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Temporal stability of the urinary proteome after kidney transplant: more sensitive than protein composition?

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Abstract

The temporal urinary proteome was examined in 4 groups of individuals in order to determine the temporal stability of diverse individuals with apparently good kidney health. The groups consisted of: 1) healthy volunteers at zero time, one and six months, 2) kidney donors before and after surgery, 3) recipients immediately after surgery and 4) successful kidney transplant recipients from one month to 4 years after transplant. Proteins were detected by reverse phase extraction of urine followed by MALDI-TOF profile and by iTRAQ analysis. Unusual components of the MALDI-TOF profiles found only in transplant subjects occurred at $m/z=3370$, 3441 and 3385 (human neutrophil defensins), 4303 , 10350 , and 11732 (beta-2 microglobulin, B2M). The peaks at $m/z=4303$ and 11732 were also quite intense among kidney donors following surgery. The peaks at $m/z=4303$ and 10350 in transplant recipients were associated with higher serum creatinine. Several additional proteins detected by iTRAQ were up-regulated in a manner that correlated closely with B2M. Overall, despite large differences between protein composition in different transplant recipients, there was remarkable stability for each individual as detected by either MALDI-TOF or iTRAQ analyses. These results suggested that, within limits, stability of profile components may be as important as protein content for definition of kidney health. Longitudinal study of urinary proteins from kidney recipients may demonstrate instability as a sensitive biomarker of adverse kidney health.

Synopsis—A stable urinary proteome may best signify stable function for kidney transplant recipients. MALDI-TOF profiles of urine collected from a kidney recipient 2 and 4 years after transplant show only one peak common to controls ($m/z=9746$). The shared property of transplant recipients with stable function was remarkable consistency of the urine proteome during follow-up, despite the frequent presence of unusual components. These properties were confirmed by iTRAQ analysis.

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SUPPORTING INFORMATION: Two more examples of sequential MALDI-TOF profiles from transplant recipients; tables of proteins identified by iTRAQ technology and accompanying information.

Keywords

Kidney transplant; Proteomics; MALDI-TOF; iTRAQ; Temporal stability; urine proteome

Kidney transplant function is frequently followed by changes in serum creatinine. Although highly useful, this marker is a relatively poor indicator of graft dysfunction until significant damage has occurred. Moreover, many transplant centers do not routinely use protocol biopsies to observe changes at earlier stages of graft failure. The long-term benefits of this procedure are unknown and carries some risk to the graft (1). Another approach is the study of urine proteins to identify non-invasive biomarkers of kidney disease. These biomarkers may assist providers in tailoring immunosuppression regimens to optimize graft survival for each individual.

Proteomics is increasingly used in the discovery of biomarkers. Examples include cancer screening and various kidney diseases. Potential biomarkers have been reported for glomerular diseases in native kidneys (2) and for acute rejection (3-5) and BK virus nephropathy (6) in kidney transplants. These studies were mostly cross-sectional in nature and did not test intra-individual variability over time. Plasma protein profiles of healthy individuals were found to be remarkably stable over time for each individual but, as a group, a normal value was difficult to establish because of inter-individual variability (7). If these properties extend to the urinary proteome, it is possible that longitudinal analyses of persons with kidney disease may identify biomarkers that are characterized by temporal variability.

Several methods have been used to search for biomarkers in urine samples. The most commonly reported methods include two-dimensional electrophoresis (2,8,9), surface-enhanced laser desorption/ionization time-of-flight (SELDI (5,10,11)), and capillary electrophoresis (12). Oetting, et al. (4) successfully used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) to identify beta-2 microglobulin (B2M) in the urine as a potential biomarker in acute rejection for kidney transplant recipients. Another method recently introduced is iTRAQ. This technique uses isobaric peptide tags to modify all primary amines to provide accurate identification and quantification of proteins in multiple samples. The use of this method on kidney tissue was recently reported in a mouse model of autosomal-dominant polycystic kidney disease (13). However, we are unaware of iTRAQ studies of kidney transplant recipients.

To study the stability of serial urinary profiles and variation within different groups of individuals, we examined serial samples from four groups of subjects: 1) healthy volunteers at zero time and 6 months, 2) kidney donors before and after surgery, 3) kidney transplant recipients immediately after surgery and 4) successful kidney transplant recipients from one month to 4 years after transplant. The results show large inter-person differences but remarkable intra-person temporal stability of urinary proteome among healthy individuals and stable transplant recipients.

Materials and Methods

All urine samples were collected after informed consent and the protocols were approved by the institutional review boards at the University of Minnesota Medical Center and Hennepin County Medical Center.

Laboratory Measures

Serum creatinine was measured using the Beckmann Rate Jaffe/CXR Synchron method which was calibrated to the MDRD core laboratory (14). Urine total protein was measured from a 24 hour urine collection. The urine albumin/creatinine ratio was determined from early morning voided samples from two consecutive days using nephelometry for albumin concentration and an auto analyzer for creatinine concentration (15). Glomerular filtration rate (GFR) was measured using iothalamate clearance. Renal biopsies were read by an experienced renal pathologist.

Kidney Donors

Urine samples from kidney donors were collected at the University of Minnesota Medical Center as part of a prospective study being done at our institution. These donors present to the clinic the day prior to surgery for a final evaluation. A mid-stream, clean-catch urine specimen was collected as their baseline sample (day minus 1). After surgery, the day 0 sample is collected as a catheter specimen. Urine samples were collected daily either from an indwelling catheter or as a clean-catch specimen until the subject was discharged from the hospital. Several individuals returned to the surgery clinic for follow up and provided urine samples 2-4 weeks after donation.

