and 10783.05Da, respectively. The observed masses by SELDI-TOF-MS are 10650.7Da (-3.2Da) and 10782.1Da (-1Da). By theoretically removing the disulphide bond (C25-C80) from the S61-, T68- and E69-cleaved β2-microglobulin, three peptides will result:

[I]

I1QRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSF SKDWS61 with a predicted molecular weight of 7047.83Da (SELDI-TOF-MS mass 7042.9Da (-4.9Da))

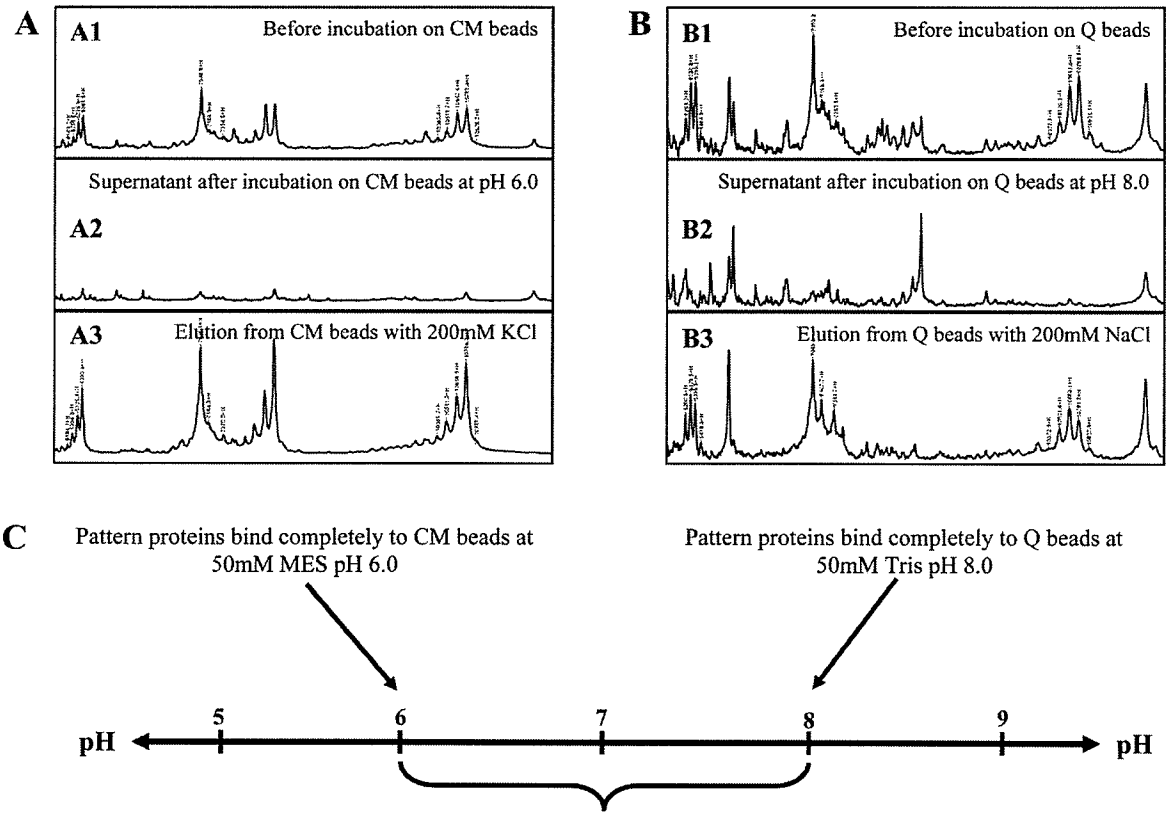


Figure 21. Determination of pI of pattern proteins. [A] Urine sample with the pattern proteins was dialysed against 50mM MES pH 6.0 (profile A1) and then incubated with cation-exchange beads. The supernatant was checked for the presence of the pattern protein (profile A2). Subsequently, proteins were eluted from the beads with increasing KCl concentrations. The major fraction containing pattern proteins eluted with 200mM KCl (profile A3). **[B]** Urine sample with the pattern proteins was dialysed against 50mM Tris pH 8.0 (profile B1) and then incubated with anion-exchange beads. The supernatant was checked for the presence of the pattern protein (profile B2). Subsequently, proteins were eluted from the beads with increasing NaCl concentrations. The major fraction containing pattern proteins eluted with 200mM NaCl (profile B3). **[C]** Proteins bind completely to ion exchange sorbents about one pH unit below (cation-exchange sorbents) or above (anion-exchange sorbents) their pI. Therefore, the pI of the pattern proteins can be estimated to be around 7.0.

I

Protein	MWt [kDa]	Theoretical pI	Percent of total protein*
Uromodulin	85-1000	5.05	
Transferrin	79.6	6.8	2
Albumin	66.0	5.67	
α1-Microglobulin	39	5.95	
Ig light chains (and IgG)	23 (150)	5.75	
Retinol-binding protein	20.9	5.27	
Cystatin C	13.3	8.75	
β2-Microglobulin	11.7	6.07	
Pattern proteins	7-11	7.0-7.2	
Hepcidin, α- & β-Defensins	2.1-5.1	>8.2	<1

* Based on healthy individuals

II

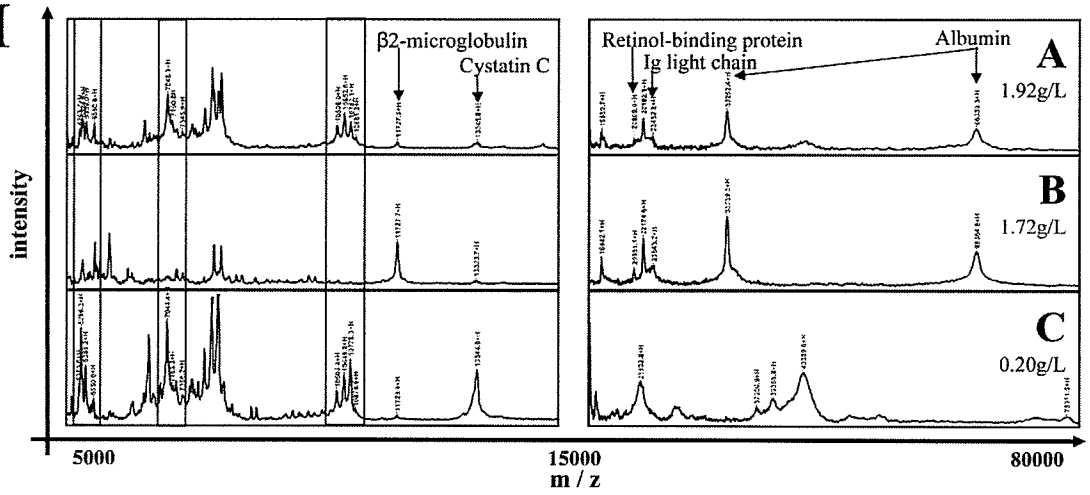


Figure 22: First step of purification of pattern proteins with cation-exchange beads. **[I]**, Major components of the urine proteome, their pI's and molecular weights. Red boxes indicate proteins which should not or only partially bind to cation-exchange beads incubated at pH 6.2 due to their pI's. **[II]**, Purification of pattern proteins on cation-exchange beads at pH 6.2. The box frames indicate the pattern proteins. They are present before incubation to cation-exchange beads (profile A). The supernatant after incubation on cation-exchange beads shows many of the major components of the urine proteome but not the pattern proteins (profile B). These elute with 200mM KCl, notably without a significant contamination with albumin, Ig light chain, retinol-binding protein and β2-microglobulin (profile C). As expected from their contribution to the total protein content of urine, the purification on the cation-exchange beads at pH 6.2 resulted in a significant decrease of the total protein concentration in the elution fraction from 1.92g/L (before incubation on beads, A) to 0.2g/L (C) (measured with the BCA protein assay, Pierce, Rockford, IL, USA). Therefore, the use of the cation-exchange beads as a first step of purification can not only concentrate the pattern proteins but can also separate them from many of the major protein components in urine (e.g. albumin, Ig light chain, retinol-binding protein, β2-microglobulin).

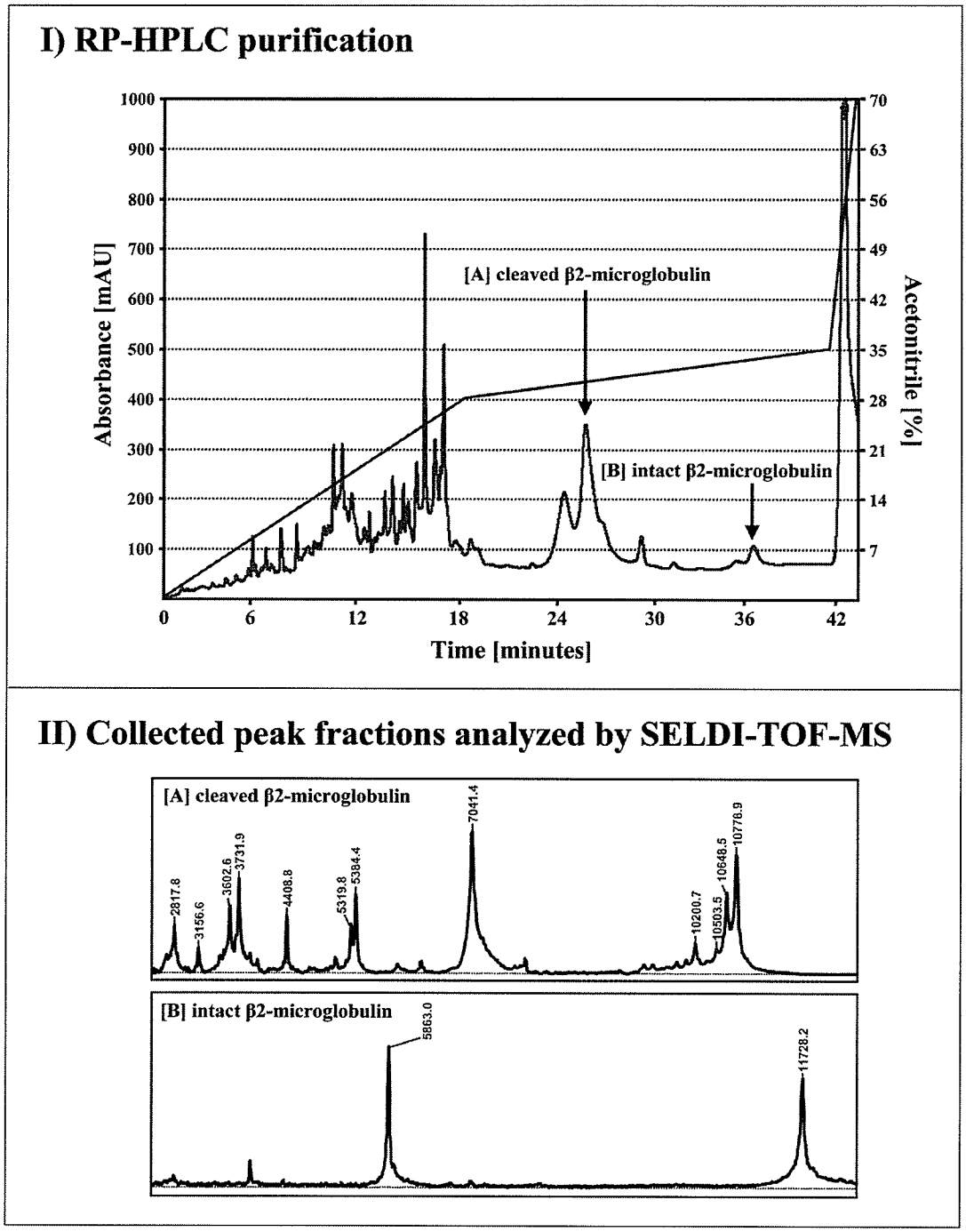
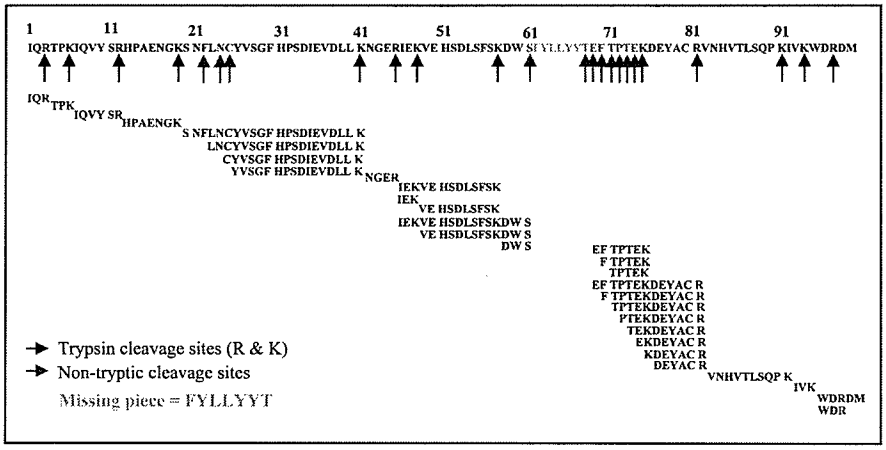


Figure 23: Second step of purification by RP-HPLC. [I], Concentrated and partially purified sample after cation-exchange separation was applied to RP-HPLC. The peak fraction containing the rejection pattern proteins (i.e. cleaved β2-microglobulin) eluted at 31% acetonitrile. Intact β2-microglobulin eluted at 33% acetonitrile. **[II]**, Collected peak fractions were analyzed by SELDI-TOF-MS. Fraction [A] confirmed the presence of the rejection pattern proteins (subsequently identified as cleaved β2-microglobulin), whereas fraction [B] showed the intact β2-microglobulin.

A



B

AS #	Fragments	Mass calculated (Da)	Mass found by MS (Da)	Mass found by LC-MS (Da)	Sequence confirmed by MS/MS	Comment
1-3	IQR	416.262	-	416.266	+	N-terminal peptide
4-6	TPK	345.214	-	345.213	-	
7-12	IQVYSR	765.426	765.431	765.43	+	
13-19	HPAENGK	752.369	753.358	753.354	+	N17 deamidation
20-41	SNFLNCYVSGFHPSDIEVDLLK	2554.229	2554.244	2554.24	+	
23-41	LNCYVSGFHPSDIEVDLLK	2206.085	2206.101	2206.112	+	non-tryptic
25-41	CYVSGFHPSDIEVDLLK	1978.958	1978.964	1978.972	+	non-tryptic
26-41	YVSGFHPSDIEVDLLK	1818.928	1818.936	1818.943	+	non-tryptic
42-45	NGER	475.226	-	476.217	+	N42 deamidation
46-58	IEKVEHSDLSFSK	1518.78	-	1518.79	+	
49-58	VEHSDLSFSK	1148.559	1148.561	1148.566	+	
46-48	IEK	389.24	-	389.247	+	
46-61	IEKVEHSDLSFSKDWS	1906.919	-	1906.927	+	non-tryptic
49-61	VEHSDLSFSKDWS	1536.697	-	1536.692	+	non-tryptic
59-61	DWS	407.157	-	407.16	+	non-tryptic
69-75	EFTPTEK	851.415	-	851.416	+	non-tryptic
70-75	FTPTEK	722.372	-	722.372	+	non-tryptic
71-75	TPTEK	575.304	-	575.298	+	non-tryptic
69-81	EFTPTEKDEYACR	1645.717	1645.721	1645.72	+	non-tryptic
70-81	FTPTEKDEYACR	1516.674	1516.681	1516.682	+	non-tryptic
71-81	TPTEKDEYACR	1369.606	1369.614	1369.614	+	non-tryptic
72-81	PTEKDEYACR	1268.558	-	1268.576	+	non-tryptic
73-81	TEKDEYACR	1171.505	-	1171.518	+	non-tryptic
74-81	EKDEYACR	1070.458	-	1070.461	+	non-tryptic
75-81	KDEYACR	941.415	-	941.424	+	non-tryptic
76-81	DEYACR	813.32	813.323	813.324	+	
82-91	VNHVTLSQPK	1122.627	1122.627	1122.631	+	
92-94	IVK	359.266	-	359.27	+	
95-99	WDRDM	722.293	-	722.297	+	C-terminal peptide
95-97	WDR	476.226	-	476.226	+	

Figure 24: Identification of the rejection pattern proteins as cleaved β2-microglobulin. Identification by LC-MS and MS/MS confirmed the rejection pattern proteins as a cleaved form of β2-microglobulin. All peptides covering the whole β2-microglobulin sequence were detected with the exception of the non-trypsin cleaved fragment F62YLLYYT68 (A). MS/MS confirmed the amino acid sequence of all detected peptides (B). Non-tryptic cleavages sites were found at F22, N24, C25, S61, T68, E69, F70, T71, P72, T73 and E74.

[II] E68FTPTEKDEYACRVNHVTLSQPKIVKWDRDM99 with a predicted molecular weight of 3737.22 Da (SELDI-TOF-MS mass 3733.0Da (-4.2Da))

[III] F69TPTEKDEYACRVNHVTLSQPKIVKWDRDM99 with a predicted molecular weight of 3608.10 Da (SELDI-TOF-MS mass 3603.6Da (-4.5Da))

Therefore, these three cleavage sites combined with or without the theoretical removal of the disulphide bond explain five of the seven major peaks detected by SELDI-TOF-MS (**Figure 25A**). The remaining two peaks at 5322.6Da and 5387.2Da are consistent with the double changed ions of the 10650.7Da and 10782.1Da protein fragments. This explanation is further supported by the disappearance of the SELDI-TOF-MS peaks at 5322.6Da, 5387.2Da, 10650.7Da and 10782.1Da after reduction and alkylation, while maintaining the peaks at 7042.9Da, 3733.0Da and 3603.6Da (with an additional ~65Da due to reaction with iodoacteamide) (data not shown).

However, the initial described rejection pattern had prominent peak clusters at 5.27-5.55kDa (5 peaks), 7.05-7.36kDa (3 peaks), and 10.53-11.1kDa (5 peaks). The unaccounted SELDI-TOF-MS peaks at 7.2kD and 7.36kD, as well as the concomitant appearing or disappearing peaks at 10.95kD and 11.1kD (double charged ions at 5.48kDa and 5.55kDa) can be explained by a different initial cleavage site (Y63 instead of S61) with subsequent partial removing of Y63 and F62 (**Figure 25B**). The two last remaining unaccounted peaks of the original rejection pattern (5.27kDa and 10.53kDa) can most likely be explained by removing F70, which was an observed cleavage site (**Figure 24**). However, this has not been proven in an experiment yet.

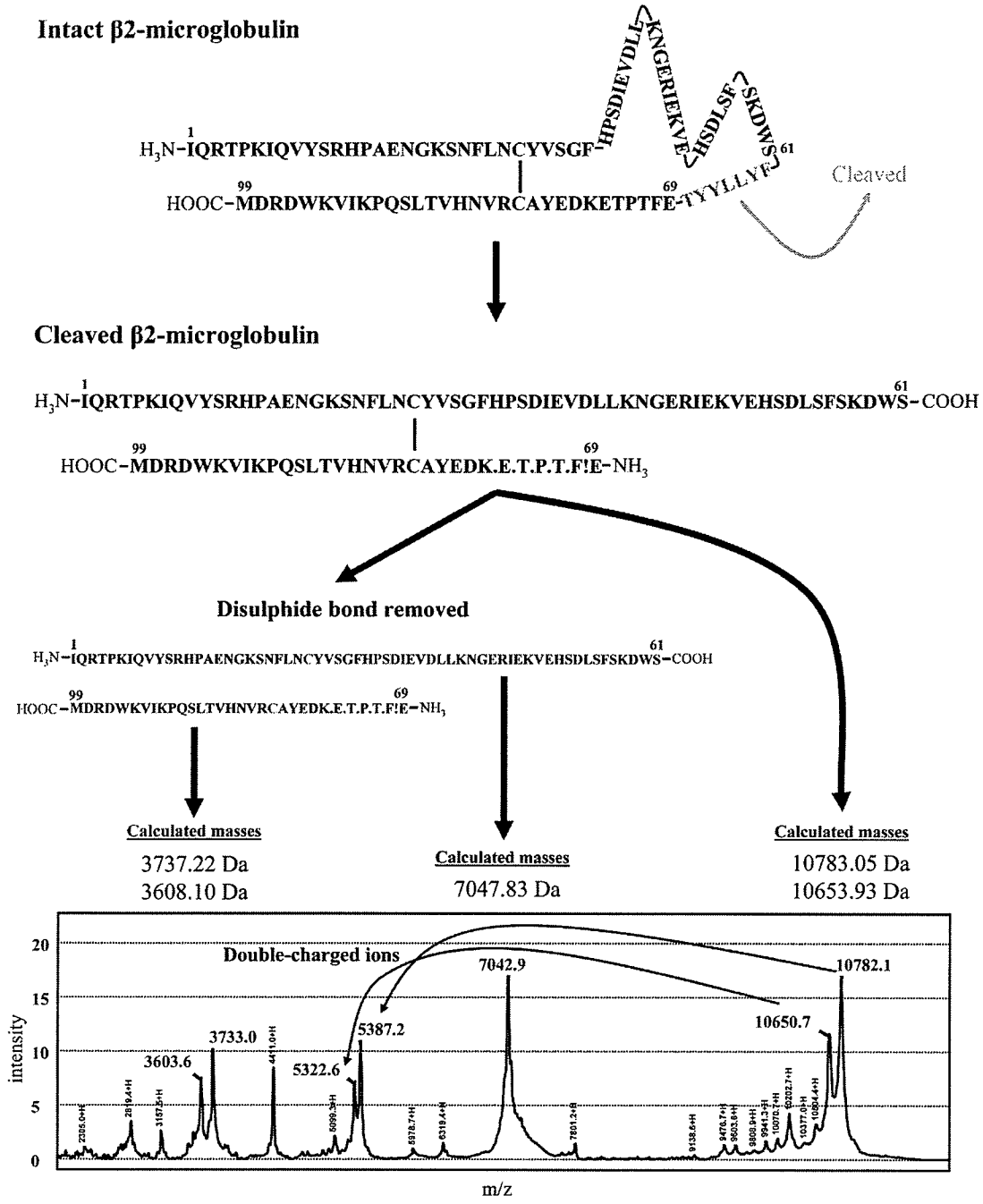


Figure 25A: Confirmed SELDI-TOF-MS detected peaks of the rejection pattern as deriving from cleaved β2-microglobulin. Initially, the peptide piece F62YLLYYT68 is cleaved from intact β2-microglobulin by proteinase(s) resulting in the cleaved form of β2-microglobulin. An additional major cleavage site at E69 leads to two major components with calculated masses of 10783.05Da and 10653.93Da, respectively. Calculated masses are within a few Da from the actual masses observed by SELDI-TOF-MS. During analysis by SELDI-TOF-MS (most likely due to energy application from the laser) the disulphide bond breaks in some proteins, resulting in the detection of fragments with actual SELDI-TOF-MS masses of 7042.9Da, 3733.0Da and 3603.6Da. These masses are consistent with the calculated masses of the resulting peptides after theoretically removing the disulphide bond of cleaved β2-microglobulin (7047.83Da, 3737.22Da and 3608.1Da, respectively). ! denotes major cleave site; . denotes additional observed cleavage sites, which no corresponding proteins were detectable by SELDI-TOF-MS.

