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Author Footnote: Some of these data were presented in abstract form at the 2002 American Society of Nephrology Meetings. Portions of this study were supported by the National Institutes of Health (HL105649, HL071556 and HL1090145). The investigators would also like to acknowledge generous support from Brickstreet Insurance and the Huntington Foundation. David Kennedy is supported by a Scientist Development Award from the American Heart Association.

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Use of Surface-Enhanced Laser Desorption/Ionization with Time of Flight (SELDI-TOF) of the Urine in the Assessment of Acute Kidney Injury (AKI)

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All authors have no conflicts of interest to disclose.

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ABSTRACT

Background: Urinalysis is an important component in the assessment of acute kidney injury (AKI). Proteonomics is a rapidly developing approach in the analysis of physiological states. Several techniques have been developed to screen for protein populations. In this regard SELDI-TOF is a technique based on mass spectroscopy that is being utilized in proteonomics research.

Methods: For this study, clean catch or catheterized urine was collected from normals (n=18) and patients referred to the renal service with AKI. Based upon urine and serum chemistries, clinical parameters, and microscopic urinalysis, the urines were separated into those consistent with prerenal azotemia (n=17) and acute tubular necrosis (ATN) (n=29). Initially, 5 samples each were chosen from the pre-renal and ATN who had no preexisting renal disease. Other etiologies of AKI were not included in this analysis. The urine specimens were diluted 1:5 and deposited onto an H4 ProteinChip array using 50% acetonitrile as the binding buffer. This system captured the greatest spectral range with the SELDI-TOF evaluation (compared to SAX, WCX2, IMAC, and NP1 ProteinChips). Low (250) and high (300) laser intensities were utilized to ionize and desorb the protein molecules; the spectra were collected in a positive ion mode and analyzed with Ciphergen Peaks software (v 3.0).

Results: Five peaks with the high laser power were identified as potential candidates to discriminate between AKI due to prerenal or ATN causes. Those urines from the prerenal subjects were associated with detectable masses at 22.6 and 44.8 kilodaltons (KD); whereas subjects with ATN were noted to have urine with substantial masses at 11, 11.7, and 14.6 KD. The intensity of these peaks were then added together and normalized with the individual components of the discriminate peaks representing a percentage of the total. The prerenal and ATN subjects were then randomized in a training set consisting of 23 subjects and a testing set consisting of 23 subjects. Multiple linear regression was performed on the training set, and this allowed for 65% accuracy when applied to the testing set. Feed forward neural networks with hidden neuron layers ranging from 2-10 achieved similar predictive capability on the training set and testing sets.

Conclusions: Although the SELDI-TOF methodology may be a useful adjunct in the assessment of AKI and renal disease, we suggest that larger training sets will be necessary to effectively exploit this strategy.

Key Words: Acute kidney injury, urine proteomics, SELDI, mass spectroscopy
INTRODUCTION

The accurate etiological diagnosis of acute kidney injury continues to require the visual inspection of the urine sediment as well as the cognitive skills of a well trained clinician. To assist in the distinction between pre-renal azotemia (PRA) and parenchymal acute kidney injury, most notably acute tubular necrosis (ATN), differences in renal sodium and water handling in these conditions have been utilized. While the urinary sodium, creatinine and the calculated indices (e.g., fractional sodium excretion) have been employed with some success to differentiate acute tubular necrosis (ATN) from pre-renal azotemia (PRA), the ability of these measurements to distinguish between ATN and PRA is limited in a number of settings including contrast nephropathy, pigment nephropathy, ATN in patients with severe liver disease and patients with non-oliguric ATN.

It has been known for many years that tubular protein metabolism is altered by conditions associated with acute kidney injury. This has been clearly demonstrated in the case of beta 2 microglobulin where marked elevations in urine beta 2 microglobulin anticipate increases in serum creatinine with aminoglycoside toxicity and renal allograft rejection. In fact, the criticism of the use of urinary beta 2 microglobulin is that it is “too sensitive” for reliable distinction of ATN or other parenchymal acute kidney injury from easily reversible renal injury and/or non-significant renal impairment whereas other markers such as Kim1 and NGal have shown greater promise. Hampel and coworkers demonstrated that analysis of urine proteins with surface-enhanced laser desorption/ionization (SELDI) – time of flight (TOF) spectroscopy could differentiate those patients who developed contrast nephropathy from those who did not develop nephropathy following radiocontrast. From the exciting data that these workers presented, it seemed clear to us that the pattern of urine proteins observed in patients with nephropathy might be used to differentiate among the causes of acute kidney injury without necessarily identifying which proteins were contributing to these patterns. As neural networks have been employed in the sorting of these types of patterns, we decided to pursue the following studies.