Kidney Recipients — Peri-operative Samples

Urine samples from kidney recipients immediately after transplantation were collected at the University of Minnesota. These recipients, also enrolled in the same study for kidney donors, received a kidney from one of the kidney donors described above. The first urine sample was collected within 6 hours of surgery as a catheter specimen. Urine samples were collected daily from an indwelling urinary catheter until this was discontinued and the subject was discharged.

Kidney Recipients — Long-term Samples

Urine samples for long-term follow up for kidney recipients were obtained from the Angiotensin II Blockade in Chronic Allograft Nephropathy (ABCAN) study, an ongoing, randomized, double-blinded, placebo-controlled trial (16). This study enrolled stable kidney or kidney-pancreas recipients 1 month after transplant and were randomized to either losartan or placebo to study the effects of angiotensin blockade in the progression of interstitial fibrosis/tubular atrophy. Urine samples were collected at the beginning of the study (1 month after surgery) and yearly thereafter during the 5 year study. Upon entry of the study and during annual visits, the subjects had their GFR, urine protein, urine albumin/creatinine, and serum creatinine measured. Kidney deterioration in this study was defined as a halving of glomerular filtration rate (GFR) or a doubling of protein excretion. None of the individuals in this study met either of these criteria and all were considered successful transplant recipients defining a healthy kidney. Samples were collected yearly and microalbuminuria determined. Seven of the 16 individuals showed persistent microalbuminuria (defined as 2 consecutive samples with microalbuminuria) at some time during the 4 years of the study. iTRAQ analysis included only one individual with microalbuminuria, subject 14 who showed microalbuminuria at both time points assayed.

Urine Storage

All urine samples were immediately centrifuged for 10 min at 5,000 rpm and the supernatant stored at -80C until further analysis. No protease inhibitors were added to the urine prior to freezing. At time of analysis, the original stock samples were thawed and aliquoted to minimize freeze-thaw cycles.

MALDI-TOF Profile Analysis

The procedure for analysis of urine was adapted from the method described for diluted blood plasma (17). Briefly, urine samples were thawed (a single freeze-thaw cycle) and 0.1 mL was acidified to pH <3.0 by sequential additions (0.5 microliters) of 10% trifluoroacetic acid (TFA). A reverse phase C4 ZipTip (Millipore Corp.) containing approximately 1 microliter of reverse phase column resin was activated by drawing and extruding two 10 microliter volumes of water:acetonitrile:TFA (50:50:0.1) and washed with two 10 microliter changes of water:TFA (100:0.1). The acidified urine was slowly drawn into and extruded from the tip for 2 minutes. The tip was washed 7 times with 10 microliter changes of water:TFA (100:0.1) and peptides eluted by aspiration and extrusion of 1.6 microliters of water:acetonitrile:TFA (25:75:0.1, eight times). The eluted proteins (0.75 microliters) were applied to a MALDI target along with 0.75 microliters of 85% saturated sinapinic acid in water:acetonitrile:TFA (50:50:0.1). The solution was stirred with the pipette tip until crystallization fully developed. The target was dried and analyzed in a Bruker Biflex III mass spectrometer at a laser attenuation of 39-46, depending on the age of the laser. Five hundred laser shots were collected from at least 10 locations on the target. The spectra were smoothed (15 units) and background was subtracted. Peaks were labeled at the time that the spectra were collected. A defined peak had an intensity of at least 100 counts over a background of approximately 50 counts. The resulting profiles showed a high level of reproducibility with respect to the ratio of saposin B isoforms and the other components observed. For example, replicate assays of the 12 samples gave an average coefficient of variation of 7% for the saposin B peak ratio. Up to four freeze-thaw cycles did not alter the observed ratio. For novel peaks found only in transplant recipients or donors following surgery, saposin B offered an internal standard to ensure that the assay was successful. Reproducibility of these peaks was tested by two different individuals who performed MALDI-TOF analysis on the same 25 samples at a 12-month interval. The first analysis followed one freeze-thaw cycle and the second followed two. Intermediate storage

was at -80 degrees C. The sum of the targeted peaks (B2M, the components at 4303, 10350 and 9480) was 104. Ninety-four of the peaks were identified in both assays.

iTRAQ® Analysis

Urine samples (1.5mL or 2mL) were dialyzed against 20 mM NH_4HCO_3 solution (pH = 7.8) using dialysis tubing with a MW cutoff of 3.5 K (Spectrum Laboratories, Inc., Rancho Dominguez, CA) to remove salts. They were then frozen and lyophilized. The protein powders were dissolved in 50 microliters of iTRAQ® dissolution buffer (20-fold diluted, final concentration = 25 mM, pH 8.5), and protein concentrations were measured using a protein assay kit (BioRad, Hercules, CA). Equivalent amounts of protein (12.5 μg) from each of eight urine samples were labeled for each iTRAQ® experiment (Applied Biosystems, Inc., Foster City, CA., ABI) following manufacturer's instructions. The 8-plex iTRAQ labeling procedure is very similar to 4-plex¹ except the incubation time during labeling was two hours instead of one hour. The final mixture of labeled samples was desalted and purified using MCX cartridge (Mixed-mode Cation Exchange, Oasis® solid phase extraction, 30 μm , 3 cc, 60 mg, Waters, Milford, MA). They were then subjected to HPLC fractionation and mass spectrometry analysis.