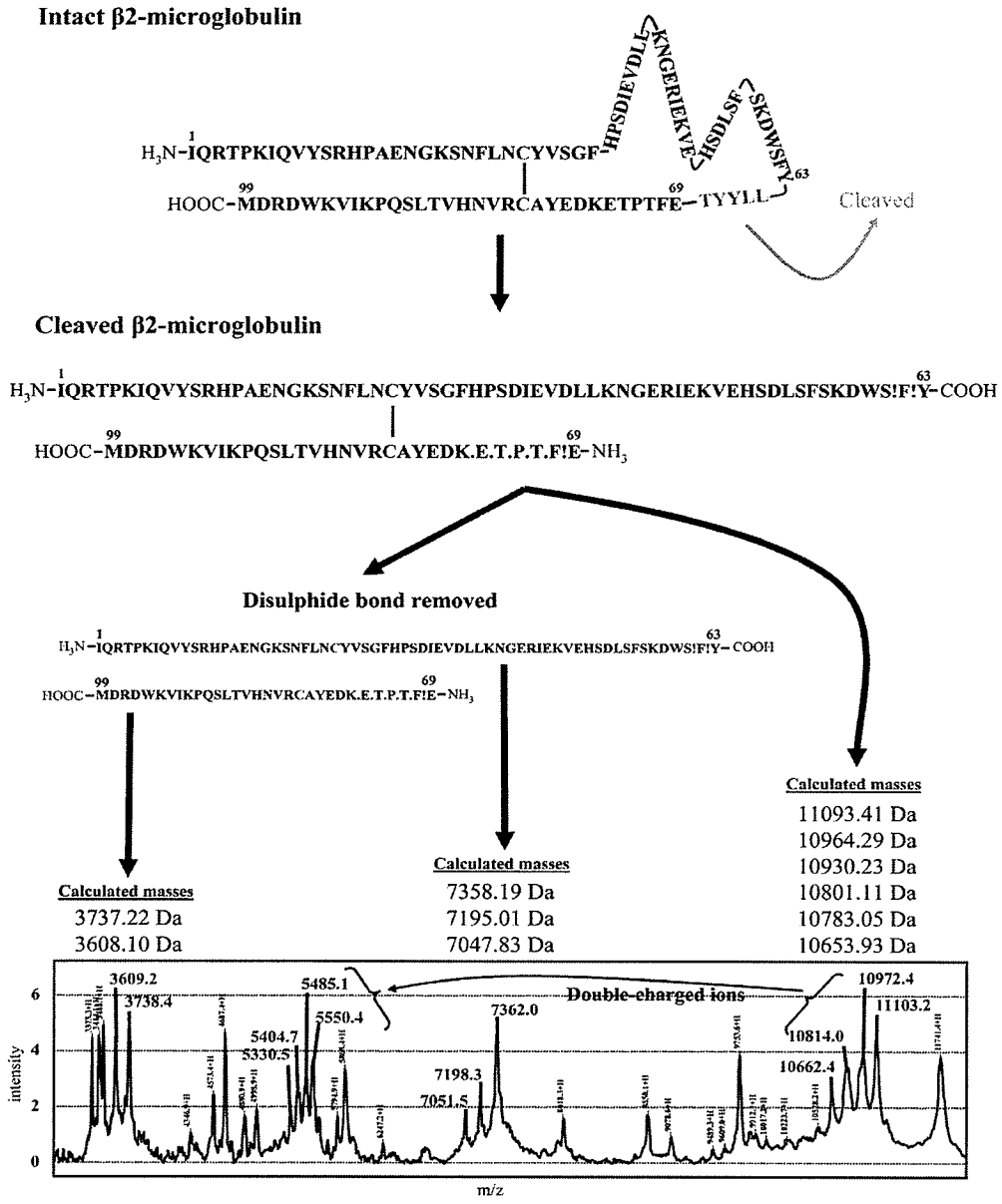


Figure 25B: Postulated fragmentation of β2-microglobulin explaining SELDI-TOF-MS detected peaks of the rejection pattern. As described in **Figure 16**, the rejection pattern consisted of five peaks at 5.27 to 5.55kDa, three peaks at 7.05 to 7.36kDa and five peaks at 10.53 to 11.1kDa. In the sample which the identification of cleaved β2-microglobulin was made from (**Figure 25A**), six peaks at 5.48, 5.55, 7.2, 7.36, 10.95 and 11.1kDa were not detected and accounted for. Based on the SELDI-TOF-MS detected β2-microglobulin fragments, we postulate the initial non-tryptic cleavage sites to be Y63 and T68 (removing L64LYYT68), which results in two chains connected by the disulphide bond (C25-C80). Thereafter additional partial cleavages occur at S61, F62 and E69. During analysis by SELDI-TOF-MS disulphide bonds can break resulting in the detection of the two single chains. The different forms of cleaved β2-microglobulin (with or without intact disulphide bond) explain 11 of 13 peaks contributing to the rejection pattern. The two last remaining unaccounted peaks of the original rejection pattern (5.27 and 10.53kDa) can be explained by removing F70, which was an observed cleavage site. ! denotes major cleave site; . denotes additional observed cleavage sites, which no corresponding proteins were detectable by SELDI-TOF-MS. The expected mass accuracy of SELDI-TOF-MS as given by the manufacturer is <0.1%. Most observed peaks are within this range compared to the calculated masses.

Chapter 6

6) Discussion

6.1) Urine protein profiling with SELDI-TOF-MS

6.2) Detection and identification of urine proteins associated with acute renal allograft rejection

6.1) Urine protein profiling with SELDI-TOF-MS

In order to use SELDI-TOF-MS as a high throughput urine protein profiling methodology, it is critical to define those factors that affect reproducibility, as well as identify the confounding variables that affect the detection of proteins that are known to be present in the sample.

6.1.1) Extrinsic factors

The most important extrinsic factors that influence reproducibility and peak detection are the matrix composition and the instrument settings. Matrix allows for efficient ionization and vaporization of proteins (83). The most popular matrices for the SELDI-TOF-MS system are SPA and CHCA. Saturated SPA is preferable for looking at masses above 10-20 kDa, while 10-20% CHCA provides the best resolution for proteins/peptides up to about 5 kDa. For urine protein profiling from 2-25 kDa, more peaks and a higher degree of resolution were observed with 35% CHCA. Instrument settings such as detector sensitivity, detector voltage, and laser intensity have to be determined individually. The higher the detector sensitivity and voltage or the laser intensity is, the better the detection of high mass proteins will be. This is accompanied by an increase in background noise, which limits detection of low intensity peaks.

The number of positions sampled on a spot is an important parameter for optimal peak detection. Ideally, all proteins are distributed homogeneously on the chip and are crystallized homogeneously in the matrix. If so, one would expect to generate the same spectra at every position. From the three spot sampling protocols it is clear, that there are

'hot positions', where proteins are clustered on the spot leading to the detection of an abundance of peaks with a high intensity. Similarly, there are 'cold positions', where only few or even no peaks are detected. Unfortunately, 'hot position' sampling may not accurately profile low abundant proteins due to ion suppression that can occur due to high abundance proteins. Therefore, the most representative spectra for a given urine sample is achieved by sampling many different spot positions and combining the data. This is especially true for dilute urine samples.

The stability of urine proteins under various storage conditions is important to know. Recent studies have found little or no changes in albumin-, retinol-binding protein-, N-acetyl glucosaminidase-, IgG- and kappa/lambda light chain concentrations after storage at room temperature, 4°C, -20°C and -70°C (84,85,86,87). Our experiments using SELDI-TOF-MS found that up to four freeze-thaw cycles at -80°C did not alter the urine protein profile significantly; thereafter, peak intensities became weaker. The protein profiles of all mid-stream urine samples remained almost unchanged after storage for three days at 4°C, whereas three of six first-void urines underwent major changes. First-void urine can have significant bacterial contamination resulting in either urine protein degradation and/or contamination with bacterial proteins within a few days.

6.1.2) Intrinsic factors

Mid-stream urine is the standard for almost all urine analysis. In a clinical setting, there are always urine samples that are not mid-stream urines. Therefore, knowing the variation in urine protein profiles that may occur between first-void and mid-stream urines is important. There were no clear differences between first-void and mid-stream urine in

males, whereas in females, variations were easily detectable. For example, a peak cluster between 3.3 and 3.5 kDa (consistent with the masses of the α-defensins) is present in female first-void urine in high intensity. This peak cluster is either absent or of low intensity in female mid-stream urine samples. Indeed, α-defensins, which are an important part of the human antimicrobial defense (88,89), have been detected by SELDI-TOF-MS technology in urine (90), as well as in culture supernatants of human $CD8^+$ T-cells (91). The differences in the protein profile between first-void and mid-stream urines may be explained by urethral secretion of these proteins, which are then washed away by the first-void urine. Therefore, consistent urine protein profiling requires mid-stream urine samples for analysis, because first-void urine has a different protein composition than mid-stream urine and is more prone to protein degradation.

Blood was observed to be a major confounding variable affecting the normal urine protein profile. Not only did new peaks appear (i.e. peaks consistent with the masses of hemoglobin and albumin), but many of the normal peaks observed became undetectable. This is likely due to ion suppression by the blood proteins. Notably, even with a dilution of 10 μL blood in 64 ml diluted urine (1:6400 dilution), the peaks consistent with hemoglobin remained dominant. Clearly, such contamination invalidates any interpretation of the urine protein profile. Although centrifugation of the urine sample removes RBC, contamination with serum proteins will still continue to confound the urine protein profile.

Depending on fluid intake the kidneys can concentrate urine to an output as low as 0.5 L/day, or dilute urine to almost 20 L/day. Under normal conditions, about 1-2L urine are excreted per day. In a very dilute urine sample (urine creatinine 0.9 mmol/L), most of the proteins could not be detected on a NP20-chip. The threshold for a stable urine protein

profile on a NP20-chip was a urine output of 2 L/day. Because every ProteinChip type has different binding capacities, the detection threshold has to be determined for every chip type individually.

6.1.3) Protein quantification and detection limits with SELDI-TOF-MS

Peak height and area under the peak have been used to reflect protein abundance (90,92). Although good correlation between the amount of a single protein alone or in a mixture with four other proteins and the peak intensity was found, it is questionable whether this remains true in a complex protein mixture (e.g. urine) due to many factors like ion suppression and competition for binding sites on the ProteinChips. Therefore, care should be taken in comparing relative peak heights between two different urine protein profiles as an indicator of change in protein abundance under different circumstances (i.e. normal versus pathologic state).

The detection of a protein by SELDI-TOF-MS is critically determined by its concentration in the sample, its binding to the chromatographic surface and its ionization process within the mass spectrometer. For single proteins, the detection threshold for α-defensins (3371Da) was 10-100ng/L (90), for ubiquitin (8565Da) was 100ng/L to 1μg/L, and for albumin (66500Da) was 1-6mg/L, respectively. The increased detection threshold for high molecular weight proteins is well known and thought to be related to inferior ionization of large proteins. In a complex protein mixture (e.g. urine, serum), however, the detection threshold increases by roughly 10-1000 fold compared to the detection threshold for single proteins. This decrease in sensitivity is mainly caused by competition for

binding sites (i.e. binding competition) on the ProteinChips and competition for ionization (i.e. ion suppression). Whereas the former is unique to the SELDI-TOF-MS platform, the later is a common problem for all mass spectrometers. By changing the conditions for protein binding to different chromatographic surfaces, some proteins may be selected and enriched, whereas others may be excluded, allowing the detection limit to drop. However, the detection limit might be at best 10 times above the detection threshold for a single protein.

Based on these experiments, the potentially detectable urine proteins by SELDI-TOF-MS can approximately be defined by their concentration and their molecular weight (**Figure 26**). This 'accessible' part of the proteome becomes even smaller when the inferior sensitivity of the SELDI-TOF-MS system for proteins above 25kDa is taken into account. Therefore, profiling strategies involving clinical samples where potential biomarkers are at a concentration and in a molecular weight range detectable by SELDI-TOF-MS may be more successful. Specifically, urine protein profiling using SELDI-TOF-MS may be sensitive enough to detect potential biomarkers in kidney diseases, because the affected cells 'drain' selectively into urine. In contrast, several groups use SELDI-TOF-MS for serum protein profiling in order to detect new biomarkers for early cancer detection (93,94). However, serum consists of a few high abundance proteins that account for 99% of the total protein amount (95), which may increase the detection threshold of SELDI-TOF-MS even above the one outlined for urine. It is therefore not surprising that identified potential cancer biomarker found by SELDI-TOF-MS were all in concentrations ranging form mg/L to g/L (96), representing more likely cancer epiphenomena (e.g. liver metabolism changes) than specific cancer related proteins.

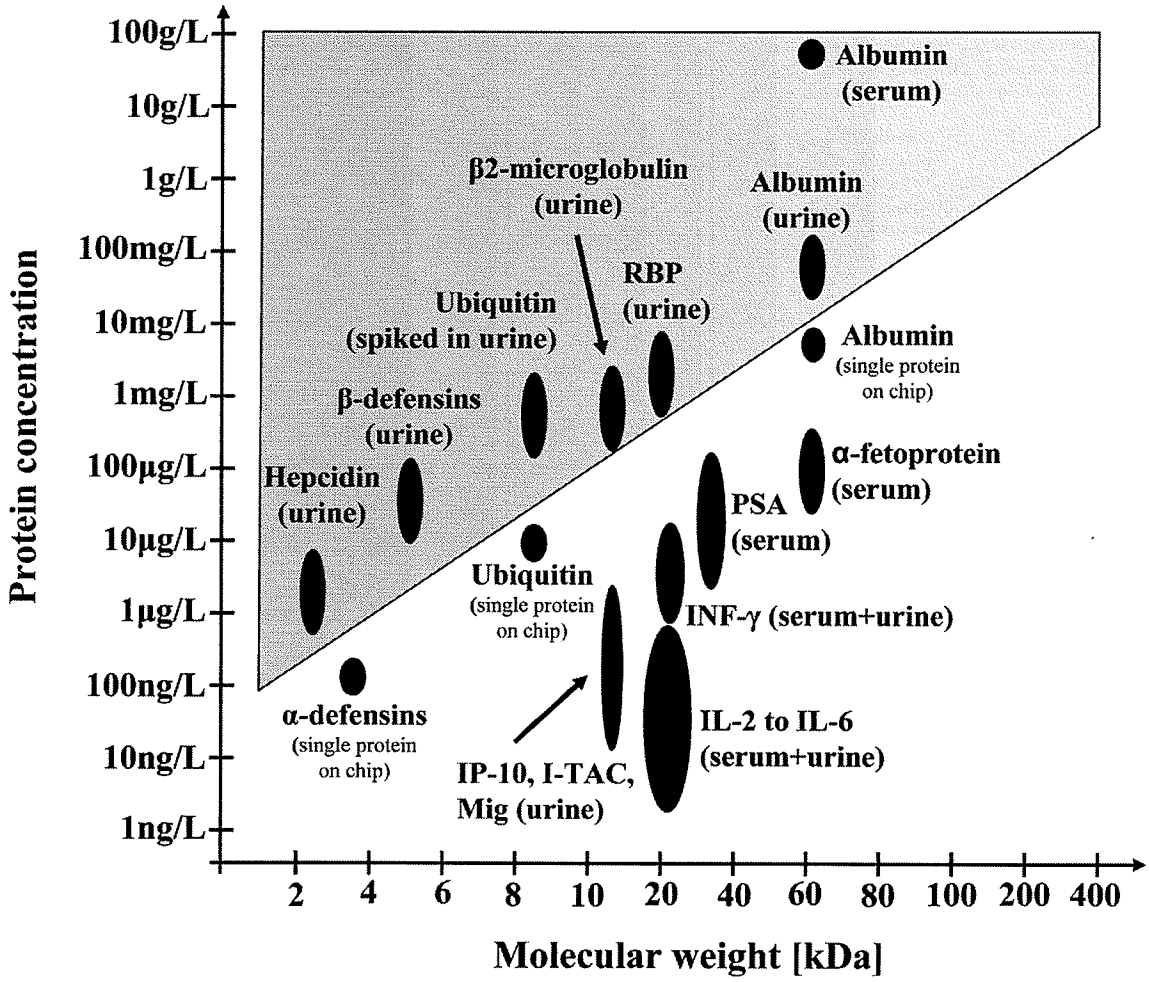


Figure 26. Detection limits of SELDI-TOF-MS. Ovals represent normal (hepcidin, β-defensins, β2-microglobulin, retinol-binding-protein (RBP) and albumin), and pathological concentrations (IL-2 to IL-6, INF-γ, IP-10, I-TAC, Mig, PSA and α-fetoprotein) of known urine and serum proteins measured by ELISA or equal quantitative tests. Hepcidin, β-defensins, β2-microglobulin and albumin are detectable by SELDI-TOF-MS in urine from healthy individuals. From dilution experiments, it is known that in a 1:4-1:16 urine dilution these proteins are not detectable anymore. In addition, ubiquitin (spiked in normal urine) was detectable until 100000pmol/L but not at 10000pmol/L. Therefore, the SELDI-TOF-MS-detection threshold of these proteins in urine can be approximated (light grey area). Single proteins (α-defensins, ubiquitin and albumin) are detectable by SELDI-TOF-MS at roughly 100 times lower concentrations than in urine. However, even this threshold is above the level of cytokines, chemokines and currently used tumour maker (i.e. PSA and α-fetoprotein). This graphic also shows the high dynamic range of urine and serum proteins, which spans over 7-10 log units.

In addition, it seems unlikely that a small tumor, which weighs only a few grams, produces proteins detectable in the mg/L to g/L range in serum. This is underscored by the serum protein concentration range of currently used cancer biomarkers, which is in the 0.1-100μg/L range (96).

6.1.4) Summary of advantages and limitations of SELDI-TOF-MS for urine protein profiling

SELDI-TOF-MS offers many advantages for protein profiling in urine. First, only 5 to 10 μL of sample is needed for one analysis. Second, due to the simple chip preparation, many samples can be analyzed quickly. Third, the washing step removes most of the salts, which otherwise interfere with mass spectrometric analysis. And fourth, the impact of different chromatographic chemistries can be analyzed, which may allow one to find optimal purification conditions for a protein of interest in a short time with small amounts of sample. However, sensitivity is moderate (especially in a complex mixture), and resolution above 25kDa is low, resulting in a limited part of the urine proteome been detectable by SELDI-TOF-MS. In addition, standardization of analysis conditions is essential, and both extrinsic and intrinsic factors must be taken into account for accurate data interpretation.

6.2) Detection of urine proteins associated with acute renal allograft rejection

We used a proteomic technique to determine whether the urine of renal transplant patients undergoing acute allograft rejection had a characteristic profile. As discussed above urine can be very heterogeneous making standardization of urine collection and storage critical (97). In the design of the current study, we therefore required mid-stream urines that were collected immediately before the allograft biopsy and were stored the same day at -70°C. Next it was necessary to determine the urine protein profile of a 'normal' kidney transplant, and this was done by selecting urines from patients with immediate and persistent good graft function that had normal graft histology on protocol biopsy. This stringently defined control group is unique as it includes histology; other groups attempting similar studies have inferred normal histology from a stable serum creatinine (58,57,98). Indeed, adherence to this stringent definition of 'normal' demonstrates that the urine protein profile from 18 of 22 patients (82%) in the stable transplant group was similar to the urine profile of normal non-transplanted individuals.