METHODS

Patient selection:

Written informed consent was obtained from the patient prior to enrollment for the protocol, which was approved by the Institutional Review Board. Patients from the nephrology service at a tertiary care center (the Medical College of Ohio) were prospectively enrolled. Co-morbid conditions, medication usage, serum electrolytes, blood urea nitrogen, serum and urine creatinine were assessed. The clinical diagnosis was discussed and agreed upon by a committee of 4 nephrologists without knowledge of the SELDI-TOF results (vida infra).

Criteria for acute tubular necrosis (ATN) were:

1) compatible premorbid clinical course (hypotension, exposure to nephrotoxic agents)
2) no evidence of obstructive etiology
3) urine sediment consistent with ATN (e.g., showing renal tubular epithelial cells (RTE), RTE and “muddy” casts)
Criteria for prerenal disease was:
   1) compatible premorbid conditions
   2) objective evidence of intravascular volume depletion
   3) clinical improvement with adequate volume repletion
   4) absence of characteristic urine sediment findings for ATN

For the purpose of this report, we focus on those 49 patients who presented with acute kidney injury. In order to develop our model, we initially studied 5 patients with acute tubular necrosis (ATN) and 5 patients with prerenal azotemia to identify different patterns on the SELDI-TOF spectrum. Once these were identified, we studied all of the remaining patients with AKI, created training and testing sets for the neural network analysis.

SELDI-TOF Proteomic Analysis.

Initial determination of optimum array surface

Surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry was used to generate protein profile spectra from the urine specimens. In order to determine the optimal conditions and array surface for subsequent pattern profiling, urine specimens from different clinical conditions (e.g., control, acute tubular necrosis (ATN), prerenal azotemia) were applied under varied conditions to 5 different ProteinChip array (Ciphergen Biosystems, Palo Alto, CA) surfaces. After some optimization work (Appendix 1), the H4 ProteinChip array (hydrophobic) consisting of 16 methylene groups which bind molecules through interactions with hydrophobic amino acids (alanine, valine, phenyalanine, tryptophan, tyrosine, leucine, isoleucine) was employed for subsequent studies using the “high” laser setting.

Clean-catch urine specimens (frozen and stored –20°C) were thawed, diluted 1:5 and deposited directly onto a 2 mm spot of the H4 ProteinChip arrays. The arrays were allowed to incubate in a humidity chamber as specified in the manufacturer’s protocol. The spots were then washed with the appropriate washing solution. Twice, one half microliter of energy absorbing matrix (EAM, a saturated solution of 3,5-Dimethoxy-4- hydroxycinnamic acid in aqueous 50% acetonitrile and .5% trifluoroacetic acid) was applied to the sample and allowed to air dry. The H4 ProteinChip array was transferred to a ProteinChip reader and a laser (N2 320 nm- UV) was focused on the sample in a vacuum chamber. After 2 warming laser shots, proteins absorbed to the matrix were ionized and desorbed from the array surface. Ionized proteins were detected and molecular masses were determined using time of flight (TOF) analysis. The TOF mass spectra were collected in the positive ion mode with a ProteinChip System (PBSII series, Ciphergen) using Ciphergen Peaks (version 2.1b) software.

Real-time signal averages of 65 laser shots were averaged to generate each spectrum. Data were collected without filters and were later used for analyses. Patient samples and controls were run at the same time and intermixed on the same chip and on multiple chips.

Regression analysis and neural network design and implementation:

Data analysis was performed using the program MatlabTM (Release R2014a, TheMathWorks, Inc.).
Multiple logistic regression was performed using the program “stepwise” from within the curve-fitting toolbox.