LC-MALDI-MS

The strong cation exchange (SCX) (column size = 150 $\times$ 1.0mm, 5 μm , 300 $\text{\AA}$, flow rate = 35 $\mu\text{L}/\text{min}$, The Nest Group Inc., Southborough, MA) fractionation followed a standard protocol developed in the Center for Mass Spectrometry and Proteomics. Selected fractions (14 to 24) were subjected to the Tempo™ LC-MALDI spotting system (ABI, Foster City, CA) coupled with capillary LC using in-house packed C18 columns with a length of ~12 cm using 100 μm Integrafrit™ tubings (New Objective, Woburn, MA; packed with Magic C18AQ, Michrom BioResources, Auburn, CA, 5 μm 200 $\text{\AA}$). Peptides were then loaded onto a ProteoCol™ C18 trap cartridge (SGE Analytical Science, Victoria, Australia, column size = 10mm length $\times$ 300 μm internal diameter, 300 $\text{\AA}$ pore size) and washed for 18 minutes at 9 microliters/min with load buffer (98:2, water:ACN, 0.1% formic acid). Gradient separation of peptides at 500 nL/min was performed with the following profile: 0 – 5 min 5 – 15% Buffer B, 5 – 52 min 15 – 35% Buffer B, 52 – 54 min 35 – 80% Buffer B, 54 – 64 min 80% Buffer B followed by re-equilibration for 8 minutes to 95% A Buffer (Buffer A = 2:98, ACN:H₂O, 0.1% trifluoroacetic acid (TFA) and Buffer B = 98:2, ACN:H₂O, 0.1% TFA). Peptide elution was monitored by UV absorbance at 214 nm. LC eluate was deposited onto LC-MALDI targets (1232-spot format). A matrix solution of 6 mg/mL α -cyano-4-hydroxycinnamic acid (Fluka brand from Sigma Aldrich) in 75:25, ACN:H₂O, 0.1% TFA and 10 mM ammonium phosphate was added post-UV in a mixing tee at 1 microliter/min using a Harvard Apparatus (Holliston, MA) syringe pump. Spotting interval was 24 seconds and the plate voltage applied during each spotting event was 2.8 kV.

Peptide-containing LC spots were submitted to 4800 MALDI TOF/TOF™ analyzer (ABI) with a 200 Hz repetition rate (Nd:YAG laser). MS full scan spectra were acquired from 800 – 4000 m/z . A total of either 800 or 1000 (sample dependent) laser shots were accumulated for each TOF-MS spectrum at an optimized fixed laser setting. Tandem MS mode was operated with 2kV collision energy with CID gas (air) over a range of 10 m/z to 95% of the precursor mass value. Precursor mass window was 250 ppm (FWHM) in relative mode. A minimum of 800 and a maximum of 4000 laser shots were accumulated with laser stop conditions set at six product ion peaks of S/N (signal to noise, S/N, ratio) > 60 at an optimized, fixed laser setting with metastable suppressor option on. Data dependent tandem MS settings included acquisition of up to 20 most intense ion signals per spot. If two or more consecutive spots in an LC run with precursor m/z were within 200 ppm tolerance, the spot with the maximum S/N was subjected to tandem MS analysis.

iTRAQ® Data Analysis

Peptide identification and quantification were obtained with ProteinPilot™ software (Version 1.1, ABI). The MS data was searched against NCBI Human Reference Sequence database (October, 2007, 32,850 entries) using the thorough search option with fixed MMT modifications on Cys residues. Only proteins identified with 95% identification confidence (> 1.3 Unused Score) were selected. The protein identification information and the number of peptides used for identification is shown in supplemental data. Proteins with >95% confidence and Error Factor < 2 (EF, manufacturer's criteria) were subjected to further quantification analysis.

Statistical Analysis

Continuous variables are reported as mean±standard deviation. Comparisons between groups were analyzed with the two-tailed Student's t-test in Microsoft EXCEL (Office EXCEL 2003, Microsoft Corporation, Redmond, WA) for comparisons between groups. Error estimates represent standard deviation. Correlation of components in the MALDI-TOF profile (binary response, peak present/absent) with continuous variables including GFR, protein and albumin excretion, and creatinine were evaluated using mixed linear models with repeated measures with SAS (Version 9.1, SAS Institute Inc., Cary, NC). Variance of protein ratios in different samples was determined with ANOVA (Analysis of Variance).

RESULTS

Kidney donors before and after donation

Urine specimens from 20 potential kidney donors (average age 41.0±12.0 years, 11 males and 9 females) were obtained. Their average creatinine at time of donation was 1.0±0.14 mg/dL. MALDI-TOF profiles of pre-surgery controls showed two ubiquitous components at m/z=9073 and 9746 with few others detected (light line, Figure 1A, B). These represented saposin B glycoisoforms (18). The lower mass protein contains one sugar residue while the higher mass contains 5. Superimposed on the pre-surgery samples is an averaged profile of 12 kidney donors at 1 to 3 days post-surgery (heavy line, Figure 1A, B). New intense peaks at m/z=4303 and 11732 (beta-2-microglobulin, B2M (4)) appeared in all individuals. The average intensities of these components had not declined by day 3. A sub-group of these individuals showed a component at m/z=10350. Whether these components arose from the kidney or other source is not known. An impact of catheterization on protein content seemed unlikely but could not be ruled out. It was of interest that the same components characterized some of the transplant recipients several years following surgery (see below).