The reliable identification of the urine protein pattern of the normal kidney transplant allowed for the clear differentiation, on visual inspection alone, of a distinct urine protein profile in the group with acute rejection (Figure 2). Other groups have used SELDI-TOF-MS to compare the protein profiles between different clinical outcomes, but required bioinformatic analysis to assign protein peaks to a specific outcome (99,100). In a similar study to ours, Clarke et al. (99) reported differences in the urine profiles between rejection and stable transplants; however, their requirement of bioinformatics to do so may relate to the fact that their definition of 'stable' transplants was less stringent than ours (i.e. based on serum creatinine alone). Interestingly, the protein peaks reported in their

paper as specific to rejection, are different from those found by our group. This may be related to the different protein chip surfaces and experimental conditions that were utilized; but also, to the fact that Clarke et al. (99) failed to include any control populations (e.g. ATN, recurrent or *de novo* glomerulopathies, UTI, CMV) in the analysis, the importance of which is discussed below. In another study, Petricoin et al. (100) have used SELDI-TOF-MS to compare the protein profiles between different clinico-pathological diagnoses in cases of ovarian cancer, but also required bioinformatic analysis to assign peaks to specific outcomes. In their study the analysis involved serum samples which is clearly a more complex biological fluid than urine. Indeed, the urine-based proteomics has the advantage of excluding most of the serum proteins from the urine due to the size/charge selectivity of the glomerular basement membrane.

Urine profiles of the various groups could have been altered by the procedures of urine collection and storage. Due to the fact that all urine samples were stored non-centrifuged, the rejection pattern may have derived from intracellular proteins of leucocytes, RBC or tubular epithelial cells released after a freeze-thaw cycle. Interestingly, in one of the rejection cases we found that lysis of RBC prevented the detection of the rejection pattern due to ion suppression. However, pre-centrifugation to remove the RBC prior to freeze-thawing of this sample allowed the rejection pattern to be detected. Therefore, this argues that the pattern is not necessarily derived from cell lysis associated with a freeze-thaw cycle.

Although there were significant differences in the urine profiles between the stable transplant and the acute clinical rejection groups, there were also one 'false negative' and four 'false positives' samples. The only patient with the 'false negative' urine profile in the acute clinical rejection group had no specific clinical or demographic feature. He had a

course of a subclinical rejection (ai3t3g0v0) followed by a clinical rejection (ai3t3g0v1) - both treated with oral high dose steroids- and returned to normal histology (ai0t1g0v0) 15 weeks later. We found no obvious explanation for this 'false negative' result. Theoretically, a low protein concentration in dilute urine may influence the ability to detect a rejection pattern. However, the protein concentration of the urine samples from the stable transplant and the acute clinical rejection group were similar, making inadequate protein load an unlikely explanation for the absence of the rejection pattern. The four patients with 'false positive' urine profiles in the stable transplant group also had no specific clinical or demographic features at the time of the biopsy. However, one of them went on to subclinical rejection (ai1t3g0v0) 9 weeks later and one experienced an acute clinical rejection and polyomavirus type BK-nephropathy 13 weeks later. The other two patients had a normal transplant course with stable graft function. There are mainly two possible explanations for these 'false positive' results. First, they are true 'false positives' and we cannot explain why. Second, they are not 'false positives' as the urine profile maybe detecting an early rejection process that was missed by the allograft biopsy (i.e. sampling error) (101,50).

The urine protein profile in the ATN and glomerulopathy groups did not show the pattern of rejection. Both ATN and glomerulopathies are important in the differential diagnosis of allograft dysfunction, and may represent pathophysiological models of allograft injury distinct from that due to the alloimmune response. Whereas ATN can be regarded as a model of injury to the tubules due to ischemia-reperfusion, in the glomerulopathies, the injury, although presumably immune in nature, is largely centered on the glomerular capillary. As these two pathological states did not show the characteristic pattern of rejection, we infer that the urine proteins detected in acute

rejection are related to recipient immune cells infiltrating the graft and/or to tubular epithelial cells that are involved in the allo-directed inflammation. We acknowledge, however, that we can not exclude the possibility that the urine proteins associated with rejection may also be found in other causes of tubular-based pathology (i.e. calcineurin-inhibitor-toxicity, polyomavirus type BK-nephropathy, pyelonephritis). These latter outcomes are of relatively lower frequency in our patient population, such that we were unable to generate pure examples of each in sufficient number to make any reliable conclusions. Indeed, it is notable, that in our patient population (n=212) only one patient (0.5%) developed polyomavirus type BK-nephropathy, which is a much lower incidence than reported from another centre (8%) (11).

An additional potential confounder of the diagnostic specificity of the urine protein profile observed in allograft rejection is systemic inflammation that could lead to the filtration of inflammatory proteins (e.g. chemokines, cytokines) by the transplant kidney. Post-transplant CMV-viremia, which has a high incidence in kidney transplant recipients (102,103) but very rarely infects the allograft (104,105) is one of the most common causes of systemic inflammation post-transplant. Indeed, our group has previously reported that CMV-viremia is a significant confounding variable when examining activated T-cells in the circulation as a possible non-invasive correlate of biopsy proven allograft rejection (62). In the current study, we found no correlation between CMV-viremia and the urine profile of rejection, which argues against systemic inflammation associated with CMV-viremia as a significant confounding factor. While this does not rule out the possibility that other systemic inflammatory processes may mimic the urine profile seen in allograft rejection, it suggests that this is probably less likely.

It was of interest that the protein profile of rejection was similar regardless of the histological severity (Banff IA vs. IB) or type (Banff IA/B vs. IIA). This finding might represent a relative limitation of the technique of urine proteomics in identifying biomarkers specific for tubulointerstitial versus vascular rejection. However, because the assignment of histological severity/type of acute rejection is based upon a small biopsy sample of a large organ, urine profiling, which is representative of the entire allograft, may be pointing to the extent of heterogeneity of inflammation within the allograft, a fact that renal transplant pathologists are well aware of (50).

The correlation between the changes in serial urine profiles and the clinico-pathological course of the patients provided additional support that the detected proteins are related to acute allograft rejection. However, we do not propose the SELDI-TOF-MS spectra as a diagnostic test, but rather as a tool to detect proteins that are specifically involved in the pathogenesis of rejection. In addition, the patient selection criteria set for this study reflect the extremes of the rejection spectrum (stable transplant vs. acute clinical rejection in a 1:1 ratio) rather than the whole spectrum seen in regular clinics. Therefore, we have avoided calculation of parameters that characterizes a clinical test (e.g. sensitivity, specificity, PPV and NPV) as we regarded this as potentially misleading.

6.3) Identification of proteins associated with acute renal allograft rejection

Protein can separated based on [I] their molecular weight, [II] their pI and [III] their hydrophobicity. The use of ion-exchange beads as a first step to purify the target proteins with a pI of 7.0 from urine offered two advantages. First, it allows one to concentrate the target proteins, and second, many proteins with lower pI's could by excluded.

Subsequently, the high resolution ability of RP-HPLC allowed purifying the cleaved β2-microglobulin. Indeed, it was even possible to separate the cleaved form (eluted at around 31% acetonitrile) from the intact form (eluted at around 33% acetonitrile), which only differ by seven amino acids.

Identification of cleaved β2-microglobulin by LC-MS and LC-MS/MS is very reliable. Not only were all the peptides corresponding to the β2-microglobulin sequence (without the cleaved piece 'F62YLLYYT68') found and confirmed by MS/MS, but the observed and predicted cleaved forms could explain 11 of 13 peaks of the rejection pattern detected by SELDI-TOF-MS. However, the question remains, why cleaved β2-microglobulin produces the observed multiple peaks on the SELDI-TOF-MS spectra. β2-microglobulin consists of 99 amino acids and contains one disulphide bond (C25-C80). Purified human β2-microglobulin from urine is not fragmented when analysed by SELDI-TOF-MS and only the double charged species is observed beside the parent ion (**Figure 23, II; page 82**). However, after cleavage of the above-mentioned piece two chains result, which are connected by the disulphide bond (C25-C80). During the ionisation process disulphide bond may break (106,107) in some cleaved β2-microglobulin molecules resulting in additional detection of the two single chains.

Based on the SELDI-TOF-MS detected β2-microglobulin fragments, we postulate the initial non-tryptic cleavage sites to be Y63 and T68. Thereafter additional major cleavages occur at S61, F62 and E69, resulting in 11 of 13 peaks contributing to the rejection pattern (**Figure 25B; page 86**). However, more non-tryptic cleavage sites were found (F22, N24, C25, F70, T71, P72, T73 and E74) which we could not assign corresponding SELDI-TOF-MS peaks for. The proteinase(s) involved in the initial

cleavage as well as the proteinase(s) responsible for further fragmentation of β2-microglobulin are not determined yet. Cleavage of β2-microglobulin could either have happened intracellularly (i.e. lysosomal enzymes) or intraluminally (i.e. secreted proteinases from CTL, macrophages or tubular epithelial cells; release of lysosomal enzymes due to tubular epithelial cell death) (**Figure 27**).

β2-microglobulin is freely filtered through the glomerular barrier and is normally reabsorbed by proximal tubular epithelial cells to a large extent. Therefore, changes in β2-microglobulin metabolism and excretion are mainly dependent on the function of the tubular epithelial cells. In addition, proteinases in urine may mostly be derived from these cells (108,109,110). Taken together, the presence of cleaved β2-microglobulin in urine is most likely to be associated with tubular epithelial cell stress/injury. Interestingly, in patients with pure humoral rejection (n=3, data not shown), which does not target the tubular cells, cleaved β2-microglobulin was not detectable by SELDI-TOF-MS further supporting the association between tubular cell stress/injury and the presence of cleaved β2-microglobulin. Whether cleaved β2-microglobulin is specific for tubular cell stress/injury due to rejection is not known yet and needs to be addressed in further analysis of samples with different pathologies affecting the tubuli (i.e. CNI-toxicity, polyomavirus type BK-nephropathy, pyelonephritis).

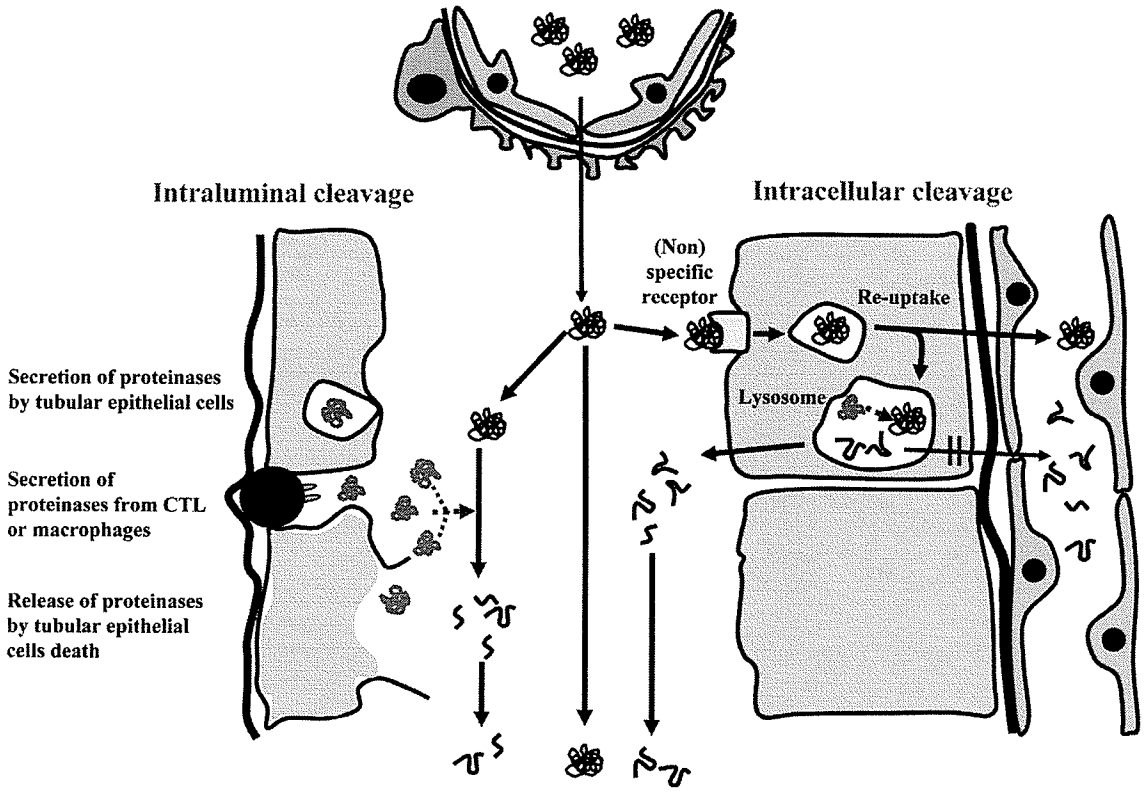


Figure 27. Suggested pathways involved in cleavage of β2-microglobulin. β2-microglobulin is freely filtered through the glomerular barrier and reabsorbed to a large (but unknown) extent by tubular epithelial cells. It can by transferred directly back into the bloodstream, but it may also by degraded in lysosomes. Resulting fragments may be brought back into the blood stream, but regurgitation of fragments into the urine is also possible. Tubular epithelial stress/injury due to rejection may enhance regurgitation of fragmented proteins into urine and decrease their transport into blood stream (grey double-line). However, β2-microglobulin may also be cleaved intraluminally by proteinases released by tubular epithelial cells, CTL and macrophages.

Chapter 7

Further research steps

7.1) Studies of cleaved β2-microglobulin and its responsible proteinase(s)

7.2) Other proteomic approaches than SELDI-TOF-MS to identify urine proteins involved in renal allograft rejection

7.1) Studies of cleaved β2-microglobulin and its responsible proteinase(s)

We have demonstrated that cleaved β2-microglobulin has the potential to be a useful biomarker for tubular epithelial cell stress/injury occurring during renal allograft rejection. However, several important questions have to be answered before cleaved β2-microglobulin can be tested for its clinical usefulness in a separate prospective study.

[I] Can we reliably measure cleaved β2-microglobulin in urine ?

As detection of cleaved β2-microglobulin in urine by SELDI-TOF-MS can by influenced by technical and sample-related limitations (i.e. binding competition, ion suppression, blood in urine), a more sensitive and less interference-prone assay is mandatory (i.e. ELISA). However, commercially available anti-β2-microglobulin antibodies may not detect the cleaved form or cross-react with both the cleaved and the intact form of β2-microglobulin. Therefore, the next step is to determine the specificity of different anti-β2-microglobulin antibodies (monoclonal and polyclonal) for their detection of both forms of β2-microglobulin by Western-blot and immunoprecipitation. Once an antibody, that recognizes only cleaved β2-microglobulin, is found, it would allow determining the quantitative differences of cleaved β2-microglobulin in all the studied groups. However, if no specific anti-cleaved β2-microglobulin antibodies are commercially available, immunizing animals with purified cleaved β2-microglobulin could produce such antibodies. Alternatively, the amount of cleaved β2-microglobulin could be calculated by subtracting the intact β2-microglobulin value (e.g. measured by monoclonal antibody that

only recognizes the intact form) from total β2-microglobulin value (e.g. measured by polyclonal antibody that recognizes intact and cleaved β2-microglobulin).

[II] Which proteinase(s) are responsible for the cleavage of urinary β2-microglobulin ?

The non-tryptic cleavage sites of the identified cleaved β2-microglobulin do not show a specific pattern that can be easily attributed to one proteinase. Therefore, the involved proteinase(s) for the suggested initial cleavage sites and the subsequent further cleavages have to be determined experimentally. Besides preferred amino acid cleavage sites, proteinases can be closer assigned to a family (i.e. aspartic proteinase, serine proteinase) by their optimal working pH and their susceptibility to specific proteinase-inhibitors. Finally, *in vitro* generation of the same fragments by incubating intact β2-microglobulin with one or more proteinases will ultimately confirm the responsible proteinase(s).

[III] What measurement is more reliable, cleaved β2-microglobulin or proteinase-activity ?

As cleaved β2-microglobulin is *in vivo* proof of proteinase activity in urine, it may be possible that under certain conditions (low urine pH, high storage temperature) proteinases will further degrade cleaved β2-microglobulin. This would result in a false negative result when measuring cleaved β2-microglobulin in urine. Therefore, measurement of the enzymatic activity of the proteinase(s) may be more reliable.

7.2) Other proteomic approaches than SELDI-TOF-MS to identify urine proteins involved in renal allograft rejection

As described and discussed above, SELDI-TOF-MS can only detect a limited subset of urine proteins. Therefore other approaches are needed to profile different subsets of urine proteins for their potential as biomarkers for renal allograft rejection. Such approaches include comparative analysis of urine samples from stable transplants and patients undergoing rejection (i.e. differential protein profiling).

The Aebersold laboratory introduced the concept of isotope coded affinity tags, ICAT, in an effort to provide a means for direct comparison of protein levels in two samples by mass spectrometry (71). The ICAT reagent is in two structurally identical forms, which only differ by the presence of heavy, H, or light, L, species of stable isotopes of carbon or hydrogen (i.e. C^{12}/C^{13} or H^1/H^2) resulting in H and L forms of ICAT. Equal amounts of the two samples to be compared are reduced, labelled separately, each with only one species of ICAT, and then pooled for processing. ICAT reacts with free -SH groups and introduces a selectable biotin affinity tag which allows for the isolation of the tag labelled peptides from the overall digest. This step was designed to reduce the overall complexity of the samples in the subsequent chromatographic and mass spectrometric steps. The tagged peptides can be separated by 1 or 2 dimensional LC on-line with a mass spectrometer. In single MS mode peptides from the same protein species but labelled with the heavy and light forms of the tag will display a predictable separation in m/z depending on the charge state of the peptides. Integration of the areas of the isotope cluster for the H and L species provides a basis for comparing their relative abundance. Subsequent analysis of the parent ion by tandem MS provides protein identification. Thus in a single

experiment it is possible to obtain information on relative protein abundance and identity of the altered expression patterns. The limitations of the approach relate to the relatively narrow dynamic range (i.e.~10 fold) and the requirement for cysteins in the proteins.

Another approach for differential protein profiling employs digestion in the presence of O^{16} or O^{18} (111). Equal quantities of urinary proteins from the groups to be compared will be dialysed and lyophilised. The samples will then be resuspended in buffer containing exclusively either O^{16} or O^{18} H_2O. During trypsin digestion peptide bonds C terminal to the basic residues are hydrolysed resulting in the incorporation of OH into the C terminal carboxyl group. Thus by digesting the two protein mixtures to be compared in different forms of H_2O sets of peptides differing by 2 mass units are generated. Combining equal quantities of the protein digests and fractionating in a similar fashion to the ICAT provides a means of performing a similar type of quantitative comparison and protein identification. This scheme labels all peptides and is only dependent on the presence of cleavage sites for trypsin rather than cysteins.

In summary, the results from the ICAT and the hydrolytic labelling offer the means to obtain broad comparative analysis of the urine samples of interest. However, both methods do not allow for high throughput analysis making the selection of few clinically well defined samples mandatory to allow meaningful interpretation.