Another indication of status change following kidney donation was the ratio of the saposin B glycoisoforms (m/z=9073/9746). As shown by previous studies, the ratio of protein isoforms of the plasma, as detected by mass spectrometry, was characteristic of each individual and very stable over time in a healthy individual (17). However, the ratio changed in response to kidney donation (Figure 2). Evaluation of 12 kidney donors showed an average ratio for m/z=9073/9746 of 0.35±0.066 before surgery and 0.53±0.16 (p=0.001) at day 2. Results for one individual at 24 days showed a return to normal with sequential values of: baseline ratio=0.34, 2 days=0.62 and 24 days=0.39. While the change of saposin B isoforms was highly significant, overlap of pre- and post-surgery values was considerable. Use of this ratio to distinguish pre- vs. post-operative patients in a population would result in high numbers of false positive and false negative readings. On the other hand, comparison of values for each subject before and after surgery would successfully identify most donors. The change provided proof of principle that 1) protein isoform ratios can serve as surrogate biomarkers of health status or challenge (7) and 2) that longitudinal study of each individual was substantially more sensitive for detection of status change than cross-sectional comparison of a population.

Profiles of kidney recipients following surgery

Four kidney transplant recipients (average age, 49.4 ± 5.2 years; 3 males, 1 female; 3 were on hemodialysis prior to transplant) were evaluated on days 1-3 following surgery. Diabetes was the underlying disease in all four individuals. Most profiles contained hemoglobin ($m/z=15126$ and 15880 , not shown) that was assumed to be temporary and to arise from surgery. Key regions of the averaged profile for samples collected from 3 of the subjects are shown in Figure 3A. Saposin B was detected in all of these samples. Other components included those common to kidney donors immediately after surgery (B2M plus peaks at $m/z=4303$ and 10350 , Figure 3A). Five of 9 samples contained detectable levels of a component at $m/z=9480$. This feature was almost ubiquitous among persons with advanced native kidney disease¹ but was a minor component of the samples in Figure 3.

The fourth individual showed a profile similar to other kidney recipients at day 1 with intense B2M and low intensity of $m/z=10350$ (Figure 3B). This individual produced no urine prior to transplant so that profile components all arose from the transplanted kidney. On day 2, intense components occurred at $m/z=4272$, 4860 , and 5007 appeared along with an unidentified peak at $m/z=2715$ along with the disappearance of B2M (Figure 3C). This was unusual as these were among the most common features in profiles of persons with advanced kidney disease or with prognosis of transplant kidney failure¹. The profile obtained from this individual on day 3 showed the same properties with increased levels of the components at $m/z=2715$ and 9480 . Clinically, on post-operative day 1, this subject showed declining serum creatinine indicating immediate graft function. On day 2 this reversed with a 23% increase and declining urine output to less than 400 cc per day. Renal ultrasound did not show any signs of hydronephrosis and the renogram suggested acute tubular necrosis (ATN). However, by day 2, MALDI-TOF profiles showed markers characteristic of advanced kidney disease (Figure 3C). A renal biopsy was done on post-operative day 3 which showed the presence of ATN but no evidence of rejection. No improvement occurred over the next week and a repeat biopsy was done on post-operative day 9 which showed signs of antibody-mediated rejection (c4d positive, diffuse) and possible microangiopathy. The patient was treated for antibody-mediated rejection and regained kidney function after about one month. However, the profile components appeared before the severity of the condition was detected by other means. These components may be associated with ATN or may be predictive of antibody-mediated rejection or thrombotic microangiopathy. Problems of this nature are quite rare, making it very challenging to obtain statistical evaluation of this biomarker for this circumstance.

Longitudinal study of individual kidney transplant recipients

Baseline characteristics as well as GFR and urine protein and albumin excretion at the start of the study and at year 4 are shown in Table 1. The MALDI-TOF profiles were conducted on those individuals with at least 4 years in the study ($n=16$). Results for specific individuals are shown in Figures 4 and 5 (also see Figures 1 and 2 of supplemental information).

The first individual gave a normal profile at 1 month (Figure 4) followed by the appearance of $m/z=4303$ at year 1 that remained stable through years 2 and 3. The intensities of the components at $m/z=8180$, 8844 , and 9746 , although unusual among kidney donors, remained very stable. A renal biopsy was done on this individual after the fourth year in the study. The biopsy showed grade 1 interstitial fibrosis/tubular atrophy (Table 1). The profiles for this individual did not change by year 3 suggesting the changes seen on biopsy did not occur until later or that changes were too small to affect the MALDI-TOF protein profiles. For contrast, Figure 5 shows the profiles of an individual who showed intense B2M throughout with almost no detectable saposin B (Figure 5). This individual showed frequent appearance of $m/z=4303$, HNP ($m/z=3371$, 3441 , 3485 (19)) and a novel component at $m/z=6124$. That no biopsy was reported during the follow-up period indicated that the transplanted kidney was stable by

clinical criteria. Thus, despite substantial elevation of some proteins, including those that appear in response to stress such as kidney transplant surgery (donors or recipients), the close follow-up over several years of this study did not suggest serious deterioration of kidney function in any case.

Other examples of novel components in the MALDI-TOF profiles and stability over time are shown in supplemental information. These included unidentified peaks at $m/z=4750$, 10840, 11011, 11350 and others. These examples illustrate the most unusual profiles found among the sequential samples of transplant recipients and show the stability over the time of this study.

iTRAQ® of stable kidney transplant recipients

Urinary proteins of transplant recipients from the clinical trial as well as healthy volunteers were also examined by iTRAQ analysis. The MALDI-TOF profiles were considered an initial survey of normal vs. unusual proteome and individuals were selected to represent the spectrum of results. Each 8-plex included at least one individual with nearly normal MALDI-TOF profile and one with unusual components. The experimental design is shown in Table 2. Runs 1 and 2 included samples from two individuals. Run 3 included four individuals with normal MALDI-TOF profiles. A single analysis from these 4 individuals consisted of pooled urine from several yearly samples. Two samples from one individual with abnormal MALDI-TOF profile were included in this experiment.

A total of 307, 175, and 236 proteins were identified with more than 95% confidence from experiments 1, 2, and 3, respectively. Proteins with at least 2 peptides were used for further analysis ($n=150$, 82 and 121, respectively). Specific information and ratios of these proteins is presented in supplemental data. The results were compared with data for healthy controls that were analyzed by similar methods for samples at times of 0, 1 and 6 months (summarized in Figure 6A and B (solid symbols) and Table 3A). Average protein ratios (relative to control 1) were obtained and difference from control 1 was expressed as the standard deviation. This illustrated the similarity or difference of a given transplant recipient sample from the same control (Figure 6A). The standard deviations for transplant recipients was similar to of the average value of 4 healthy control individuals, all expressed relative to control 1 (Figure 6A, solid diamond).

Temporal, intra-person variation was obtained by comparison of each time point to an individual subject's first sample (one month, described as 0 time, or 1 year post-transplant). Intra-person variation was much smaller (Figure 6B) and, in most cases, was similar to values obtained for comparison of longitudinal samples (6 month interval) taken from 5 healthy individuals (Figure 6B, solid diamond). Two exceptions included subject 6 who showed higher variation at year 1 vs. year 0 than at year 2 vs. year 0. Subject 6 gave no indication of clinical abnormality at year 1. The other example was subject 14 who showed increased protein change at the 4 year time point. This individual was highly unusual with 3 biopsies performed (Table 1). While GFR declined for subject 14, the final biopsy appeared normal (Table 1). While no definitive conclusion regarding kidney deterioration could be established, it was interesting to note increased standard deviation for this individual at year 4 (Figure 6). In other cases, temporal variation of proteins within each transplant recipient was similar to the coefficient of variance observed for healthy volunteers who were evaluated at a six-month interval (Figure 6B).

Differences in protein variation were also evaluated by ANOVA (Analysis of Variance) to determine actual variance and significance. In all cases but one, the variance for comparison of longitudinal samples from the same individual were lower than for inter-person comparisons (Table 6A). The average variance for inter-person comparisons was 5-times greater than the average for intra-person comparisons (Table 3A). This may suggest the increase of sensitivity

expected from biomarker analysis by comparison of samples from the same individual vs. cross-sectional population comparisons. Intra-person variances for transplant recipients were not significantly different from those of controls (Table 3B). Although low, significant differences in variance occurred between longitudinal samples from 3 of 5 controls and 4 of 6 transplant comparisons (Table 3B). This suggested small but real protein fluctuations for most individuals.

A number of proteins were approximately halved in transplant recipients relative to controls. Examples included uromodulin (Tamm-Horsfall protein, ratio of 0.84 ± 0.17 in 4 controls vs. 0.44 ± 0.18 in transplant recipients, $p=0.001$) and epidermal growth factor (beta-urogastrone, 0.80 ± 0.26 among controls vs. 0.46 ± 0.37 among transplant recipients, $p=2 \times 10^{-7}$) (Figure 7). Another example was cell adhesion molecule 4 (0.93 ± 0.13 among controls vs. 0.54 ± 0.51 among transplant recipients, $p=2.4 \times 10^{-5}$, data not shown). Approximately halved levels might occur for a kidney-derived protein produced by a single organ.

A 50% reduction of certain urinary proteins of kidney donors was also suggested by decline of peak intensities in the MALDI-TOF profiles. Among kidney donors, the intensity of saposin B ($m/z=9746$) was 1942 ± 949 counts before surgery versus 821 ± 477 ($p=0.0003$) at 2 days following surgery. Although absolute intensities must be interpreted with care, averaging of many samples that were evaluated by identical procedures may provide a valid estimate of actual protein change. A 58% reduction was expected change from loss of one kidney.

A number of proteins were increased more than 3-fold in the several individuals who showed intense B2M in the MALDI-TOF profile (Figure 7). Three individuals (subjects 6, 15 and 16) with elevated B2M as detected by MALDI-TOF profile (e.g. Figure 5) also showed elevated B2M by iTRAQ analysis (subjects 15 and 16 are illustrated in Figure 7A, B). The same individuals had consistent high levels of three other proteins: retinol-binding protein 4, zinc finger protein 262, and Cystatin M (Figure 7). Subject 6 gave similar results. The same proteins displayed a narrow range among 4 healthy controls with an average of 0.82 ± 0.36 (Figure 7, solid diamond) or among 5 transplant recipients who had normal MALDI-TOF profiles (1.24 ± 0.43 , Figure 7, solid square). Interestingly, these proteins were not elevated in subject 14 (Figure 7D), the individual who had several biopsies and who showed changes that approached the definition of kidney deterioration (Table 1). This individual had unusual components in the MALDI-TOF profile (Figure 2 of supplemental information) and the largest increase of variance among the intra-individual comparisons (Table 3). If this kidney continues to decline in function, increased variance and persistence of the novel components observed (Figure 2 of supplemental information) may prove effective biomarkers. At this time, it was only possible to conclude that a number of proteins could be elevated without implication of pathology over the time spanned by this study. Again, the most striking feature of the elevated proteins was the consistency with which they appeared in sequential samples from the same individual.