Chapter 8

References

1. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D: Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 342:605-612, 2000
2. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B: Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *Am J Transplant* 4:378-383, 2004
3. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 346:580-590, 2002
4. Meier-Kriesche HU, Ojo AO, Hanson JA, Cibrik DM, Punch JD, Leichtman AB, Kaplan B: Increased impact of acute rejection on chronic allograft failure in recent era. *Transplantation* 70:1098-1100, 2000
5. Almond PS, Matas A, Gillingham K, Dunn DL, Payne WD, Gores P, Gruessner R, Najarian JS: Risk factors for chronic rejection in renal allograft recipients. *Transplantation* 55:752-756, 1993
6. Gourishankar S, Hunsicker LG, Jhangri GS, Cockfield SM, Halloran PF: The stability of the glomerular filtration rate after renal transplantation is improving. *J Am Soc Nephrol* 14:2387-2394, 2003
7. Hariharan S, McBride MA, Cohen EP: Evolution of endpoints for renal transplant outcome. *Am J Transplant* 3:933-941, 2003
8. Kreis HA and Ponticelli C: Causes of late renal allograft loss: chronic allograft dysfunction, death, and other factors. *Transplantation* 71:SS5-SS9, 2001

9. Kasiske BL, Snyder JJ, Gilbertson DT, Wang C: Cancer after kidney transplantation in the United States. *Am J Transplant* 4:905-913, 2004

10. Kiberd B, Keough-Ryan T, Panek R: Cardiovascular disease reduction in the outpatient kidney transplant clinic. *Am J Transplant* 3:1393-1399, 2003

11. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Steiger J: Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med* 347:488-496, 2002

12. Mylonakis E, Goes N, Rubin RH, Cosimi AB, Colvin RB, Fishman JA: BK virus in solid organ transplant recipients: an emerging syndrome. *Transplantation* 72:1587-1592, 2001

13. Kirk AD: Less is more: maintenance minimization as a step toward tolerance. *Am J Transplant* 3:643-645, 2003

14. Le Moine A, Goldman M, Abramowicz D: Multiple pathways to allograft rejection. *Transplantation* 73:1373-1381, 2002

15. Baldwin WM, III, Larsen CP, Fairchild RL: Innate immune responses to transplants: a significant variable with cadaver donors. *Immunity* 14:369-376, 2001

16. Pratschke J, Wilhelm MJ, Kusaka M, Beato F, Milford EL, Hancock WW, Tilney NL: Accelerated rejection of renal allografts from brain-dead donors. *Ann Surg* 232:263-271, 2000

17. Paul WE and Seder RA: Lymphocyte responses and cytokines. *Cell* 76:241-251, 1994

18. Sayegh MH, Akalin E, Hancock WW, Russell ME, Carpenter CB, Linsley PS, Turka LA: CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med* 181:1869-1874, 1995

19. Simeonovic CJ, Townsend MJ, Karupiah G, Wilson JD, Zarb JC, Mann DA, Young IG: Analysis of the Th1/Th2 paradigm in transplantation: interferon-gamma deficiency converts Th1-type proislet allograft rejection to a Th2-type xenograft-like response. *Cell Transplant* 8:365-373, 1999

20. Chan SY, DeBruyne LA, Goodman RE, Eichwald EJ, Bishop DK: In vivo depletion of CD8+ T cells results in Th2 cytokine production and alternate mechanisms of allograft rejection. *Transplantation* 59:1155-1161, 1995

21. Le Moine A and Goldman M: Non-classical pathways of cell-mediated allograft rejection: new challenges for tolerance induction? *Am J Transplant* 3:101-106, 2003

22. Hongwei W, Nanra RS, Stein A, Avis L, Price A, Hibberd AD: Eosinophils in acute renal allograft rejection. *Transpl Immunol* 2:41-46, 1994

23. Kormendi F and Amend WJ, Jr.: The importance of eosinophil cells in kidney allograft rejection. *Transplantation* 45:537-539, 1988

24. Nolan CR, Saenz KP, Thomas CA, III, Murphy KD: Role of the eosinophil in chronic vascular rejection of renal allografts. *Am J Kidney Dis* 26:634-642, 1995

25. Poulin LF, Richard M, Le Moine A, Kiss R, McKenzie AN, Goldman M, Renauld JC, Van Snick J, Braun MY: Interleukin-9 promotes eosinophilic rejection of mouse heart allografts. *Transplantation* 76:572-577, 2003

26. Ten RM, Gleich GJ, Holley KE, Perkins JD, Torres VE: Eosinophil granule major basic protein in acute renal allograft rejection. *Transplantation* 47:959-963, 1989

27. Lakkis FG, Arakelov A, Konieczny BT, Inoue Y: Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat Med* 6:686-688, 2000

28. Racusen LC, Colvin RB, Solez K, Mihatsch MJ, Halloran PF, Campbell PM, Cecka MJ, Cosyns JP, Demetris AJ, Fishbein MC, Fogo A, Furness P, Gibson IW, Glotz D, Hayry P, Hunsickern L, Kashgarian M, Kerman R, Magil AJ, Montgomery R, Morozumi K, Nickeleit V, Randhawa P, Regele H, Seron D, Seshan S, Sund S, Trpkov K: Antibody-mediated rejection criteria - an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant* 3:708-714, 2003

29. Saisu K, Morozumi K, Suzuki K, Fujita K: Significance of interstitial lesions as the early indicator for acute vascular rejection in human renal allografts. *Clin Transplant* 13 Suppl 1:17-23, 1999

30. Weir MR, Hall-Craggs M, Shen SY, Posner JN, Alongi SV, Dagher FJ, Sadler JH: The prognostic value of the eosinophil in acute renal allograft rejection. *Transplantation* 41:709-712, 1986

31. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Yamaguchi Y, .: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55:713-723, 1999

32. Macdonald FI, Ashraf S, Picton M, Dyer PA, Parrott NR, Short CD, Roberts IS: Banff criteria as predictors of outcome following acute renal allograft rejection. *Nephrol Dial Transplant* 14:1692-1697, 1999

33. Feucht HE: Complement C4d in graft capillaries -- the missing link in the recognition of humoral alloreactivity. *Am J Transplant* 3:646-652, 2003

34. Halloran PF: The clinical importance of alloantibody-mediated rejection. *Am J Transplant* 3:639-640, 2003

35. Nickeleit V, Zeiler M, Gudat F, Thiel G, Mihatsch MJ: Detection of the complement degradation product C4d in renal allografts: diagnostic and therapeutic implications. *J Am Soc Nephrol* 13:242-251, 2002

36. Regele H, Bohmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S, Watschinger B, Kerjaschki D, Exner M: Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 13:2371-2380, 2002

37. Meehan SM, Domer P, Josephson M, Donoghue M, Sadhu A, Ho LT, Aronson AJ, Thistlethwaite JR, Haas M: The clinical and pathologic implications of plasmacytic infiltrates in percutaneous renal allograft biopsies. *Hum Pathol* 32:205-215, 2001

38. Charney DA, Nadasdy T, Lo AW, Racusen LC: Plasma cell-rich acute renal allograft rejection. *Transplantation* 68:791-797, 1999

39. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, Salvatierra O, Jr.: Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 349:125-138, 2003

40. Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA: Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. *J Am Soc Nephrol* 15:390-397, 2004

41. Libby P and Pober JS: Chronic rejection. *Immunity* 14:387-397, 2001

42. Halloran PF: Call for revolution: a new approach to describing allograft deterioration. *Am J Transplant* 2:195-200, 2002

43. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR: The natural history of chronic allograft nephropathy. *N Engl J Med* 349:2326-2333, 2003

44. Rush D, Nickerson P, Gough J, McKenna R, Grimm P, Cheang M, Trpkov K, Solez K, Jeffery J: Beneficial effects of treatment of early subclinical rejection: a randomized study. *J Am Soc Nephrol* 9:2129-2134, 1998

45. Rush DN, Henry SF, Jeffery JR, Schroeder TJ, Gough J: Histological findings in early routine biopsies of stable renal allograft recipients. *Transplantation* 57:208-211, 1994

46. Shapiro R, Randhawa P, Jordan ML, Scantlebury VP, Vivas C, Jain A, Corry RJ, McCauley J, Johnston J, Donaldson J, Gray EA, Dvorchik I, Hakala TR, Fung JJ, Starzl TE: An analysis of early renal transplant protocol biopsies--the high incidence of subclinical tubulitis. *Am J Transplant* 1:47-50, 2001

47. Gloor JM, Cohen AJ, Lager DJ, Grande JP, Fidler ME, Velosa JA, Larson TS, Schwab TR, Griffin MD, Prieto M, Nyberg SL, Sterioff S, Kremers WK, Stegall MD: Subclinical rejection in tacrolimus-treated renal transplant recipients. *Transplantation* 73:1965-1968, 2002

48. Grimm PC, Nickerson P, Gough J, McKenna R, Stern E, Jeffery J, Rush DN: Computerized image analysis of sirius red-stained renal allograft biopsies as a surrogate marker to predict long-term allograft function. *J Am Soc Nephrol* 14:1662-1668, 2003

49. Nickerson P, Jeffery J, Gough J, McKenna R, Grimm P, Cheang M, Rush D: Identification of clinical and histopathologic risk factors for diminished renal function 2 years posttransplant. *J Am Soc Nephrol* 9:482-487, 1998

50. Nicholson ML, Wheatley TJ, Doughman TM, White SA, Morgan JD, Veitch PS, Furness PN: A prospective randomized trial of three different sizes of core-cutting needle for renal transplant biopsy. *Kidney Int* 58:390-395, 2000

51. Hernandez-Fuentes MP, Warrens AN, Lechler RI: Immunologic monitoring. *Immunol Rev* 196:247-264, 2003

52. Gebel HM, Bray RA, Nickerson P: Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: contraindication vs. risk. *Am J Transplant* 3:1488-1500, 2003

53. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, Tary-Lehmann M: Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 163:2267-2275, 1999

54. Hricik DE, Rodriguez V, Riley J, Bryan K, Tary-Lehmann M, Greenspan N, Dejelo C, Schulak JA, Heeger PS: Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* 3:878-884, 2003

55. Roberti I, Panico M, Reisman L: Urine flow cytometry as a tool to differentiate acute allograft rejection from other causes of acute renal graft dysfunction. *Transplantation* 64:731-734, 1997

56. Roberti I and Reisman L: Serial evaluation of cell surface markers for immune activation after acute renal allograft rejection by urine flow cytometry--correlation with clinical outcome. *Transplantation* 71:1317-1320, 2001

57. Ding R, Li B, Muthukumar T, Dadhania D, Medeiros M, Hartono C, Serur D, Seshan SV, Sharma VK, Kapur S, Suthanthiran M: CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. *Transplantation* 75:1307-1312, 2003

58. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, Serur D, Mouradian J, Schwartz JE, Suthanthiran M: Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 344:947-954, 2001

59. Simon T, Opelz G, Wiesel M, Ott RC, Susal C: Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. *Am J Transplant* 3:1121-1127, 2003

60. Sadeghi M, Daniel V, Wiesel M, Hergesell O, Opelz G: High urine sIL-6R as a predictor of late graft failure in renal transplant recipients. *Transplantation* 76:1190-1194, 2003

61. Hu H, Aizenstein BD, Puchalski A, Burmania JA, Hamawy MM, Knechtle SJ: Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. *Am J Transplant* 4:432-437, 2004

62. Karpinski M, Rush D, Jeffery J, Pochinco D, Milley D, Nickerson P: Heightened Peripheral Blood Lymphocyte CD69 Expression is Neither Sensitive nor Specific as a Noninvasive Diagnostic Test for Renal Allograft Rejection. *J Am Soc Nephrol* 14:226-233, 2003

63. McLean A: What goes around (in kidney transplant rejection) does not necessarily come around (in the blood). *Am J Transplant* 3:1045-1046, 2003

64. Lachenbruch PA, Rosenberg AS, Bonvini E, Cavaille-Coll MW, Colvin RB: Biomarkers and Surrogate Endpoints in Renal Transplantation: Present Status and Considerations for Clinical Trial Design. *American Journal of Transplantation* 4:451-457, 2004

65. Lang T and Secic M: How to report statistics in medicine. ACP - Medical writing and communications. 147-169, 1997

66. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van d, V, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R: A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347:1999-2009, 2002

67. Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P, Hochstrasser DF, Sanchez JC: A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. *Proteomics* 3:1486-1494, 2003

68. Guillaume E, Zimmermann C, Burkhard PR, Hochstrasser DF, Sanchez JC: A potential cerebrospinal fluid and plasmatic marker for the diagnosis of Creutzfeldt-Jakob disease. *Proteomics* 3:1495-1499, 2003

69. Knepper MA: Proteomics and the kidney. *J Am Soc Nephrol* 13:1398-1408, 2002

70. Hanash S: Disease proteomics. *Nature* 422:226-232, 2003

71. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17:994-999, 1999

72. Zhou H, Ranish JA, Watts JD, Aebersold R: Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry. *Nat Biotechnol* 20:512-515, 2002

73. Shevchenko A, Chernushevich I, Ens W, Standing KG, Thomson B, Wilm M, Mann M: Rapid 'de novo' peptide sequencing by a combination of nanoelectrospray, isotopic labeling and a quadrupole/time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 11:1015-1024, 1997

74. Stewart II, Thomson T, Figeys D: 18O labeling: a tool for proteomics. *Rapid Commun Mass Spectrom* 15:2456-2465, 2001

75. Yao X, Afonso C, Fenselau C: Dissection of proteolytic 18O labeling: endoprotease-catalyzed 16O-to-18O exchange of truncated peptide substrates. *J Proteome Res* 2:147-152, 2003

76. D'Amico G and Bazzi C: Pathophysiology of proteinuria. *Kidney Int* 63:809-825, 2003

77. Russo LM, Bakris GL, Comper WD: Renal handling of albumin: a critical review of basic concepts and perspective. *Am J Kidney Dis* 39:899-919, 2002

78. Marino M, Andrews D, Brown D, McCluskey RT: Transcytosis of retinol-binding protein across renal proximal tubule cells after megalin (gp 330)-mediated endocytosis. *J Am Soc Nephrol* 12:637-648, 2001

79. Gudehithlu KP, Pegoraro AA, Dunea G, Arruda JA, Singh AK: Degradation of albumin by the renal proximal tubule cells and the subsequent fate of its fragments. *Kidney Int* 65:2113-2122, 2004

80. Kokot F and Dulawa J: Tamm-Horsfall protein updated. *Nephron* 85:97-102, 2000

81. Prescott LF: The normal urinary excretion rates of renal tubular cells, leucocytes and red blood cells. *Clin Sci* 31:425-435, 1966

82. Loboda AV, Krutchinsky AN, Bromirski M, Ens W, Standing KG: A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance. *Rapid Commun Mass Spectrom* 14:1047-1057, 2000

83. Hillenkamp F, Karas M, Beavis RC, Chait BT: Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal Chem* 63:1193A-1203A, 1991

84. Tencer J, Thysell H, Andersson K, Grubb A: Stability of albumin, protein HC, immunoglobulin G, kappa- and lambda-chain immunoreactivity, orosomucoid and alpha 1-antitrypsin in urine stored at various conditions. *Scand J Clin Lab Invest* 54:199-206, 1994

85. Froom P, Bieganiec B, Ehrenrich Z, Barak M: Stability of common analytes in urine refrigerated for 24 h before automated analysis by test strips. *Clin Chem* 46:1384-1386, 2000

86. Innanen VT, Groom BM, de Campos FM: Microalbumin and freezing. *Clin Chem* 43:1093-1094, 1997

87. Schultz CJ, Dalton RN, Turner C, Neil HA, Dunger DB: Freezing method affects the concentration and variability of urine proteins and the interpretation of data on microalbuminuria. The Oxford Regional Prospective Study Group. *Diabet Med* 17:7-14, 2000

88. Ganz T: Defensins in the urinary tract and other tissues. *J Infect Dis* 183 Suppl 1:S41-S42, 2001

89. Kunin CM, Evans C, Bartholomew D, Bates DG: The antimicrobial defense mechanism of the female urethra: a reassessment. *J Urol* 168:413-419, 2002

90. Hampel DJ, Sansome C, Sha M, Brodsky S, Lawson WE, Goligorsky MS: Toward proteomics in uroscopy: urinary protein profiles after radiocontrast medium administration. *J Am Soc Nephrol* 12:1026-1035, 2001

91. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, Pham T, Mei J, Ho JJ, Zhang W, Lopez P, Ho DD: Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 298:995-1000, 2002

92. Cazares LH, Adam BL, Ward MD, Nasim S, Schellhammer PF, Semmes OJ, Wright GL, Jr.: Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. *Clin Cancer Res* 8:2541-2552, 2002

93. Wright GL, Jr.: SELDI proteinchip MS: a platform for biomarker discovery and cancer diagnosis. *Expert Rev Mol Diagn* 2:549-563, 2002

94. Yip TT and Lomas L: SELDI ProteinChip array in oncoproteomic research. *Technol Cancer Res Treat* 1:273-280, 2002

95. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD: Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics* 2:1096-1103, 2003

96. Diamandis EP: Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: opportunities and potential limitations. *Mol Cell Proteomics* 3:367-378, 2004

97. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int* 65:323-332, 2004

98. Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH: Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: potential novel assay for prediction of outcomes for renal transplant recipients. *J Am Soc Nephrol* 13:252-259, 2002

99. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP: Characterization of renal allograft rejection by urinary proteomic analysis. *Ann Surg* 237:660-665, 2003

100. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA: Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359:572-577, 2002

101. Sorof JM, Vartanian RK, Olson JL, Tomlanovich SJ, Vincenti FG, Amend WJ: Histopathological concordance of paired renal allograft biopsy cores. Effect on the diagnosis and management of acute rejection. *Transplantation* 60:1215-1219, 1995

102. Sagedal S, Nordal KP, Hartmann A, Degre M, Holter E, Foss A, Osnes K, Leivestad T, Fauchald P, Rollag H: A prospective study of the natural course of cytomegalovirus infection and disease in renal allograft recipients. *Transplantation* 70:1166-1174, 2000

103. Abecassis MM, Koffron AJ, Kaplan B, Buckingham M, Muldoon JP, Cribbins AJ, Kaufman DB, Fryer JP, Stuart J, Stuart FP: The role of PCR in the diagnosis and management of CMV in solid organ recipients: what is the predictive value for the

development of disease and should PCR be used to guide antiviral therapy? *Transplantation* 63:275-279, 1997

104. Andersen CB, Ladefoged SD, Lauritsen HK, Hansen PR, Larsen S: Detection of CMV DNA and CMV antigen in renal allograft biopsies by in situ hybridisation and immunohistochemistry. *Nephrol Dial Transplant* 5:1045-1050, 1990

105. Kashyap R, Shapiro R, Jordan M, Randhawa PS: The clinical significance of cytomegaloviral inclusions in the allograft kidney. *Transplantation* 67:98-103, 1999

106. Jones MD, Patterson SD, Lu HS: Determination of disulfide bonds in highly bridged disulfide-linked peptides by matrix-assisted laser desorption/ionization mass spectrometry with postsource decay. *Anal Chem* 70:136-143, 1998

107. Patterson SD and Katta V: Prompt fragmentation of disulfide-linked peptides during matrix-assisted laser desorption ionization mass spectrometry. *Anal Chem* 66:3727-3732, 1994

108. Kudo S, Miyamoto G, Kawano K: Proteases involved in the metabolic degradation of human interleukin-1beta by rat kidney lysosomes. *J Interferon Cytokine Res* 19:361-367, 1999

109. Mori K, Shimizu H, Konno A, Iwanaga T: Immunohistochemical localization of napsin and its potential role in protein catabolism in renal proximal tubules. *Arch Histol Cytol* 65:359-368, 2002

110. Goto M, Mizunashi K, Kimura N, Furukawa Y: Decreased sensitivity of distal nephron and collecting duct to parathyroid hormone in pseudohypoparathyroidism type I. *J Am Soc Nephrol* 12:1965-1970, 2001

111. Krokhin O, Li Y, Andonov A, Feldmann H, Flick R, Jones S, Stroeher U, Bastien N, Dasuri KV, Cheng K, Simonsen JN, Perreault H, Wilkins J, Ens W, Plummer F, Standing KG: Mass Spectrometric Characterization of Proteins from the SARS Virus: A Preliminary Report. *Mol Cell Proteomics* 2:346-356, 2003

Chapter 9

Appendix

- Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 65(1):323-32, 2004
- Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, Nicolle L, Karpinski M, Jeffery J, Nickerson P: Proteomic-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 15(1):219-27, 2004

Kidney International, Vol. 65 (2004), pp. 323–332

TECHNICAL NOTE

Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry

STEFAN SCHAUB, JOHN WILKINS, TRACEY WEILER, KEVIN SANGSTER, DAVID RUSH, and PETER NICKERSON

Immunogenetics Laboratory, Faculty of Medicine, Winnipeg, Manitoba, Canada; and Manitoba Centre for Proteomics, Faculty of Medicine, Winnipeg, Manitoba, Canada

Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry.

Background. In the last few years there has been an increasing interest in exploring the human proteome. In particular, efforts have focused on developing strategies to generate reproducible protein maps of normal cells, tissues, and biologic fluids, from which studies can then compare protein expression between different groups (e.g., healthy individuals vs. those with a specific pathologic state).

Methods. Various extrinsic factors (instrument settings, matrix composition, urine storage post void, freeze-thaw cycles) and intrinsic factors (blood in urine, urine dilution, first-void vs. midstream urine) were analyzed with respect to their impact on urine protein profiling using surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).

Results. Extrinsic factors that critically influenced reproducibility and peak detection of urine protein profiling were matrix composition and instrument settings, while freeze-thaw cycles had minimal impact. Midstream urines samples did not undergo changes in their protein profile when stored for three days at 4°C. Intrinsic factors that influenced normal urine protein profiling were blood in the urine and urine dilution. Female first-void urine had a significantly different ratio of proteins present compared to a midstream urine sample. Limitations of the SELDI-TOF-MS technique included ion suppression and quantification of individual proteins when protein composition was complex.

Conclusion. SELDI-TOF-MS offers a unique platform for high throughput urine protein profiling; however, standardization of analysis conditions is critical, and both extrinsic and intrinsic factors must be taken into account for accurate data interpretation.

In the last few years there has been an increasing interest in exploring the proteome of human urine. A catalog of these proteins cannot only improve our knowledge of kidney physiology [1], but can also allow the identification of novel proteins associated with pathologic states. Indeed, it may be possible to identify potential biomarkers to diagnose and/or monitor renal disease [2–4].

At present, there are several techniques to identify and compare the expression of proteins, each with advantages and disadvantages. The most established method is protein separation by two-dimensional gel electrophoresis (2-DE) followed by in-gel digestion and peptide mass fingerprinting by mass spectrometry. This method allows for the comparison of the relative abundance of proteins. In recent literature, Thongboonkerd et al [5] identified 67 protein forms of 47 unique proteins in normal urine by 2-DE. However, there are several limitations of 2-DE as a separation method for proteomic studies. The resolvable range of molecular weights is limited at both ends, with a bias toward high-abundance proteins. In addition, the technique requires relatively large amount of sample, is labor-intensive, and good gel-to-gel reproducibility can be hard to achieve [2, 6]. Thus, this approach is not optimal for high throughput profiling. An alternative approach uses liquid chromatography as the separation step upstream from the mass spectrometer (LC-MS). Using this approach, Spahr et al [7] identified 124 gene products (proteins and translations of expressed sequence tags) in normal urine samples. While this technique provides information about the protein content of the samples, little information about their relative abundance can be obtained unless the proteins/peptides are labeled first by isotope-coded affinity tags [8, 9] or other protein/peptide labeling techniques. Furthermore, this method is still labor-intensive and has limited throughput.

Surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) addresses some of the limitations of both 2-DE and LC-MS. It combines matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to surface chromatography. Specifically, a sample is applied

Key words: proteomics, urine protein profiling, SELDI-TOF-MS, mass spectrometry.

Received for publication May 29, 2003
and in revised form July 30, 2003
Accepted for publication August 6, 2003

to a chip surface carrying a functional group. After incubation, proteins that do not bind to the surface are removed by a simple wash step, and bound proteins are analyzed by mass spectrometry. This approach, in contrast to the others described, allows for high throughput profiling of multiple clinical samples. In this article, technical issues, pitfalls, and limitations of this technique in protein profiling normal urine samples are described and discussed.