Correlation of profile components with laboratory data

Four peaks of interest from the MALDI-TOF profiles were B2M, HNP, and the unidentified peaks $m/z=4300$ and 10350 . Correlation with clinical laboratory measurements was accomplished by comparing individuals containing a given component to those without. To be classified as positive for a particular component, its intensity in the profile had to be greater than that of saposin B ($m/z=9746$). Individuals positive for a given component had to display that component in at least 3 of the 5 samples. For purposes of analysis by a mixed linear effects model, the components were defined as a categorical variable with a fixed effect. These effects were correlated to GFR, urine total protein and albumin excretion, and serum creatinine as repeated measures over time (yearly up to 4 years). The least square means and standard deviations for each measure of interest are reported in Table 4. Serum creatinine was significantly different among individuals characterized by the components at $m/z=4303$ and

10350. GFR was lower in individuals with HNP. Urine total protein and albumin excretion did not differ significantly with any of the four peaks of interest.

DISCUSSION

This study examined longitudinal urinary protein composition of several groups with the objective of learning how kidney transplant influences the urinary proteome. The sample base was available as part of an ongoing clinical trial that has enrolled 150 subjects. The first 16 enrollees were used for our analysis as they approached the 5-year time of the study. This resource provided the opportunity to determine important information for biomarker discovery. This included knowledge of the range of proteins and the distribution that characterizes the stable transplant population. This critical information is needed in order to recognize aberrant proteomes that may arise during early stages of kidney disease. Two approaches were used. A rapid and inexpensive MALDI-TOF profile analysis was applied to all samples and observed a small number of proteins that were common to healthy controls and kidney donors. Each individual had a characteristic ratio of saposin B isoforms. Changes in isoform ratio therefore constituted a biomarker. The actual changes were highly significant and provided proof of principle for the suggested diagnosis by protein isoform ratios.

A surprising feature of stable kidney transplant recipients was the number of novel components in the MALDI-TOF profiles of some individuals. While only a few of the profile components were identified, the results suggested that protein content varied substantially without suggestion of an impending kidney failure. Several novel components correlated with higher serum creatinine but without statistically relevant indication of worsening condition over the time of this study. A second surprising finding was that persons with large protein differences had very stable profiles. This extended to proteins identified by iTRAQ technology. These findings suggested that longitudinal stability of the urinary proteome was at least as important as proteome content for individuals, both controls and healthy kidney recipients who are characterized by good kidney health. This suggestion had limits. Some profile features such as degradation products of alpha-1-antitrypsin were only observed in an individual who experienced temporary kidney failure. These were also frequent components of persons with advanced stage kidney disease or transplant failure¹.

B2M was a very intense component in the MALDI-TOF profiles of several subjects. While elevated B2M was corroborated by iTRAQ analysis, the increase appeared to be less than expected. Visual inspection of the MALDI-TOF profiles suggested greater than 20-fold increase over controls (Figure 5) while iTRAQ analysis gave 3 to 6-fold increase (Figure 7). Several explanations may be considered. It is possible that B2M in normal urine is present as degradation peptides that are detected by iTRAQ but not by a MALDI-TOF peak at $m/z=11732$. It is also possible that B2M is present in normal urine as a complex that is not extracted for the MALDI-TOF profile. Production of excess B2M could give rise to detectable B2M, creating the impression of an large increase. Of course, the methods themselves may contribute to the apparent difference. In any event, additional knowledge of protein state and associations in urine are needed to fully understand the urinary proteome.

Immediately following surgery, similar components appeared in the MALDI-TOF profiles of kidney donors and recipients. Prior study of immediate post-operative protein changes found increases of urinary B2M (as corroborated in this study) and retinol binding protein (20). Our study found both to be elevated long after surgery in the same post-transplant recipients. It is possible that these proteins had not resolved in all individuals or they may have increased at a later time. While this study did not show a correlation between specific elevated proteins and organ deterioration, it is possible that evaluation over longer time will find that some of the elevated proteins represent the earliest biomarkers with prognostic value.

The presence of B2M was consistent with previous studies of acute rejection (4,21) and medication-induced nephrotoxicity (22). B2M is freely filtered by the glomerulus and is reabsorbed in the proximal tubule. In the presence of proximal tubular injury, B2M excretion increases. The proximal tubule is also involved in creatinine secretion into the urine. Thus, proximal tubule damage should lead to both increased B2M excretion and increased serum creatinine. While individuals with elevated B2M showed increased serum creatinine (Table 4), it was not significant. Inverse correlation of HNP with GFR might be expected. HNP is associated with anti-microbial activity within the kidney (23) but it also has chemotactic properties for immune cells (24,25).

The need for improved biomarkers of kidney health was illustrated by subject 14 who had three renal biopsies. The first showed acute rejection that was treated, the second showed signs of chronic disease, which should be irreversible. However, the third was read as normal. Sampling error is a concern for renal biopsy. The expectation is that urinary protein biomarkers, when found, will sample the entire kidney and be a more reliable measure of kidney health.

Sample size of the current study (n=16) was small relative to the failure rate for kidney transplant. The high rate of success was illustrated by the fact that only 4 individuals had renal biopsies over 5 years. Kidney losses only accumulate over extended time. Thus, future work will examine the entire 150 subjects of this clinical trial in a prospective study. Subject recruitment and 5-year analysis will be completed by 2011 and analysis sometime later. This sample base should be adequate to observe at least some kidney failures or worsening conditions within the 5-year time of the clinical trial. Longer-term follow-up of the subjects from the current study will also allow determination of any prognostic value of unusual MALDI-TOF profiles or of elevated proteins observed by iTRAQ. Nevertheless, the results to date allowed some important observations regarding kidney function. Most striking was that, despite variable content, a stable urinary proteome suggested little deterioration or clinical problem over a 4-year period. This suggested that longitudinal analysis of each individual will provide the most sensitive biomarker of kidney health. This approach is already used for longitudinal serum creatinine measures where changes over time are used to indicate the need for follow-up analysis. Protein biomarkers must improve on the creatinine outcomes in order to provide value for transplant recipients. Long term follow-up that includes de-coding of subject therapy will also allow determination of any effects of the clinical trial, specifically losartan, on the urinary proteome and kidney health.