METHODS

Urine collection and storage

Second-morning urine from healthy men and women were collected in two different containers. The first 10–20 mL of urine was considered as first-void urine, the following 50–80 mL as midstream urine. Unless otherwise stated, urine was centrifuged in a fixed-angle centrifuge for 10 minutes at 2000 rpm (900*g*), the supernatant was transferred into 2 mL cryo-tubes (Gordon Technologies, Inc., Missisauga, Ontario, Canada), and stored at –70°C. All samples were obtained with informed consent and ethics approval of the University of Manitoba IRB.

Urine sediments

Ten mL of freshly collected urine was centrifuged for 10 minutes at 2000 rpm. The pellet was analyzed with a phase-contrast microscope at 400× augmentation and is reported as cells per high-power field (hpf).

Protein chip preparation and reading

Normal phase chips (ProteinChip NP20; Ciphergen, Freemont, CA, USA), which bind proteins through hydrophilic and charged residues (including serine, threonine, and lysine), were used for the analysis. Urine samples were thawed on ice, shortly vortexed, and centrifuged for 5 minutes at 10,000 rpm (to remove remaining cell particles). Five μL of urine supernatant were applied in duplicate to the chip and incubated for 20 minutes in a humidity chamber. Spots were then washed three times with 5 μL high-performance liquid chromatography (HPLC)-grade water and air-dried for 10 minutes. Saturated α-cyano-4-hydroxycinnamic acid (CHCA; Ciphergen) and sinapinic acid (SPA; Ciphergen) were prepared in 50% acetonitrile/0.5% trifluoroacetic acid according to the manufacturer's instructions. Dilutions of 20%, 35%, and 50% were used. One μL of matrix solution (35% CHCA, unless otherwise specified) was applied to each spot and air-dried. Unless stated otherwise, chips were read with the following SELDI-TOF-MS instrument (ProteinChip Reader II; Ciphergen) settings: Laser intensity 230; detector sensitivity 6; detector voltage 1700 V; positions 15 to 85 were read with an increment of 5 (resulting in 15 different sampling positions); 16 laser shots were collected on each position (total shots collected and averaged: 240/sample); eight warming shots were fired at each position, which were not included in the collection; the acquired mass range was from a mass-over-charge (m/z) ratio of 0 to 80,000; lag time focus of 900 ns. Calibration was done externally with a mixture of 4 proteins with masses ranging from 2 to 16 kD. After baseline subtraction and normalization, peak labeling was performed with the ProteinChip Software (version 3.1) for peaks with a signal-to-noise (S/N) ratio of ≥3 in the m/z range from 2000–25,000.

RESULTS

Evaluation of reproducibility

It is critical to evaluate the reproducibility of urine protein profiling using the SELDI-TOF-MS approach before establishing whether the urine protein profiles differ in various clinical states. Reproducibility was evaluated by applying one urine sample to 14 spots and reading the spots using the protocol described above. The total number of detected peaks with an S/N-ratio ≥3 was 25 peaks/spectrum (range, 23–29). Fourteen peaks common to all spectra were selected and compared with regard to their peak intensity by calculating the coefficient of variation. They ranged from 8% to 30%, with the lowest variation seen in the high intensity peaks and the higher variation seen in lower intensity peaks (Fig. 1A). This is expected, as small differences in low intensity peaks (e.g., 1.0 vs. 0.5) have a large influence on the calculated coefficient of variation. Independent of the software assignment of protein peaks, it is important to conduct manual inspection of the spectra, to determine whether a specific peak is present. Low intensity peaks with an S/N-ratio near the selected detection threshold (i.e., ≥3) can be unlabelled and undetected by the software (Fig. 1B).

Impact of extrinsic factors on reproducibility and peak detection of urine protein profiles

The impact of matrix on the urine protein profile was determined by comparing different dilutions of CHCA and SPA (20%, 35%, 50%, and 100%) with the otherwise unchanged protocol stated above. In the range from 2–25 kD, 22, 26, 19, and 16 peaks were detected using 20%, 35%, 50%, and 100% CHCA, respectively. In contrast, 13, 19, 11, and 10 peaks were detected using 20%, 35%, 50%, and 100% SPA. Peak intensity below 8–10 kD was higher with CHCA, whereas SPA yielded higher peak intensities above 8–10 kD (urine protein profiles not shown).

The impact of spot sampling protocols was determined by comparing three different spot sampling protocols with respect to peak detection in undiluted and diluted

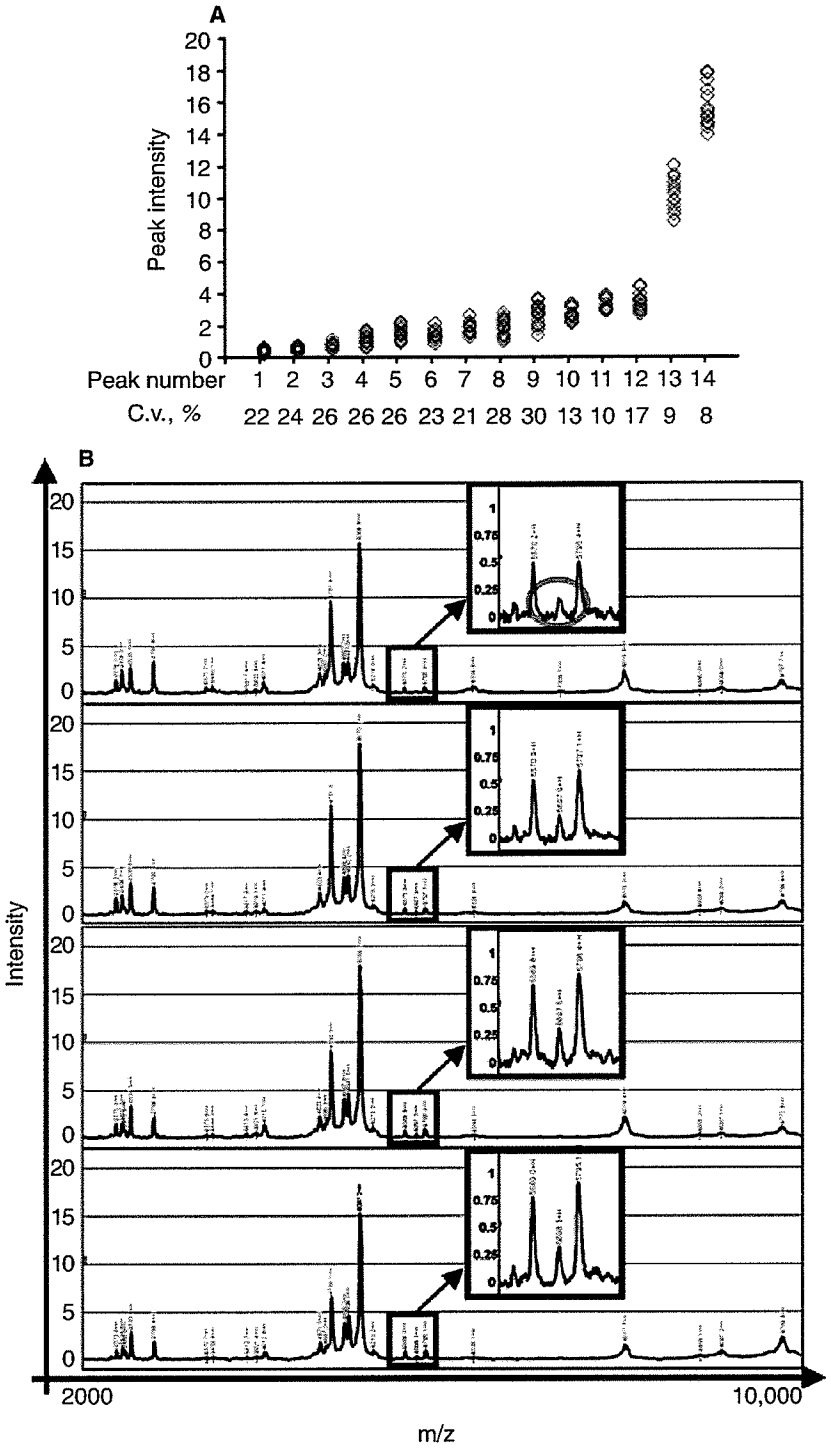


Fig. 1. Reproducibility of urine protein profiles. One urine sample was applied to 14 spots and analyzed. (*A*) Fourteen peaks common to all spectra were selected and compared with regard to their peak intensity by calculating the coefficients of variation. They ranged from 8% to 30%, with the lowest variation seen in the high-intensity peaks and the higher variation seen in lower intensity peaks. (*B*) Parts of 4 randomly selected spectra from the obtained 14. Manual inspection of the spectra showed the presence of an unlabelled peak (circle in insert), although the spectra look the same by "eyeball."

urine: protocol 1 (standard protocol; see above); protocol 2 (standard protocol modified to sample on only 5 different positions for a total of 80 shots/sample); protocol 3 [standard protocol modified to use a higher detector sensitivity (10 instead of 6)]. Protocol 1 detected 34 peaks in undiluted urine, whereas protocols 2 and 3 detected only 21 and 26 peaks, respectively. In diluted urine (urine creatinine 3.75 mmol/L) the peak counts were 20, 11, and 13, respectively (urine protein profiles not shown).

If the SELDI-TOF-MS approach is to be used in the assessment of clinical samples, it is important to assess the stability of the urine proteins before analysis.

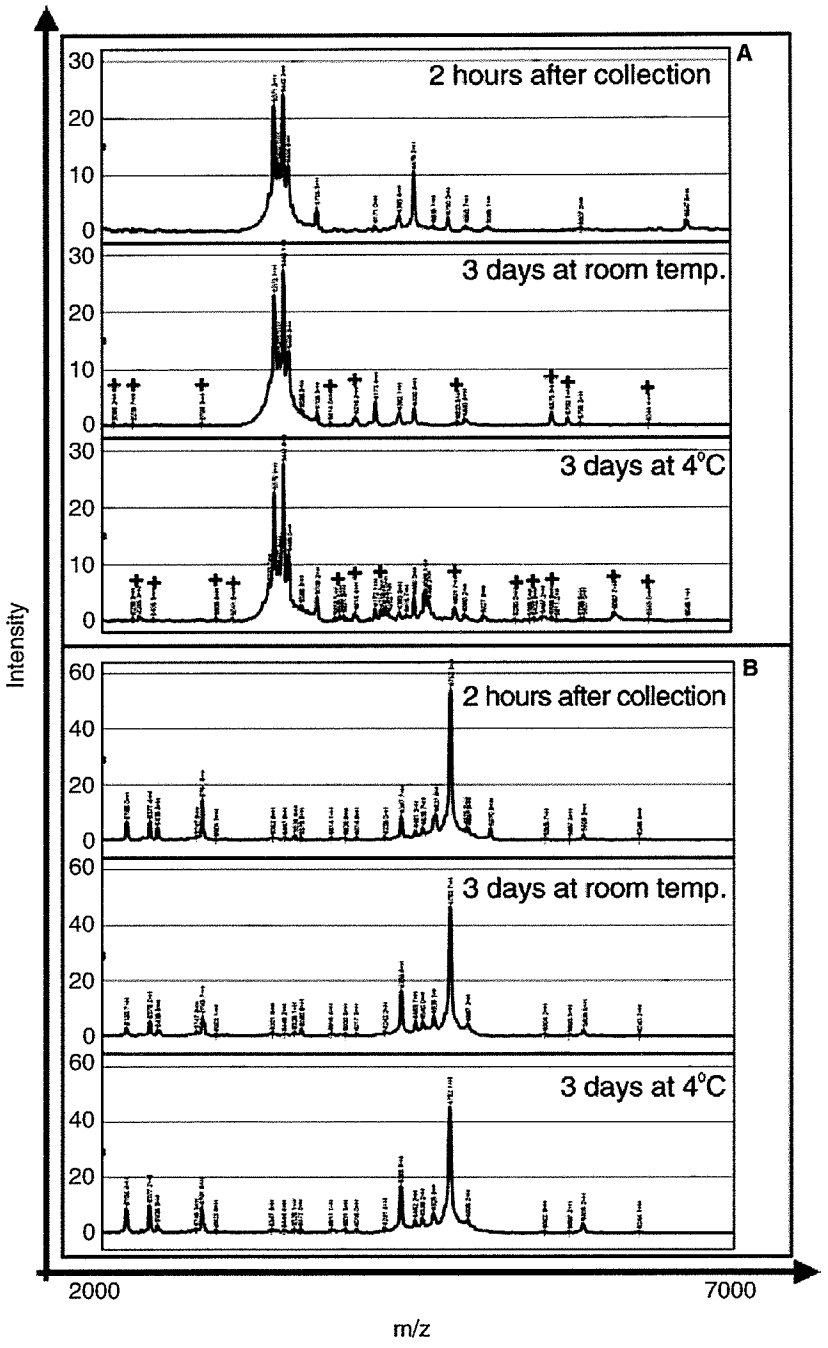


Fig. 2. Impact of storage on urine protein profiles. (*A*) Representative female first-void urine showing the appearance of new peaks (+) in the 2–6kD range after storage for 3 days at room temperature or at 4°C. (*B*) Representative male midstream urine protein profile, which showed only minor changes, whether it was analyzed 2 hours after collection or after storage for 3 days at room temperature or at 4°C, respectively.

First-void and midstream urine samples from three females and three males were analyzed within 2 hours from the time of collection, after storage for three days at room temperature, and after three days at 4°C. In all six samples, only minor differences in the midstream urine protein profiles could be detected after storage for three days at 4°C. However, in three first-void urines (two female, one male), storage for three days at room temperature or at 4°C changed the spectra considerably. A series of new peaks in the low-molecular weight range were detected (Fig. 2). Storage of the urine samples at −70°C did not change the spectra compared with those obtained before freezing. Furthermore, almost the same spectra could be generated after four freeze-thaw cycles; however, a loss of peaks was observed after the fifth freeze-thaw cycle (Fig. 3).

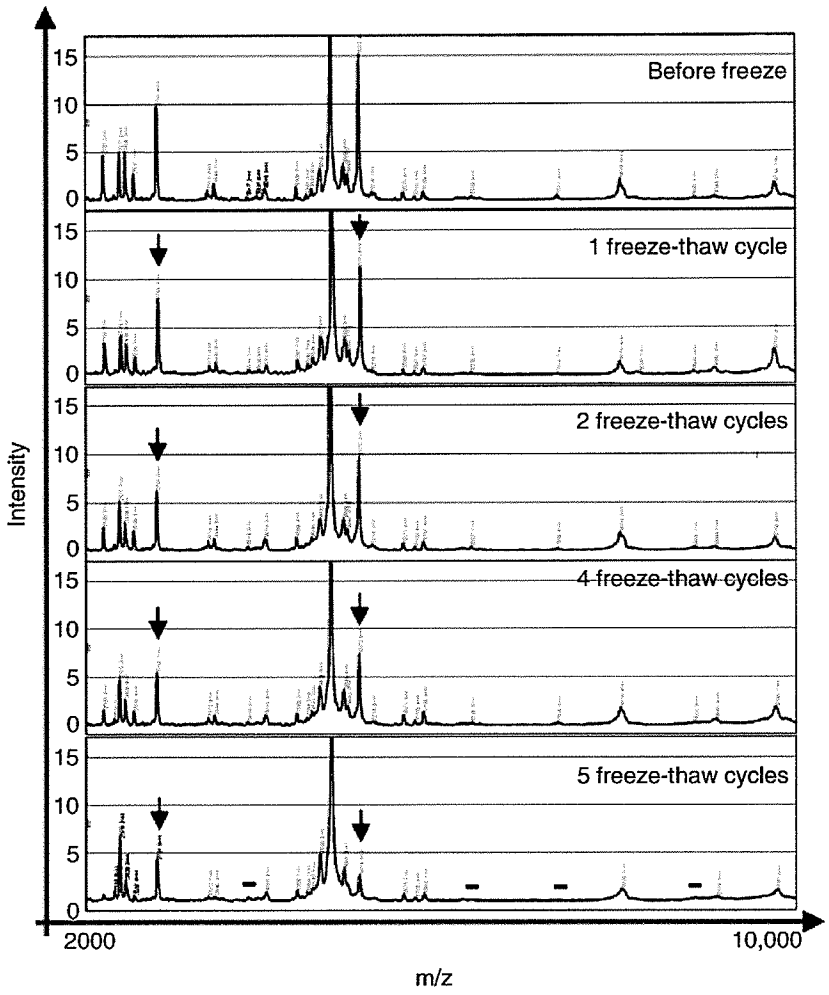


Fig. 3. Impact of freeze-thaw cycles on urine protein profiles. Urine protein profiles obtained before freeze and after 1 to 4 freeze-thaw cycles were unchanged, but an increasing loss of intensity in some peaks was detected (↓). After the fifth freeze-thaw cycle some weak intensity peaks were not detected (–).

Impact of intrinsic factors on normal urine protein profiling

A potential confounding variable in the clinical setting is if a urine sample is first-void or midstream. In all three urine samples from males, there were almost no differences between the protein profile of first-void and midstream urine (Fig. 4A). However, in all three urine samples from females, there are prominent peaks between 3.3 and 3.5 kD in the first-void urine fraction. These peaks are greatly diminished in the midstream urine sample, together with other changes in peak intensities (Fig. 4B). Three of these peaks with average masses of 3370.3, 3441.2, and 3484.3 d are consistent with the masses of the α-defensins 2, 1, and 3, respectively.

Another confounding variable in urine proteomic analysis is the presence of blood in urine. It can be present in urine under normal conditions (e.g., menstruation) or in association with urogenital tract pathologies. To investigate the impact of blood on the normal urine profile, we spiked 500 μL urine with 10 μL blood, which resulted in a red coloring of the sample [sediment analysis showed >100 red blood cells (RBC)/hpf]. In the subsequent analysis by SELDI-TOF-MS, four major peaks were detected (Fig. 5B), which are consistent with the masses of the hemoglobin α- and β-chains and their doubly charged ions. Based on the virtual disappearance of these peaks after sample centrifugation before SELDI-TOF-MS analysis, it is likely that these peaks represent hemoglobin. They were easily detectable as the most intense peaks up to a 1:128 dilution of this sample, corresponding to 10 μL blood in 64 mL diluted urine (urine protein profiles not shown). However, even when the RBC were removed by centrifugation, the urine was still contaminated with serum proteins. This is suggested by the presence of peaks with masses consistent with albumin in the urine protein profile (Fig. 5C).

A dilute urine sample may limit the ability to detect the normal urine protein profile. To address the issue of urine concentration, urine was sampled from a healthy male with a body weight of 75 kg after 20 hours of no fluid intake. The measured urine creatinine was 15 mmol/L and the total protein was 0.11 g/L. At another time point,

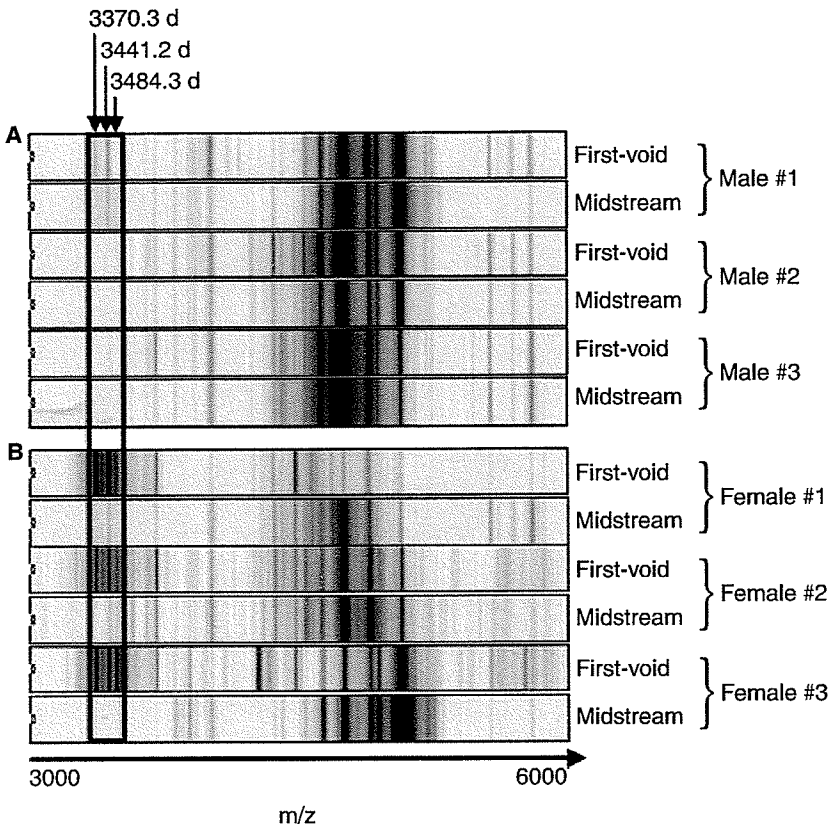


Fig. 4. Comparison between first-void and midstream urine protein profiles (gel-view). First-void and midstream urine protein profiles obtained from three females and three males. In males, both urine samples had similar protein profiles, whereas in females there are significant differences. The most prominent difference in female first-void urine are three peaks at 3370.3, 3441.2, and 3484.3 d (↓), which are consistent with the masses of the α-defensins 2, 1, and 3, respectively (Swiss-Prot P59665+P59666; 3371.9, 3442.5, 3486.5 d). The calculated mass accuracy of the surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in this example is < 0.07%, which is within the limits given by the manufacturer (<0.1%).

the same individual was challenged with 4 L of fluid over 2 hours, leading to dilute urine with a creatinine of 0.9 mmol/L and a total protein of 0.03 g/L. While the concentrated urine showed the normal peak profile (Fig. 6A), the dilute urine sample showed only three peaks in the range from 2–25 kD (Fig. 6E). To determine the detection threshold of the normal urine profile, the concentrated urine sample was serially diluted. At a 1:2 dilution, which corresponds to a urine output of 2 L/day [calculated in our test person by: creatinine production/day (0.2 mmol/kg/day × 75 kg) divided by urine creatinine (7.5 mmol/L) = 2 L/day] the profile remained unchanged (Fig. 6B). A progressive loss of urine profile peaks started with a 1:4 dilution. The 1:16 dilution showed a spectrum similar to the urine profile obtained after the fluid challenge (Fig. 6E).

Protein quantification with SELDI-TOF-MS

To determine if either the spectral peak intensity or area provides a means for reliable protein quantification, serial dilution of a single protein (ubiquitin, 8565 d) was performed. There was an excellent correlation between the amount of protein in the sample and peak intensity ($r^2 = 0.95$) or the area under the peak ($r^2 = 0.98$) in non-normalized spectra (Fig. 7A). Even in a mixture containing four other proteins, the correlation was maintained ($r^2 = 0.99$ for peak intensity and for the area under the peak), but the peak intensities were 10 times lower with the same amount of ubiquitin (Fig. 7B). When a complex protein mixture (i.e., normal urine with a protein concentration of 110 mg/L) was spiked with 1.0, 0.1, and 0.01 pmol/μL ubiquitin, only the first two concentrations of ubiquitin were detectable (Fig. 7C). The peak intensity dropped from 0.32 (1.0 pmol/μL) to 0.09 (0.1 pmol/μL), which is only a 3.5 times decrease instead of the expected 10 times. Because only two measurements of peak intensity were obtainable, no correlation was calculated.

DISCUSSION

In order to use SELDI-TOF-MS as a high throughput urine protein profiling methodology, it is critical to define those factors that affect reproducibility, as well as identify the confounding variables that affect the detection of proteins that are known to be present in the sample.

Extrinsic factors

The most important extrinsic factors that influence reproducibility and peak detection are the matrix composition and the instrument settings. Matrix allows for efficient ionization and vaporization of proteins [10]. The most popular matrices for the SELDI-TOF-MS

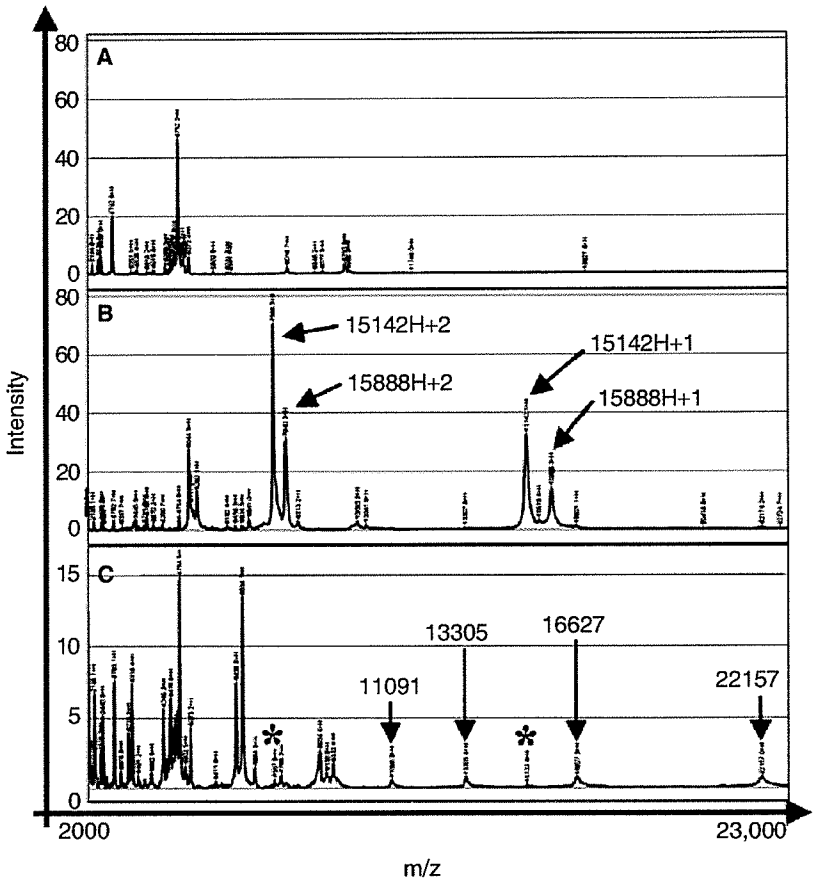


Fig. 5. Impact of blood in urine on urine protein profiles. (*A*) Protein profile of urine sample from a healthy male. (*B*) Protein profile after spiking the same sample from (A) with blood (10 µL blood in 500 µL urine). Four peaks appear which are consistent with the masses of singly and doubly charged hemoglobin α- and β-chains (Swiss-Prot P01922: 15126 d; P02023: 15867 d). The calculated mass accuracy of the surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in this example is <0.13%, which is slightly above the limits given by the manufacturer (<0.1%). (*C*) Protein profile after centrifugation of the same blood-spiked urine sample from (B). Only trace amounts of two of these peaks were detectable (*); however, contamination with serum proteins was obvious (e.g., peaks consistent with masses of serum albumin were detected). Albumin has a molecular weight of 66472 d with its multiply charged ions at an m/z of 33236 (double-charged), 22157 (triple-charged), 16618 (quadruple-charged), 13294 (quintuple-charged), and 11079 (sextuple-charged).

system are SPA and CHCA. Saturated SPA is preferable for looking at masses above 10–20 kD, while 10%–20% CHCA provides the best resolution for proteins/peptides up to about 5 kD. For urine protein profiling from 2–25 kD, more peaks and a higher degree of resolution were observed with 35% CHCA. Instrument settings such as detector sensitivity, detector voltage, and laser intensity have to be determined individually. The higher the detector sensitivity and voltage or the laser intensity, the better the detection of high-mass proteins. This is accompanied by an increase in background noise, which limits detection of low intensity peaks.

The number of positions sampled on a spot is an important parameter for optimal peak detection. Ideally, all proteins are distributed homogeneously on the spot and are crystallized homogeneously in the matrix. If so, one would expect to generate the same spectra at every position. From the three spot sampling protocols it is clear that there are "hot positions," where proteins are clustered on the spot leading to the detection of an abundance of peaks with a high intensity. Similarly, there are "cold positions," where only few or even no peaks are detected. Unfortunately, "hot position" sampling may not accurately profile low abundant proteins due to ion suppression that can occur due to high abundant proteins. Therefore, the most representative spectra for a given urine sample is achieved by sampling many different spot positions and combining the data. This is especially true for dilute urine samples.

The stability of urine proteins under various storage conditions is important to know. Recent studies have found no or just small changes in albumin-, retinol-binding protein-, N-acetyl glucosaminidase-, IgG- and kappa/lambda light-chain concentrations after storage at room temperature, 4°C, −20°C, and −70°C [11–14]. Our experiments using SELDI-TOF-MS found that up to four freeze-thaw cycles at −70°C did not alter the urine protein profile significantly; thereafter, peak intensities became weaker. The protein profiles of all midstream urine samples remained almost unchanged after storage for three days at 4°C, whereas three of six first-void urines underwent major changes. First-void urine can have significant bacterial contamination, resulting in either urine protein degradation and/or contamination with bacterial proteins within a few days.

Intrinsic factors

Midstream urine is the standard for almost all urine analysis. In a clinical setting, there are always urine samples that are not midstream urines. Therefore, knowing

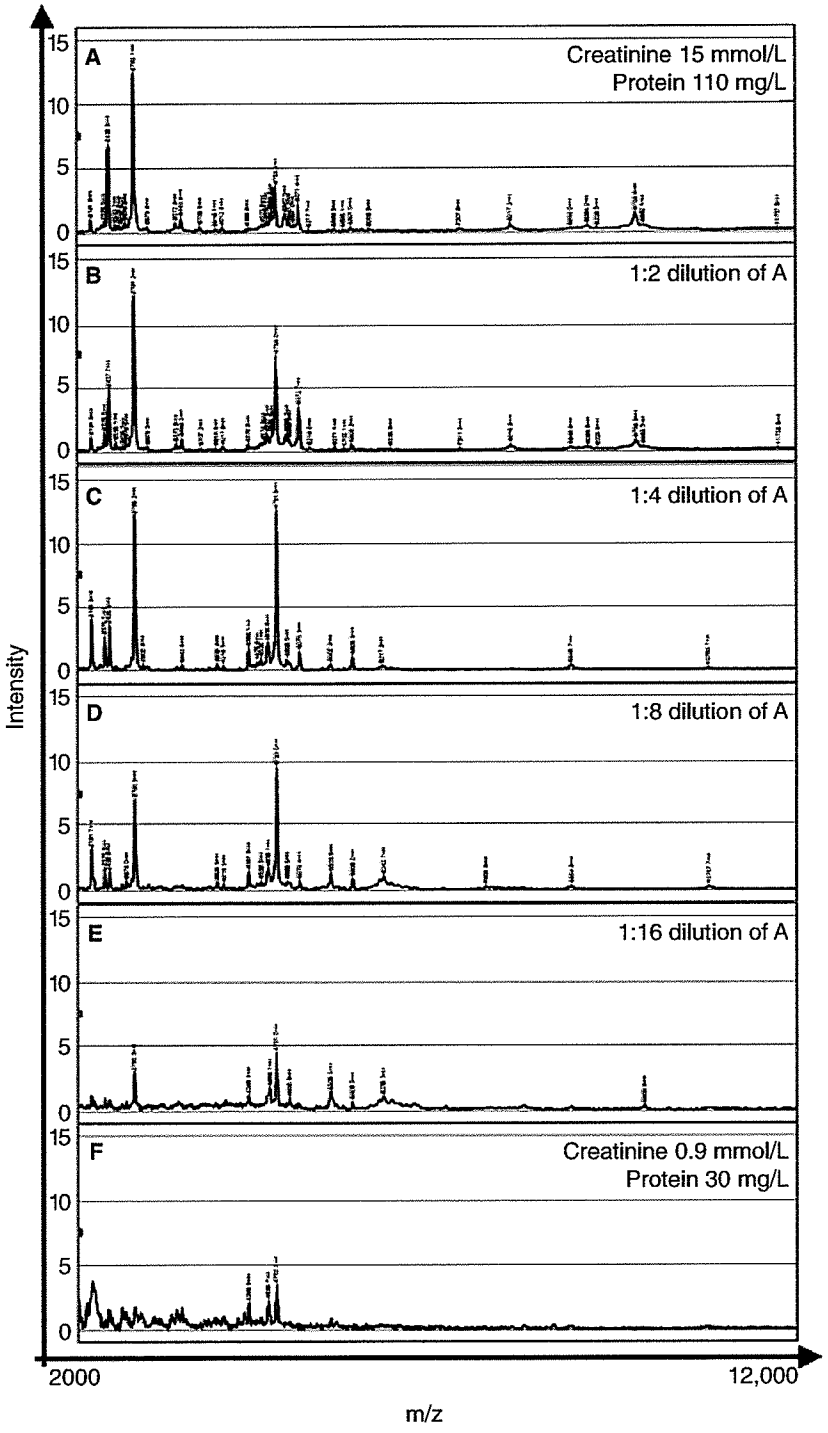


Fig. 6. Impact of dilution on urine protein profiles. Protein profile obtained from (*A*) Urine collected after a 20-hour period of no fluid intake. (*B-E*) Serial dilution of urine sample (A). (*F*) Urine collected after a 4-L fluid challenge. Starting with a 1:4 dilution, a continuous loss of peaks was observed.

the variation in urine protein profiles that may occur between first-void and midstream urines is important. There were no clear differences between first-void and midstream urine in males, whereas in females, variations were easily detectable. For example, a peak cluster between 3.3 and 3.5 kD (consistent with the masses of the α-defensins) is present in female first-void urine in high intensity. This peak cluster is either absent or of low intensity in female midstream urine samples. Indeed, α-defensins, which are an important part of the human antimicrobial defense [15, 16], have been detected by SELDI-TOF-MS technology in urine [3], as well as in culture supernatants of human

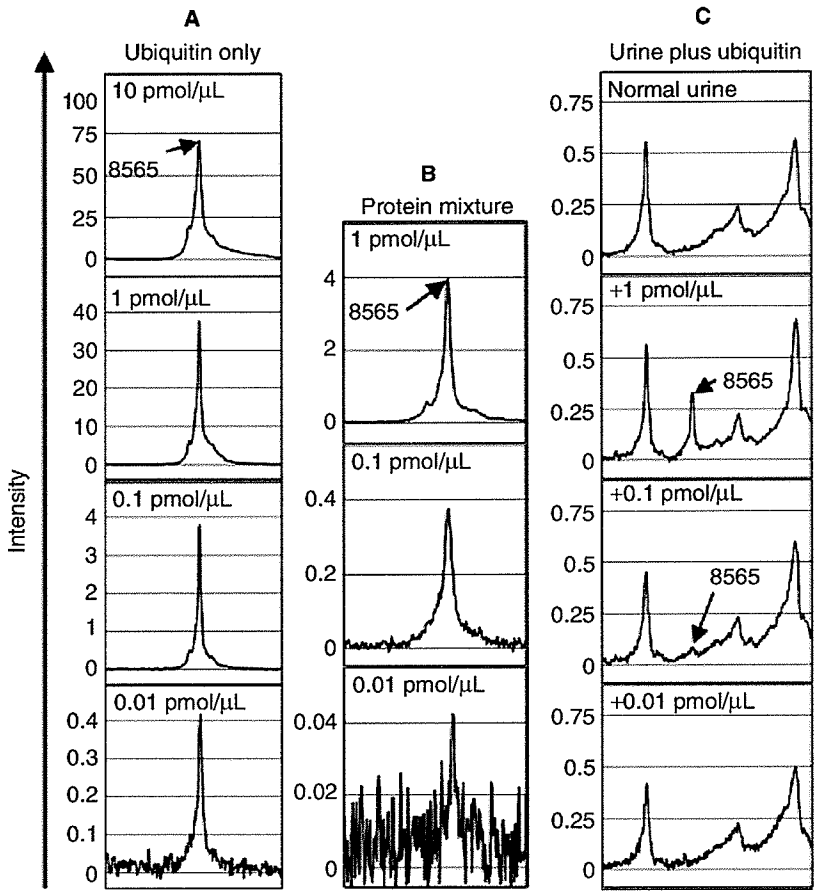


Fig. 7. Impact of protein concentration on peak intensity. (*A*) Dilution series of a single protein (ubiquitin, 8565 d) from 10 pmol/μL to 0.01 pmol/μL (equals 85.6, 8.56, 0.85, and 0.08 ng/μL, respectively). (*B*) Dilution series of ubiquitin from 1 pmol/μL to 0.01 pmol/μL in a mixture of four other proteins with constant concentrations (1.5 pmol/μL dynorphin A, 1 pmol/μL insulin, 0.3 pmol/μL cytochrome C and 0.3 pmol/μL superoxide dismutase). (*C*) Dilution series of ubiquitin from 1 pmol/μL to 0.01 pmol/μL spiked into normal male urine with a protein concentration of 110 mg/L.

$CD8^+$ T-cells [17]. The differences in the protein profile between first-void and midstream urines may be explained by urethral secretion of these proteins, which are then washed away by the first-void urine. Therefore, consistent urine protein profiling requires midstream urine samples for analysis, because first-void urine has a different protein composition than midstream urine and is more prone to protein degradation.

Blood was observed to be a major confounding variable affecting the normal urine protein profile. Not only did new peaks appear (i.e., peaks consistent with the masses of hemoglobin and albumin), but many of the normal peaks observed became undetectable. This is likely due to ion suppression by the blood proteins. Notably, even with a dilution of 10 μL blood in 64 mL diluted urine (1:6400 dilution), the peaks consistent with hemoglobin remained dominant. Clearly, such contamination invalidates any interpretation of the urine protein profile. Although centrifugation of the urine sample removes RBC, contamination with serum proteins will still continue to confound the urine protein profile.

Depending on fluid intake, the kidneys can concentrate urine to an output as low as 0.5 L/day, or dilute urine to almost 20 L/day. Under normal conditions, about 1–2 L urine are excreted per day. In a very dilute urine sample (urine creatinine 0.9 mmol/L), most of the proteins could not be detected on an NP20-chip. The threshold for a stable urine protein profile on an NP20-chip was a urine output of 2 L/day. Because every ProteinChip type has different binding capacities, the detection threshold has to be determined for every chip type individually.

In healthy individuals and under normal conditions, urine dilution, contamination with blood, and the portion of the urine specimen (first-void versus midstream) are the most obvious intrinsic factors that influence reproducibility and detection of proteins in profiles acquired by SELDI-TOF-MS. In addition, several transient "benign" states (e.g., fever, exercise) are known to increase the amount of proteins in urine [18, 19] by changing the size/charge selectivity of the glomerular barrier or by changing protein reabsorption/degradation through tubular cells [20, 21]. Whether different proteins appear in urine during these conditions has not yet been determined. However, these transient "benign" factors will need to be taken into account when comparing urine protein profiles from individuals in which such processes may be observed.

Protein quantification

Peak height and area under the peak have been used to reflect protein abundance [3, 22]. Although good correlation between the amount of a single protein alone or in a mixture with four other proteins and the peak intensity was found, it is questionable whether this remains true in a complex protein mixture (e.g., urine) due to many influencing factors like ion suppression and competition for binding sites on the ProteinChips. Therefore, care should be taken in comparing relative peak heights between two different urine protein profiles as an indicator of change in protein abundance under different circumstances (e.g., normal vs. pathologic state).

CONCLUSION

SELDI-TOF-MS offers many advantages for protein profiling in urine. First, only 5 to 10 μL of sample is needed for one analysis. Second, due to the simple chip preparation, many samples can be analyzed quickly. Third, the washing step removes most of the salts, which otherwise interfere with mass spectrometric analysis. And fourth, the impact of different chromatographic chemistries can be analyzed, which may allow one to find optimal purification conditions for a protein of interest in a short time with small amounts of sample. However, standardization of analysis conditions is essential, and both extrinsic and intrinsic factors must be taken into account for accurate data interpretation.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institute for Health Research (PN, JW, DR). SS is supported by training awards from the Swiss National Foundation (81-BS-68421) and the Novartis Stiftung (Basel, Switzerland).

Reprint requests to Dr. Peter Nickerson, Immunogenetics Laboratory, 777 William Avenue, Winnipeg, Manitoba, Canada R3E 3R4.
E-mail: peter.nickerson@bloodservices.ca

REFERENCES

1. Thongboonkerd V, Klein JB, Pierce WM, *et al*: Sodium loading changes urinary protein excretion: A proteomic analysis. *Am J Physiol Renal Physiol* 284:F1155–F1163, 2003
2. Knepper MA: Proteomics and the kidney. *J Am Soc Nephrol* 13:1398–1408, 2002
3. Hampel DJ, Sansome C, Sha M, *et al*: Toward proteomics in uroscopy: Urinary protein profiles after radiocontrast medium administration. *J Am Soc Nephrol* 12:1026–1035, 2001
4. Cutillas PR, Norden AG, Cramer R, *et al*: Detection and analysis of urinary peptides by on-line liquid chromatography and mass spectrometry: Application to patients with renal Fanconi syndrome. *Clin Sci (Lond)* 104:483–490, 2003
5. Thongboonkerd V, McLeish KR, Arthur JM, *et al*: Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int* 62:1461–1469, 2002
6. Hanash S: Disease proteomics. *Nature* 422:226–232, 2003
7. Spain CS, Davis MT, McGinley MD, *et al*: Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. *Proteomics* 1:93–107, 2001
8. Gygi SP, Rist B, Gerber SA, *et al*: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17:994–999, 1999
9. Zhou H, Ranish JA, Watts JD, *et al*: Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry. *Nat Biotechnol* 20:512–515, 2002
10. Hillenkamp F, Karas M, Beavis RC, *et al*: Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal Chem* 63:1193A–1203A, 1991
11. Tencer J, Thysell H, Andersson K, *et al*: Stability of albumin, protein HC, immunoglobulin G, kappa- and lambda-chain immunoreactivity, orosomucoid and alpha 1-antitrypsin in urine stored at various conditions. *Scand J Clin Lab Invest* 54:199–206, 1994
12. Froom P, Bieganiec B, Ehrenrich Z, *et al*: Stability of common analytes in urine refrigerated for 24 h before automated analysis by test strips. *Clin Chem* 46:1384–1386, 2000
13. Innanen VT, Groom BM, de Campos FM: Microalbumin and freezing. *Clin Chem* 43:1093–1094, 1997
14. Schultz CJ, Dalton RN, Turner C, *et al*: Freezing method affects the concentration and variability of urine proteins and the interpretation of data on microalbuminuria. The Oxford Regional Prospective Study Group. *Diabet Med* 17:7–14, 2000
15. Ganz T: Defensins in the urinary tract and other tissues. *J Infect Dis* 183(Suppl 1):S41–S42, 2001
16. Kunin CM, Evans C, Bartholomew D, *et al*: The antimicrobial defense mechanism of the female urethra: A reassessment. *J Urol* 168:413–419, 2002
17. Zhang L, Yu W, He T, *et al*: Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 298:995–1000, 2002
18. Bergstein JM: A practical approach to proteinuria. *Pediatr Nephrol* 13:697–700, 1999
19. Kashif W, Siddiqi N, Dincer AP, *et al*: Proteinuria: How to evaluate an important finding. *Cleve Clin J Med* 70:535–4, 546, 2003
20. Poortmans JR, Geudvert C, Schorokoff K, *et al*: Postexercise proteinuria in childhood and adolescence. *Int J Sports Med* 17:448–451, 1996
21. Russo LM, Bakris GL, Comper WD: Renal handling of albumin: A critical review of basic concepts and perspective. *Am J Kidney Dis* 39:899–919, 2002
22. Cazares LH, Adam BL, Ward MD, *et al*: Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by surface enhanced laser desorption/ionization mass spectrometry. *Clin Cancer Res* 8:2541–2552, 2002

J Am Soc Nephrol 15: 219–227, 2004

Proteomic-Based Detection of Urine Proteins Associated with Acute Renal Allograft Rejection

STEFAN SCHAUB,*† DAVID RUSH,*† JOHN WILKINS,*† IAN W. GIBSON,* TRACEY WEILER,*† KEVIN SANGSTER,*† LINDSAY NICOLLE,* MARTIN KARPINSKI,* JOHN JEFFERY,* and PETER NICKERSON*†

**Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; and †Manitoba Centre for Proteomics, Winnipeg, Manitoba, Canada.*

Abstract. At present, the diagnosis of renal allograft rejection requires a renal biopsy. Clinical management of renal transplant patients would be improved by the development of non-invasive markers of rejection that can be measured frequently. This study sought to determine whether such candidate proteins can be detected in urine using mass spectrometry. Four patient groups were rigidly defined on the basis of allograft function, clinical course, and allograft biopsy result: acute clinical rejection group ($n = 18$), stable transplant group ($n = 22$), acute tubular necrosis group ($n = 5$), and recurrent (or *de novo*) glomerulopathy group ($n = 5$). Urines collected the day of the allograft biopsy were analyzed by mass spectrometry. As a normal control group, 28 urines from healthy individuals were analyzed the identical manner, as well as 5 urines from non-transplanted patients with lower urinary tract infection. Furthermore, sequential urine analysis was performed in patients in the acute clinical rejection and the stable transplant group. Three prominent peak clusters were found in 17 of 18 patients (94%) with acute rejection episodes, but only in 4 of 22 patients (18%) without clinical and histologic evidence for rejection and in 0 of 28 normal controls ($P < 0.001$). In addition, the presence or absence of these peak clusters correlated with the clinicopathologic course in most patients. Acute tubular necrosis, glomerulopathies, lower urinary tract infection, and cytomegalovirus viremia were not confounding variables. In conclusion, proteomic technology together with stringent definition of patient groups can detect urine proteins associated with acute renal allograft rejection. Identification of these proteins may prove useful as non-invasive diagnostic markers for rejection and the development of novel therapeutic agents.