Overall, this study illustrated large, short-term changes of the urinary proteome occur as a result of kidney transplant surgery. A significant portion of kidney transplant recipients also showed sustained differences in urinary proteins over years. The specific proteins identified in this study did not have apparent value for prognosis of kidney failure over the 4 years of the study. Value for longer-term prognosis must await extended outcomes. The most immediate finding was the stability of the urinary proteome of individuals who have stable function. Further studies may show that change itself may provide the most sensitive detection of challenges to kidney health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Profiles of pre- and post-kidney donors. Panel A/B. Important regions of the profiles for post- (bold line) and pre-surgery donors (light line). In panel B the profile is offset to better visualize the components at $m/z=9746$ and 9073 . The rising baseline at low m/z arose from background that was not subtracted. The profiles are averaged (ClinProTools, Bruker Daltonics) from 29 controls and 12 kidney donors.

Figure 2.

Changes in saposin B isoform ratios after surgery. The ratio of m/z=9073/9746 are shown for kidney donors before (day -1) and two days following single nephrectomy (day 2). Each line represents a different individual. Averages and standard deviations are shown at -1.5 and 2.5, respectively. The difference was significant ($p=0.001$).

Figure 3.

Profiles of kidney transplant recipients at 1-3 days following surgery. Panel A. Averaged profiles (ClinProTools software) from 3 individuals show the presence of intense B2M and other peaks associated with transplant. Panel B. A fourth individual at day 1. Panels C and D. The individual of Panel B at days 2 and 3 following surgery, respectively.

Figure 4.

Sequential samples from subject 2 who developed the $m/z=4303$ component at year 1. Microalbumiuria occurred at baseline and year 1 but not at other times. Background was subtracted by software provided with the Biflex mass spectrometer.

Figure 5.
Yearly profiles of subject 16 who showed intense B2M throughout the post-transplant time. Microalbuminuria occurred at years 1 and 2 but not at other times. Background was subtracted.

Figure 6.

iTRAQ analysis of inter-person and temporal protein ratios. Panel A. Comparison of different individuals to the control. The average protein ratio and standard deviation of all proteins with error factor <2 is shown. The time since transplant (in years) and subject number are given below the values. Subject 6 had unusually high and variable levels of seminogelin I and II at the different time points. These were not included in the averages but are presented in supplemental information. Panel B. Temporal changes for individuals. Protein ratios for each individual were compared from different time points for the same individual. Averages and standard deviations are presented.

Figure 7.

Ratios of specific proteins. Panel A. Selected protein ratios for subject 16 who had abnormal MALDI-TOF profiles. The proteins included uromodulin (open circles), Retinol binding protein 4 (open squares), cystatin M (open triangles), zinc finger protein 262 (X), B2M (open diamonds) and epidermal growth factor (beta-urogastrone, +). Panel B. The same protein ratios for subject 15, who also had abnormal profiles including high B2M. Panels C and D. The same protein ratios for subjects 9 and 14, respectively. In all graphs, the solid diamond is the average and standard deviation for the four elevated proteins from Panel A but from 4 healthy control subjects. The solid square is the average and standard deviation for the elevated proteins of Panel A from subjects 1, 2, 9, 10 and 13 who showed normal MALDI-TOF profiles. Panel D shows individual 14 who displayed microalbuminuria at both time points shown and interim biopsies showing acute changes that progressed to chronic changes but ended with a normal biopsy (Table 1).

Table 1

ABCAN Subject Characteristics

Subject ID	Native Kidney Disease ^a	Donor Type	GFR 1 mo.	GFR 4 year	Protein 1 mo.	Protein 4 years	Time to Biopsy (yr)	Biopsy Results
1	SLE	DD	25	54	0.40	0.42 ^b		
2	IGA	LD	68	55	0.38	0.11	4.50	IF/TA grade 1.
3	PKD	LD	41	35	0.51	0.39		
4	PKD	DD	41	81	1.01	1.37	0.45	Borderline changes
5	PKD	DD	64	46 ^b	0.39	0.25 ^b		
6	DM	LD	33	39	0.55	0.53		
7	IGA	LD	63	59 ^b	0.32	0.31 ^b	1.70 1.75	Normal Minimal ATN
8	DM	LD	49	48 ^b	2.17 ^c	2.5 ^b		
9	DM	DD	65	48	0.25	0.18 ^b		
10	PKD	LD	52	52	0.68	0.41		
11	DM	LD	58	53	3.18 ^c	0.39		
12	HTN	DD	56	46	0.10	0.07		
13	SLE	LD	72	79	0.09	0.10		
14	DM	DD	77	40	0.28	0.44	1.26 3.86 4.39	Acute rejection type IIA IF/TA grade 2 with TG Normal
15	HUS	LD	52	51 ^b	0.56	0.43 ^b		
16	SLE	LD	38	29	0.50	0.43		

^a Abbreviations: Native Kidney Disease: SLE (systemic lupus erythematosus), IGA (IgA nephropathy), PKD (polycystic kidney disease), DM (diabetes mellitus), HTN (hypertension), HUS (hemolytic uremic syndrome); Donor Type: DD (deceased donor), LD (living donor); GFR: glomerular filtration rate (mL/min); Protein: urinary protein, grams/day; Biopsy: IF/TA: interstitial fibrosis/tubular atrophy; ATN: acute tubular necrosis; TG: transplant glomerulopathy.