Although short-term and long-term kidney allograft survival has improved over the last 15 yr (1), allograft failure is still one of the most common causes for end-stage renal disease (2). Both immunologic and non-immunologic (*e.g.*, calcineurin-inhibitor-toxicity, hypertension) factors contribute to a continuous deterioration of allograft function, which is referred to as chronic allograft nephropathy (3). Acute allograft rejection is the major immunologic risk factor for developing chronic allograft nephropathy (4,5).

At present, the diagnosis of acute rejection can only be made by renal biopsy, which is costly, inconvenient, and carries a small risk of complications (6,7). Therefore, biopsies cannot be obtained frequently (*e.g.*, weekly) to monitor the immune response to the allograft, which may be helpful, as rejection can develop in allografts before graft dysfunction occurs (*i.e.*, subclinical rejection) (8,9). Sampling error is an additional problem, which can be diminished in part by collecting larger or multiple core biopsy samples (10,11). A non-invasive biomarker of rejection may benefit the kidney allograft recipient by allowing frequent monitoring to optimize immunosuppressive therapy. Various approaches such as mRNA measurement in urinary lymphocytes (12,13), urine flow cytometry (14), and measurement of alloreactive peripheral blood lymphocytes (15,16) have shown promising initial results, although none of these tests have yet reached wide clinical application.

As acute rejection is a complex process involving many different cell types of the donor and recipient, analysis of global changes at the gene (17–20) or protein level may provide both insights into its pathogenesis, as well as novel non-invasive biomarkers. Recent developments in mass spectrometry make it possible to rapidly profile and compare the proteome of clinical samples (21,22). In this study, we used proteomic technology and very rigid patient selection criteria, including allograft histology, allograft function, and clinical course, to detect urine proteins associated with acute renal allograft rejection.

Materials and Methods

Urine Collection

All urine samples were stored non-centrifuged at −70°C until further analysis. All patient and control urine samples were obtained

Received August 19, 2003. Accepted September 28, 2003.

Corresponding to Dr. Peter Nickerson, Immunogenetics Laboratory, Winnipeg Blood Centre, 777 William Avenue, Winnipeg, Manitoba, Canada R3E 3R4. Phone: 204-789-1125; Fax: 204-783-6780; E-mail: peter.nickerson@bloodservices.ca

1046-6673/1501-0219
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000101031.52826.BE

with informed consent and ethics approval by the University of Manitoba institutional review board.

Transplanted Patients

From July 1997 to March 2003, 2400 serial midstream urine samples from 212 renal transplant patients were collected. Patients were treated with a triple immunosuppressive regimen consisting of calcineurin-inhibitor (cyclosporine or tacrolimus), prednisone, and mycophenolate-mofetil or azathioprine. These 212 patients underwent a total of 693 protocol or clinically indicated core needle allograft biopsies. All biopsies were analyzed by experienced renal pathologists and scored according to the Banff 1997 classification (23) (acute Banff score: interstitial (ai 0–3), tubular (at 0–3), vascular (av 0–3), glomerular (ag 0–3); chronic Banff score: ci 0–3, ct 0–3, cv 0–3, cg 0–3). A biopsy specimen was judged adequate when $\geq$ 7 glomeruli and $\geq$ 1 vessel were present. Delayed graft function (DGF) was defined as the need for hemodialysis within the first week or a drop of serum creatinine <50% from pretransplant levels by day 5 posttransplant. On the basis of allograft function, the clinical course, and the allograft biopsy result, four rigidly defined patient groups were extracted from the whole patient population (n = 212) as follows.

Stable Transplant Group. Consists of 22 midstream urine samples (from 22 patients) obtained immediately before a protocol renal allograft biopsy performed within the first 12 mo posttransplant. None of these patients had experienced DGF. All had stable allograft function (*i.e.*, serum creatinine within 110% of baseline value at the time of biopsy), and none experienced a clinical or protocol biopsy-proven rejection before the date of examination. All biopsies met the criteria for adequacy, and all were required to have an acute and chronic Banff score of zero (*i.e.* ai0t0v0g0 and ci0t0v0g0).

Acute Clinical Rejection Group. Consists of 18 midstream urine samples (from 18 patients) obtained immediately before a renal allograft biopsy performed within the first 12 mo posttransplant. All experienced an elevation in creatinine >110% from baseline, and the diagnosis of acute rejection required an acute Banff score $\geq$ai2t2v0g0. Patients with a chronic Banff score >ci1t1v0g0 were excluded to avoid chronic allograft nephropathy as a confounding variable in the analysis.

Acute Tubular Necrosis (ATN) Group. Consists of 5 midstream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed within the first 6 d posttransplant to diagnose the cause of DGF. Antibody-mediated rejection was excluded on the basis of a negative flow-crossmatch and histologic changes on the biopsy consistent with ATN. In all biopsies, the acute Banff score was ai0t0v0g0, and significant donor pathology was excluded by requiring a chronic Banff score of $\leq$ci1t1v0g0.

Recurrent (or *de novo*) Glomerulopathy Group. Consists of 5 midstream urine samples (from 5 patients) obtained immediately before a renal allograft biopsy performed to diagnose the cause of proteinuria ($\geq$1.5 g/d). The patients had diagnoses of membranous glomerulonephritis, focal-segmental glomerulosclerosis, or IgA-nephropathy, and all had acute Banff scores $\leq$ai1t1v0g0.

Non-Transplanted Control Groups

Normal Control Group. Consists of 28 midstream urine samples from 28 healthy individuals (14 women and 14 men; age, 20–50 yr).

Urinary Tract Infection (UTI) Group. Consists of 5 midstream urine samples from 5 women obtained during an episode of a lower UTI, which was defined as requiring the clinical symptoms of a UTI, a leukocyte count in the urine sediment > 40/high power field, and a positive bacterial culture ($>10^8$ colony-forming units).

Urine Protein Profiling with Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

Urine samples were thawed on ice, vortexed, and centrifuged for 5 min at 10000 × g to remove remaining cell particles. Five microliters of urine supernatant were applied in duplicate to normal phase chips (ProteinChip NP20; Ciphergen, Freemont, CA) and incubated for 20 min in a humidity chamber. Spots were then washed three times with 5 μl of HPLC-grade water and air-dried for 10 min. One microliter of 35% α-cyano-4-hydroxycinnamic acid (CHCA: Ciphergen) was applied to each spot and air-dried. Chips were read with a SELDI-TOF-MS instrument (ProteinChip Reader II: Ciphergen) in the positive ion mode with the following settings: laser intensity, 230; detector sensitivity, 6; detector voltage, 1700 V; 240 shots were collected per sample. Peak labeling was performed with the ProteinChip Software (Version 3.1) for peaks with a signal-to-noise ratio of $\geq$ 3 in the mass over charge (m/z) range from 2000 to 80000. For comparison, spectra were normalized by total ion current. Calibration was done externally with a mixture of four proteins with masses ranging from 2 to 16 kD.

Determination of Cytomegalovirus (CMV) Viremia

CMV-viremia was measured on peripheral blood buffy coat specimens using a semiquantitative PCR assay developed at the Manitoba Cadham Provincial Laboratory that is accredited by the College of American Pathologists (for details see reference 32).

Statistical Analyses

We used JMP IN software version 4.0.4 (SAS Institute Inc., Cary, NC) for statistical analyses. For categorical data, Fisher exact test or Pearson χ^2 test was used. Parametric continuous data were analyzed by Student t tests or one-way ANOVA. For nonparametric continuous data, Wilcoxon or Kruskal-Wallis rank sum tests were used. A P-value < 0.05 (two-sided test) was considered to indicate statistical significance.

Results

Patient Characteristics

The acute clinical rejection group had more HLA-mismatches and a higher mean serum creatinine level at the time of the renal allograft biopsy compared with the stable transplant group. Otherwise, there were no significant differences between these groups (Table 1).

Characterization of Urine Protein Profiles Associated with Individual Patient Groups

In the m/z range from 5000 to 12000, we observed two distinct urine protein patterns when comparing the normal control group or stable transplant group to the acute clinical rejection group. One urine protein profile (rejection pattern) had prominent peak clusters in three regions corresponding to m/z values of 5270 to 5550 (region I; 5 peaks), 7050 to 7360 (region II; 3 peaks), and 10530 to 11100 (region III; 5 peaks) that always occurred together, whereas the other urine protein profile (normal pattern) had no peak clusters in these m/z regions (Figure 1). All 28 urine samples (100%) from the normal control group, 18 of 22 urine samples (82%) from the

Table 1. Patient characteristics

Variable	Stable Transplant ($n = 22$)	Acute Clinical Rejection ($n = 18$)	ATN ($n = 5$)	Recurrent or *de novo* Glomerulopathy ($n = 5$)
Female gender, *n* (%)	12 (55)	6 (33)	2	2
Age, mean ± SD	45 ± 13	43 ± 10	40 ± 18	47 ± 9
Caucasian race, *n* (%)	14 (64)	15 (83)	3	5
Nephropathy				
diabetic, *n* (%)	6 (27)	3 (17)	1	0
glomerulonephritis, *n* (%)	6 (27)	6 (33)	3	4
others, *n* (%)	10 (46)	9 (50)	1	1
First transplant, *n* (%)	21 (95)	16 (89)	5	4
Cadaveric donor, *n* (%)	15 (68)	10 (56)	3	5
HLA-mismatches, median (range)	3 (1–5)	4 (2–5)[a]	3 (2–4)	3 (3–5)
Panel-reactive antibodies (PRA)				
peak PRA >10%, *n* (%)	2 (9)	0	0	1
current PRA >10%, *n* (%)	1 (5)	0	0	1
Cytomegalovirus serology				
recipient neg./donor pos., *n* (%)	3 (14)	3 (17)	1	1
recipient neg./donor neg., *n* (%)	7 (32)	4 (22)	0	2
recipient pos./donor pos., *n* (%)	4 (18)	9 (50)	3	1
recipient pos./donor neg., *n* (%)	8 (36)	2 (11)	1	1
Allograft biopsy				
week posttransplant, median (range)	8 (3–51)	8 (1–18)	day 5 or 6[d]	253 (7–442)
rejection type (Banff 1997)				
IA (moderate tubulitis), *n* (%)		7 (39)		
IB (severe tubulitis), *n* (%)		8 (44)		
IIA (moderate arteritis), *n* (%)		3 (17)		
Creatinine at biopsy [μmol/L], mean ± SD	91 ± 26	180 ± 59[b]	942 ± 80[e]	122 ± 29
% above baseline, median (range)		25 (11–76)		
Proteinuria at biopsy [g/L], median (range)	0.07[c] (0.03–0.17)	0.09 (0.03–0.28)		3.20 (0.58–6.00)

[a] $P = 0.003$ *versus* stable transplant group.
[b] $P < 0.001$ *versus* stable transplant group.
[c] $P = 0.14$ *versus* acute clinical rejection group. $P < 0.001$ *versus* recurrent or *de novo* glomerulopathy group.
[d] Not included for statistical analysis.
[e] Not included for statistical comparison (3 of 5 patients were on hemodialysis).

stable transplant group, and 1 of 18 urine samples (6%) from the acute clinical rejection group showed the normal pattern. The rejection pattern was detected significantly more often in the acute clinical rejection group (17 of 18; 94%) than in the stable transplant group (4 of 22; 18%) ($P < 0.0001$) (Figure 2). The ATN, the recurrent (or *de novo*) glomerulopathy, and the UTI groups had urine protein profiles that were different from both the normal and the rejection pattern (Figure 1 and Figure 2).

Influence of CMV-Viremia on Urine Protein Profile Pattern

Twenty-seven of 40 patients (68%) in the stable transplant and acute clinical rejection groups were tested for the presence of CMV viremia at the time of renal allograft biopsy. Five patients tested positive; however, none had or developed CMV disease subsequently. CMV viremia was found in 2 of 21 patients (10%) with the rejection pattern and in 3 of 19 patients (16%) with the normal pattern ($P = 0.83$) (Table 2). We could not detect any additional peaks in the urine protein profiles from patients who had CMV-viremia.

Sequential Urine Protein Profile Analysis

To further determine the specificity of the normal and rejection pattern, we examined serial urine protein profiles in the stable transplant and acute clinical rejection groups and correlated them with the clinicopathologic course of the renal allograft. In particular we were interested in four specific outcomes: (1) the stable course persisted; (2) the stable transplant patient subsequently had an acute clinical rejection; (3) acute clinical rejection resolved to a stable course; (4) acute clinical rejection recurred.

In the stable transplant group, we had sufficient urine and histology samples for sequential analysis to evaluate 12 of the

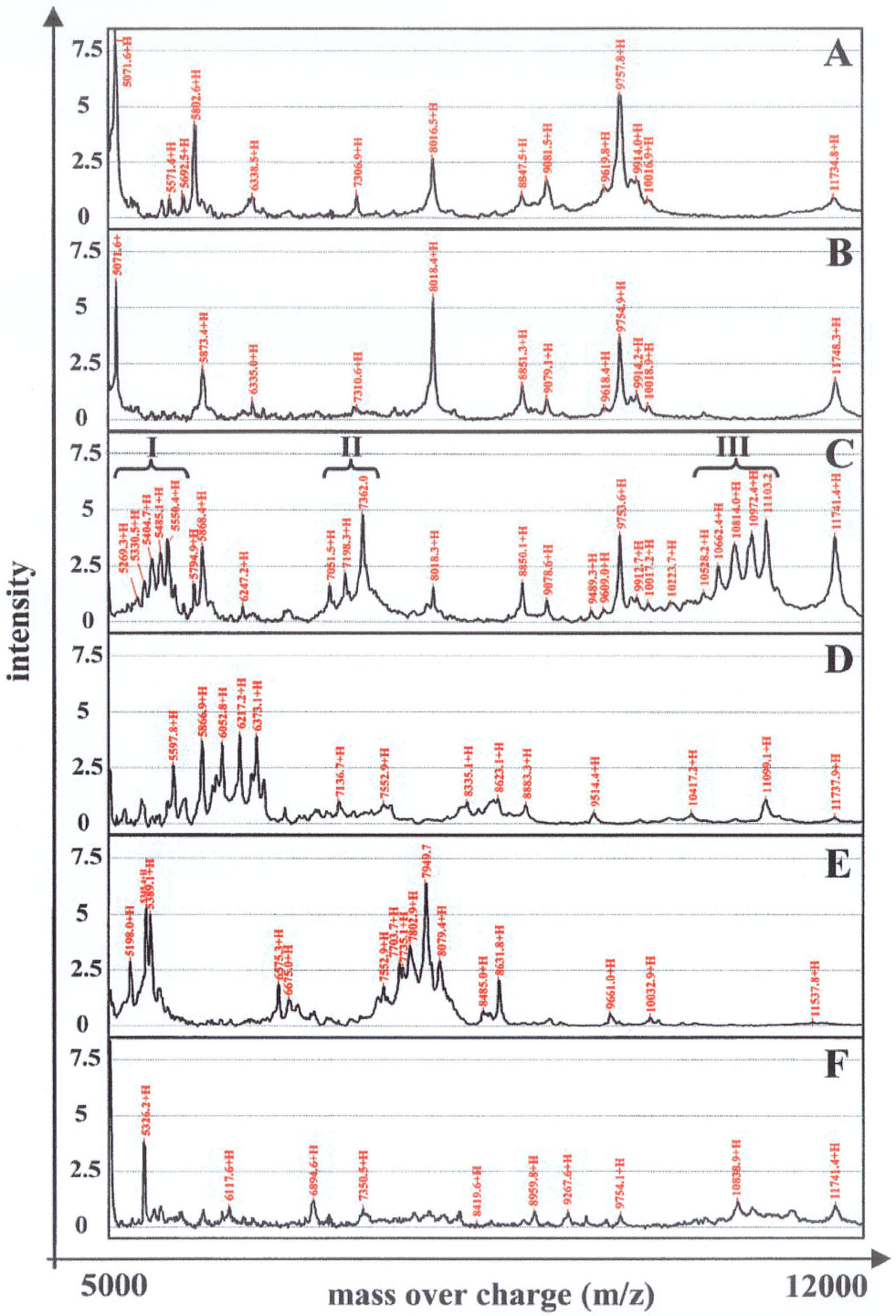


Figure 1. Representative urine protein profiles. (A) Normal control with normal pattern. (B) Stable transplant with normal pattern. (C) Acute clinical rejection with rejection pattern. (D) Glomerulopathy. (E) ATN. (F) UTI. The rejection pattern had prominent peak clusters in three regions corresponding to m/z values of 5270–5550 (region I; 5 peaks), 7050 to 7360 (region II; 3 peaks), and 10530 to 11100 (region III; 5 peaks).

18 patients who originally had a normal pattern. One patient went on to have stable allograft function and two normal protocol biopsies, but the urine profile could not be classified. One patient developed acute clinical rejection (Banff type IA) and the urine protein profile changed from the normal to the rejection pattern. In ten patients, stable allograft function per-

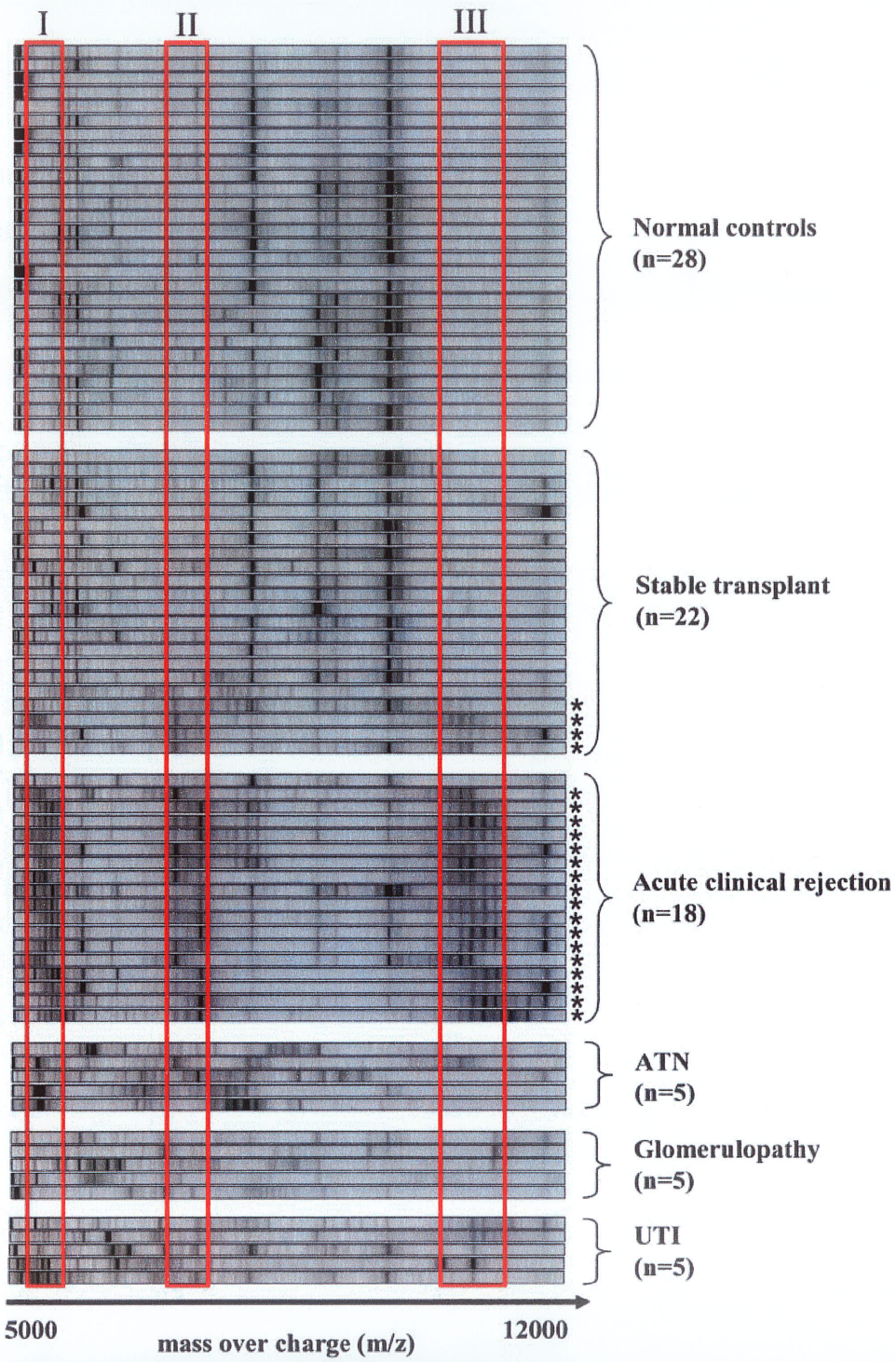


Figure 2. Software generated gel-view of urine protein profiles from all groups. Box frames represent the three regions corresponding to m/z values of 5270 to 5550 (region I), 7050 to 7360 (region II), and 10530 to 11100 (region III). * a urine sample with the rejection pattern.

sisted and 20 subsequent protocol biopsies were interpreted as normal ($n = 18$) or borderline rejection ($n = 2$). Eight of these ten patients showed the normal pattern throughout (Figure 3A), whereas two patients exhibited the rejection pattern in a single urine sample that subsequently reverted to the normal pattern.

In the acute clinical rejection group, we had sufficient urine

Table 2. Correlation between CMV-viremia and urine protein pattern

CMV-Viremia	Normal Pattern (n = 19)[a]	Rejection Pattern (n = 21)[b]	P-value
CMV-DNA positive, n	3	2	
CMV-DNA negative, n	10	12	P = 0.83
No CMV-PCR available, n	6[c]	7[d]	

[a] Consists of 18 patients from the stable transplant group plus 1 patient from the acute clinical rejection group.

[b] Consists of 4 patients from the stable transplant group plus 17 patients from the acute clinical rejection group.

[c] CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (n = 2); test was not ordered (n = 3); or only CMV pp65-antigen was evaluated (n = 1; patient tested negative).

[d] CMV-PCR was not performed for the following reasons: CMV sero-negativity of both donor and recipient (n = 3); test was not ordered (n = 3); or only CMV pp65-antigen was evaluated (n = 1; patient tested negative).

and histology samples for sequential analysis to evaluate 12 of the 17 patients who originally had a rejection pattern. One patient had two subsequent normal protocol biopsies, but the creatinine remained elevated at the level seen during the acute rejection episode (20% above baseline), and the urine always showed the rejection pattern. In six patients, the allograft function returned to baseline and subsequent protocol biopsies were interpreted as normal (n = 3) or borderline rejection (n = 3). All urine samples from these patients changed to the normal pattern (Figure 3B). Five patients had further episodes of acute clinical rejections, and all of them kept the rejection pattern throughout (Figure 3C).

Discussion

We used a proteomic technique to determine whether the urine of renal transplant patients undergoing acute allograft rejection had a characteristic profile. As urine can be very heterogeneous, standardization of urine collection and storage is critical. We have recently reported those factors that influence the reproducibility and peak detection in urines analyzed by SELDI-TOF-MS (24). In the design of the current study, we therefore required midstream urines that were collected immediately before the allograft biopsy and were stored the same day at −70°C. Next it was necessary to determine the urine protein profile of a "normal" kidney transplant, and this was done by selecting urines from patients with immediate and persistent good graft function that had normal graft histology on protocol biopsy. This stringently defined control group is unique as it includes histology; other groups attempting similar studies have inferred normal histology from a stable serum creatinine (12,13,16). Indeed, adherence to this stringent definition of "normal" demonstrates that the urine protein profile from 18 of 22 patients (82%) in the stable transplant group was similar to the urine profile of normal non-transplanted individuals.

The reliable identification of the urine protein pattern of the normal kidney transplant allowed for the clear differentiation, on visual inspection alone, of a distinct urine protein profile in the group with acute rejection (Figure 2). Other groups have used SELDI-TOF-MS to compare the protein profiles between different clinical outcomes, but required bioinformatic analysis to assign protein peaks to a specific outcome (25,26). In a similar study to ours, Clarke *et al.* (25) reported differences in the urine profiles between rejection and stable transplants; however, their requirement of bioinformatics to do so may relate to the fact that their definition of "stable" transplants was less stringent than ours (*i.e.*, based on serum creatinine alone). Interestingly, the protein peaks reported in their paper as specific to rejection are different from those found by our group. This may be related to the different protein chip surfaces and experimental conditions that were utilized; but also, to the fact that Clarke *et al.* (25) failed to include any control populations (*e.g.*, ATN, recurrent or *de novo* glomerulopathies, UTI, CMV) in the analysis, the importance of which is discussed below. In another study, Petricoin *et al.* (26) have used SELDI-TOF-MS to compare the protein profiles between different clinicopathologic diagnoses in cases of ovarian cancer, but also required bioinformatic analysis to assign peaks to specific outcomes. In their study the analysis involved serum samples, which is clearly a more complex biologic fluid than urine. Indeed, the urine-based proteomics has the advantage of excluding most of the serum proteins from the urine due to the size/charge selectivity of the glomerular basement membrane.

Urine profiles of the various groups could have been altered by the procedures of urine collection and storage. Due to the fact that all urine samples were stored non-centrifuged, the rejection pattern may have derived from intracellular proteins of leukocytes, red blood cells (rbc), or epithelial cells released after a freeze-thaw cycle. Interestingly, in one of the rejection cases, we found that lysis of rbc prevented the detection of the rejection pattern due to ion suppression. However, pre-centrifugation to remove the rbc before freeze-thawing of this sample allowed the rejection pattern to be detected (data not shown). Therefore, this argues that the pattern is not necessarily derived from cell lysis associated with a freeze-thaw cycle.

Although there were significant differences in the urine profiles between the stable transplant and the acute clinical rejection groups, there were also one "false negative" and four "false positives" samples. The only patient with the "false negative" urine profile in the acute clinical rejection group had no specific clinical or demographic feature. He had a course of a subclinical rejection (ai3t3g0v0) followed by a clinical rejection (ai3t3g0v1)—both treated with oral high dose steroids—and returned to normal histology (ai0t1g0v0) 15 wk later. We found no obvious explanation for this "false negative" result. Theoretically, a low protein concentration in dilute urine may influence the ability to detect a rejection pattern. However, the protein concentration of the urine samples from the stable transplant and the acute clinical rejection group were similar, making inadequate protein load an unlikely explana-

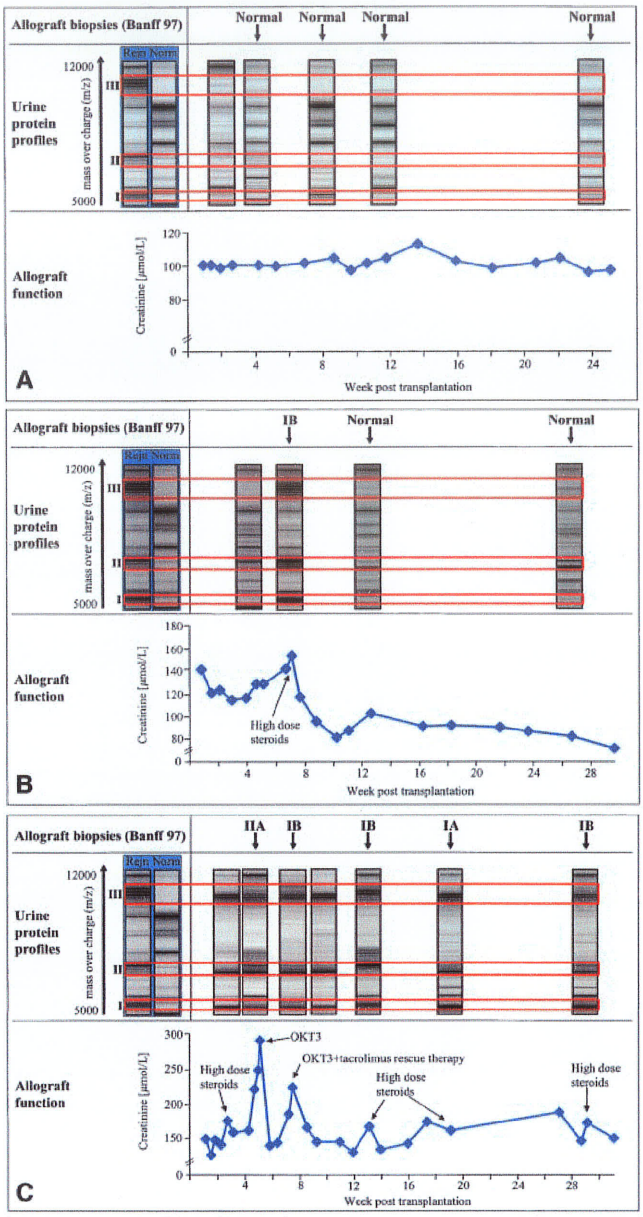


Figure 3. Sequential urine protein profiles in representative patients. Urine protein profiles highlighted in the blue box are examples of the rejection (Rejn) and the normal (Norm) pattern for comparison. Red box frames indicate the three regions corresponding to m/z values of 5270 to 5550 (region I), 7050 to 7360 (region II), and 10530 to 11100 (region III). (A) Patient with stable allograft function, normal protocol allograft biopsies, and normal pattern urine protein profiles throughout the posttransplant course. (B) Patient with acute clinical rejection (Banff IB) on week 7 posttransplant. After treatment with high-dose oral steroids, the serum creatinine normalized and remained stable. Subsequent allograft biopsies were normal. The urine protein profile showed the normal pattern 3 wk before the rejection episode, changed to the rejection pattern at the time of rejection, and returned to the normal pattern consistent with the subsequent allograft biopsies and the allograft function. (C) Patient with recurrent acute clinical rejection episodes (Banff IA to IIA). Despite treatment with OKT3, high-dose steroids and increased baseline immunosuppression, the patient always exhibited the rejection pattern.

tion for the absence of the rejection pattern. The four patients with "false positive" urine profiles in the stable transplant group also had no specific clinical or demographic features at the time of the biopsy. However, one of them went on to subclinical rejection (ai1t3g0v0) 9 wk later, and one experienced an acute clinical rejection and polyomavirus-type BK-nephropathy 13 wk later. The other two patients had a normal transplant course with stable graft function. There are mainly two possible explanations for these "false positive" results: first, they are true "false positives" and we cannot explain why; second, they are not "false positives," as the urine profile may be detecting an early rejection process that was missed by the allograft biopsy (*i.e.* sampling error) (10,11).

The urine protein profile in the ATN and glomerulopathy groups did not show the pattern of rejection. Both ATN and glomerulopathies are important in the differential diagnosis of allograft dysfunction and may represent pathophysiologic models of allograft injury distinct from that due to the alloimmune response. Whereas ATN can be regarded as a model of injury to the tubules due to ischemia-reperfusion, in the glomerulopathies, the injury, although presumably immune in nature, is largely centered on the glomerular capillary. As these two pathologic states did not show the characteristic pattern of rejection, we infer that the urine proteins detected in acute rejection are related to recipient immune cells infiltrating the graft and/or to tubular epithelial cells that are involved in the allo-directed inflammation. We acknowledge, however, that we cannot exclude the possibility that the urine proteins associated with rejection may also be found in other causes of tubular-based pathology (*i.e.*, calcineurin-inhibitor-toxicity, polyomavirus type BK-nephropathy, pyelonephritis). These latter outcomes are of relatively lower frequency in our patient population, such that we were unable to generate pure examples of each in sufficient number to make any reliable conclusions. Indeed, it is notable that only one patient (0.5%) in our patient population ($n = 212$) developed polyomavirus type BK-nephropathy, which is a much lower incidence than reported from another center (8%) (27).

An additional potential confounder of the diagnostic specificity of the urine protein profile observed in allograft rejection is systemic inflammation that could lead to the filtration of inflammatory proteins (*e.g.*, chemokines, cytokines) by the transplant kidney. Posttransplant CMV viremia, which has a high incidence in kidney transplant recipients (28,29) but very rarely infects the allograft (30,31), is one of the most common causes of systemic inflammation posttransplant. Indeed, our group has previously reported that CMV viremia is a significant confounding variable when examining activated T cells in the circulation as a possible non-invasive correlate of biopsy-proven allograft rejection (32). In the current study, we found no correlation between CMV viremia and the urine profile of rejection, which argues against systemic inflammation associated with CMV viremia as a significant confounding factor. While this does not rule out the possibility that other systemic inflammatory processes may mimic the urine profile seen in allograft rejection, it suggests that this is probably less likely.

It was of interest that the protein profile of rejection was

similar regardless of the histologic severity (Banff IA *versus* IB) or type (Banff IA/B *versus* IIA). This finding might represent a relative limitation of the technique of urine proteomics in identifying biomarkers specific for tubulointerstitial *versus* vascular rejection. However, because the assignment of histologic severity/type of acute rejection is based on a small biopsy sample of a large organ, urine profiling, which is representative of the entire allograft, may be pointing to the extent of heterogeneity of inflammation within the allograft, a fact that renal transplant pathologists are well aware of (11).

The correlation between the changes in serial urine profiles and the clinicopathologic course of the patients provided additional support that the detected proteins are related to acute allograft rejection. However, we do not propose the SELDI-TOF-MS spectra as a diagnostic test, but rather as a tool to detect proteins that are specifically involved in the pathogenesis of rejection. In addition, the patient selection criteria set for 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, we have avoided calculation of parameters that characterizes a clinical test (*e.g.*, sensitivity, specificity, positive and negative predictive value) because we regarded this as potentially misleading.

Clearly the isolation and identification of the urine proteins associated with acute clinical rejection is the next step. In terms of diagnostics, once they are identified, simple specific assays (*e.g.*, ELISA) may be developed to monitor the graft. In our study, the fact that the protein profiles were visually distinct between the normal and rejection pattern supports the possibility that an ELISA may detect significant quantitative differences. Finally, once validated in a larger patient population, the greatest utility of such a non-invasive biomarker may be to determine that the urine profile is normal, and by inference that the allograft is devoid of rejection. This may allow for tapering of immunosuppression, whereas an abnormal urine profile may warrant further investigation.

In conclusion, we have demonstrated that a proteomic technique, together with stringent patient selection based on allograft histology, allograft function, and clinical course, has the potential to detect a urine protein profile associated with acute renal allograft rejection. Our current efforts will concentrate on the identification of these proteins to develop a clinical test to non-invasively monitor the renal allograft post-transplant.

Acknowledgments

We thank Elzbieta Stern, Iga Dembinski, and the staff of the renal transplant unit for their help with urine specimen and clinical data collection. This work is supported by grants from the Canadian Institute for Health Research (PN, JW, DR). SS is supported by training awards from the Swiss National Foundation (81-BS-68421) and the Novartis Stiftung (Basel, Switzerland).

References

1. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D: Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 342: 605–612, 2000
2. Agodoa L, Eknoyan G, Ingelfinger J, Keane W, Mauer M, Mitch W, Striker G, Wilcox C: Assessment of structure and function in progressive renal disease. *Kidney Int Suppl* 63: S144–S150, 1997
3. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 346: 580–590, 2002
4. Meier-Kriesche HU, Ojo AO, Hanson JA, Cibrik DM, Punch JD, Leichtman AB, Kaplan B: Increased impact of acute rejection on chronic allograft failure in recent era. *Transplantation* 70: 1098–1100, 2000
5. Almond PS, Matas A, Gillingham K, Dunn DL, Payne WD, Gores P, Gruessner R, Najarian JS: Risk factors for chronic rejection in renal allograft recipients. *Transplantation* 55: 752–756, 1993
6. Wilczek HE: Percutaneous needle biopsy of the renal allograft. A clinical safety evaluation of 1129 biopsies. *Transplantation* 50: 790–797, 1990
7. Kolb LG, Velosa JA, Bergstralh EJ, Offord KP: Percutaneous renal allograft biopsy. A comparison of two needle types and analysis of risk factors. *Transplantation* 57: 1742–1746, 1994
8. Rush DN, Henry SF, Jeffery JR, Schroeder TJ, Gough J: Histological findings in early routine biopsies of stable renal allograft recipients. *Transplantation* 57: 208–211, 1994
9. Shapiro R, Randhawa P, Jordan ML, Scantlebury VP, Vivas C, Jain A, Corry RJ, McCauley J, Johnston J, Donaldson J, Gray EA, Dvorchik I, Hakala TR, Fung JJ, Starzl TE: An analysis of early renal transplant protocol biopsies–the high incidence of subclinical tubulitis. *Am J Transplant* 1: 47–50, 2001
10. Sorof JM, Vartanian RK, Olson JL, Tomlanovich SJ, Vincenti FG, Amend WJ: Histopathological concordance of paired renal allograft biopsy cores. Effect on the diagnosis and management of acute rejection. *Transplantation* 60: 1215–1219, 1995
11. Nicholson ML, Wheatley TJ, Doughman TM, White SA, Morgan JD, Veitch PS, Furness PN: A prospective randomized trial of three different sizes of core-cutting needle for renal transplant biopsy. *Kidney Int* 58: 390–395, 2000
12. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, Serur D, Mouradian J, Schwartz JE, Suthanthiran M: Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 344: 947–954, 2001
13. Ding R, Li B, Muthukumar T, Dadhania D, Medeiros M, Hartono C, Serur D, Seshan SV, Sharma VK, Kapur S, Suthanthiran M: CD103 mRNA levels in urinary cells predict acute rejection of renal allografts. *Transplantation* 75: 1307–1312, 2003
14. Roberti I, Reisman L, Burrows L, Lieberman KV: Urine cytology and urine flow cytometry in renal transplantation–a prospective double blind study. *Transplantation* 59: 495–500, 1995
15. Gebauer BS, Hricik DE, Atallah A, Bryan K, Riley J, Tary-Lehmann M, Greenspan NS, Dejelo C, Boehm BO, Hering BJ, Heeger PS: Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. *Am J Transplant* 2: 857–866, 2002
16. Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH: Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: Potential novel assay for prediction of outcomes for renal transplant recipients. *J Am Soc Nephrol* 13: 252–259, 2002
17. Scherer A, Krause A, Walker JR, Korn A, Niese D, Raulf F: Early prognosis of the development of renal chronic allograft rejection by gene expression profiling of human protocol biopsies. *Transplantation* 75: 1323–1330, 2003

18. Eikmans M, Sijpkens YW, Baelde HJ, De Heer E, Paul LC, Bruijn JA: High transforming growth factor-beta and extracellular matrix mRNA response in renal allografts during early acute rejection is associated with absence of chronic rejection. *Transplantation* 73: 573–579, 2002
19. Stegall M, Park W, Kim D, Kremers W: Gene expression during acute allograft rejection: Novel statistical analysis of microarray data. *Am J Transplant* 2: 913–925, 2002
20. Chua MS, and Sarwal MM: Microarrays: New tools for transplantation research. *Pediatr Nephrol* 18: 319–327, 2003
21. Knepper MA: Proteomics and the kidney. *J Am Soc Nephrol* 13: 1398–1408, 2002
22. Hanash S: Disease proteomics. *Nature* 422: 226–232, 2003
23. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Yamaguchi Y: The Banff 97 working classification of renal allograft pathology. *Kidney Int* 55: 713–723, 1999
24. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int* in press
25. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP: Characterization of renal allograft rejection by urinary proteomic analysis. *Ann Surg* 237: 660–665, 2003
26. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA: Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359: 572–577, 2002
27. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Steiger J: Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med* 347: 488–496, 2002
28. Sagedal S, Nordal KP, Hartmann A, Degre M, Holter E, Foss A, Osnes K, Leivestad T, Fauchald P, Rollag H: A prospective study of the natural course of cytomegalovirus infection and disease in renal allograft recipients. *Transplantation* 70: 1166–1174, 2000
29. Abecassis MM, Koffron AJ, Kaplan B, Buckingham M, Muldoon JP, Cribbins AJ, Kaufman DB, Fryer JP, Stuart J, Stuart FP: The role of PCR in the diagnosis and management of CMV in solid organ recipients: what is the predictive value for the development of disease and should PCR be used to guide antiviral therapy? *Transplantation* 63: 275–279, 1997
30. Andersen CB, Ladefoged SD, Lauritsen HK, Hansen PR, Larsen S: Detection of CMV DNA and CMV antigen in renal allograft biopsies by in situ hybridisation and immunohistochemistry. *Nephrol Dial Transplant* 5: 1045–1050, 1990
31. Kashyap R, Shapiro R, Jordan M, Randhawa PS: The clinical significance of cytomegaloviral inclusions in the allograft kidney. *Transplantation* 67: 98–103, 1999
32. Karpinski M, Rush D, Jeffery J, Pochinco D, Milley D, Nickerson P: Heightened peripheral blood lymphocyte CD69 expression is neither sensitive nor specific as a noninvasive diagnostic test for renal allograft rejection. *J Am Soc Nephrol* 14: 226–233, 2003