^b Values from year 3.

^c High protein arose from bladder-drained pancreas transplant that resulted in intense peaks in MALDI-TOF. This subject was not examined by iTRAQ analysis.

Table 2

ABCAN Samples for 8-plex iTRAQ

iTRAQ Label	8-plex 1		8-plex 2		8-plex 3	
	Subject ID	Year After Transplant	Subject ID	Year After Transplant	Subject ID	Year After Transplant
113	Control		Control		Control	
114	9	Baseline	14	1	1	pooled
115	9	1	14	3	2	pooled
116	9	2	14	4	3	pooled
117	15	Baseline	6	Baseline	13	pooled
118	15	1	6	1	16	1
119	15	2	6	2	16	4
121	15	3	6	4	10	Baseline
Proteins at 95% confidence (two or more peptides)	307(150)		175(82)		236(121)	
Total Proteins			389(186)			

Table 3
Intra- and inter-person variance of protein ratios for transplant and controls.

Subject No.	Comparison 1	Comparison 2	Variance 1	Variance 2	p
A. Interperson vs. Intraperson comparisons					
6	0 vs control 1	0 vs 1 yr	1.38	0.211	5.7*10 ⁻¹¹
6	0 vs control 1	0 vs 2 yr	1.38	0.207	3.8*10 ⁻¹¹
6	0 vs control 1	0 vs 4 yr	1.38	0.162	2.4*10 ⁻¹³
9	0 vs control 1	0 vs 1 yr	0.57	0.421	6.4*10 ⁻²
9	0 vs control 1	0 vs 2 yr	0.57	0.094	4.3*10 ⁻¹⁸
14	1 yr vs control 1	1 vs 3 yr	0.50	0.097	5.5*10 ⁻⁹
14	1 yr vs control 1	1 vs 4 yr	0.50	0.284	2.0*10 ⁻²
15	0 vs control 1	0 vs 1 yr	0.72	0.049	3.0*10 ⁻³⁴
15	0 vs control	0 vs 2 yr	0.72	0.088	3.4*10 ⁻²³
15	0 vs control 1	0 vs 3 yr	0.72	0.140	1.5*10 ⁻¹⁵
16	1 yr vs control 1	1 vs 4 yr	0.76	0.079	6.4*10 ⁻²³
Average			0.84	0.167	9.4*10 ⁻⁶
B. Intra-person comparisons					
6	0 vs 1 yr	0 vs 2 yr	0.211	0.207	0.47
6	0 vs 1 yr	0 vs 4 yr	0.211	0.162	0.17
9	0 vs 1 yr	0 vs 2 yr	0.421	0.107	1.2*10 ⁻¹¹
14	1 vs 3 yr	1 vs 4 yr	0.097	0.284	6.0*10 ⁻⁵
15	0 vs 1 yr	0 vs 2 yr	0.049	0.088	1.5*10 ⁻³
15	0 vs 1 yr	0 vs 3 yr	0.049	0.140	8.7*10 ⁻⁸
Average				0.169	
Control 1	0 vs 1 mo	0 vs 6 mo	0.067	0.078	1.0*10 ⁻¹
Control 2	0 vs 1 mo	0 vs 6 mo	0.032	0.154	0.00
Control 3	0 vs 1 mo	0 vs 6 mo	0.069	0.113	6.9*10 ⁻²
Control 4	0 vs 1 mo	0 vs 6 mo	0.131	0.267	1.6*10 ⁻⁴
Control 5	0 vs 1 mo	0 vs 6 mo	0.181	0.158	1.6*10 ⁻¹
Average				0.125	2.8*10 ^{-1a}

^aTest for variance values of controls vs. transplant recipients

Table 4
Correlation of profile components with laboratory measurements

Profile Peak	GFR ^a (mL/min)	Protein (g/day)	Albumin (mg/g UC)	Creatinine (mg/dL)	P Plus vs. Minus
Beta-2 Microglobulin					
Minus (n=11) ^b	56.0±8.8	0.53±0.57	33.7±65.2	1.28±0.32	
Plus (n=4)	47.5±8.8	0.63±0.56	97.9±64.6	1.63±0.32	
Human neutrophil peptides (HNP)					
Minus (n=9)	57.6±8.2	0.46±0.55	70.4±68.0	1.42±0.35	
Plus (n=6)	47.6±8.4 ^c	0.72±0.56	22.8±68.5	1.32±0.35	0.0403
m/z=4300					
Minus (n=6)	52.9±9.7	0.69±0.56	20.4±66.1	1.16±0.30	
Plus (n=9)	54.2±9.7	0.48±0.56	75.3±66.7	1.52±0.30 ^c	0.0396
m/z=10350					
Minus (n=11)	54.6±9.7	0.55±0.56	33.4±62.1	1.22±0.22	
Plus (n=4)	52.2±9.8	0.61±0.57	108.2±62.9	1.82±0.22 ^c	0.0004

^a Abbreviations: GFR: measured glomerular filtration rate; Protein: urinary protein excretion in g/day; Albumin: urinary albumin excretion in mg/g urine creatinine (UC); SC: serum creatinine in mg/dL.

^b Subject 8 could not be classified as pancreatic proteins obscured other components of the MALDI-TOF profiles.

^c Indicates significance with p value listed in the last column.