Feed forward neural networks were initially created within the neural network toolbox employing the graphical neural network interface, “nntool.” However, most manipulations of these were done from scripts written by the authors (see example in Appendix 2). Data figures were also created from within Matlab™ using resident graphing programs and the statistics toolbox.

RESULTS

Analysis of the urine from the 18 normal patients demonstrated a consistent, substantial, broad peak at 84 kD which we attributed to Tamm-Horsefall protein. This was present in every normal urine specimen. The peak which we attributed to albumin peak was consistently less than that of the adjacent Tamm-Horsefall in all normal subjects (Figure 1).

In order to discriminate amongst patients with AKI, we initially focused on 5 patients with pre-renal azotemia and 5 patients with ATN that had minimal co-morbidities. These subjects are specifically identified in Tables 1 and 2 as index patients. Five peaks with the high laser power were identified as potential candidates to discriminate between AKI due to prerenal or ATN causes. Representative SELDI spectra are shown for prerenal azotemia in Figure 2 and ATN in Figure 3. A comparison of urine spectra from prerenal and ATN subjects is shown in Figure 4.

Figure 1: SELDI spectrum obtained from the urine of a normal control subject. X axis is in units of 10 kilodaltons (KD). Insert expands region between 6 and 10 KD where Albumin (narrow peak centered at around 66.5 KD) and Tamm-Horsfall (broader peak centered around 84KD) are detected. Y axis shows arbitrary detection units.
**Figure 2:** SELDI spectrum obtained from the urine of a patient with prerenal azotemia. X axis is in units of 10 kilodaltons (KD). Insert expands region between 6 and 10 KD where Albumin (narrow peak centered at around 66.5 KD) and Tamm-Horsfall (broader peak centered around 84KD) are detected. Y axis shows arbitrary detection units.

**Figure 3:** SELDI spectrum obtained from the urine of a patient with prerenal azotemia. X axis is in units of 10 kilodaltons (KD). Insert expands region between 6 and 10 KD where Albumin (narrow peak centered at around 66.5 KD) and Tamm-Horsfall (broader peak centered around 84KD) are detected. Y axis shows arbitrary detection units.
Figure 4: Urine SELDI spectra from patients with prerenal azotemia (PRE, lower panels) and acute tubular necrosis (ATN, upper panels) contrasted over ranges between 1.1 and 1.5 KD (left panels) and 2 and 5 KD (right panels).

Table 1:

<table>
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<tr>
<th>Pre-Renal Azotemia</th>
<th>Renal Co-Morbidity</th>
<th>Age</th>
<th>Oliguric</th>
<th>FENa (%)</th>
<th>Index Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>60</td>
<td>X</td>
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<td>x</td>
</tr>
<tr>
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<td></td>
<td>36</td>
<td>X</td>
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<td>x</td>
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</tr>
<tr>
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<td>x</td>
</tr>
<tr>
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<td></td>
<td>71</td>
<td>X</td>
<td>0.82</td>
<td>x</td>
</tr>
<tr>
<td>6</td>
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<td>X</td>
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<tr>
<td>13</td>
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<td>X</td>
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<tr>
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<td></td>
<td>0.28</td>
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<tr>
<td>17</td>
<td>CKD3, Diabetes, Proteinuria</td>
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<td></td>
<td>1.15</td>
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</table>

CKD - refers to chronic kidney disease. The Staging is as per NKF guidelines with CKD3 referring to an estimated glomerular filtration rate (eGFR) < 60 ml/min and CKD4 implying the eGFR<30. FENa refers to the fractional excretion of sodium expressed in percent. Index patients were those used to identify the initial SELDI peaks used in discrimination.
Table 2:

<table>
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<th>Age</th>
<th>FENa (%)</th>
<th>Index Patient</th>
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</thead>
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<td></td>
<td>X</td>
<td>68</td>
<td>6.32</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CKD3, Proteinuria</td>
<td>X</td>
<td>62</td>
<td>2.52</td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>50</td>
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<tr>
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<td>X</td>
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<td></td>
<td></td>
<td>56</td>
<td>0.82</td>
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</table>
Linear regression was performed using the “regress.m” function (see Appendix A). Although a variety of feed forward neural networks were tried, best results occurred with 10 hidden neurons, a tansig transfer function and the trainlm (Levenberg-Marquardt) algorithm. Details regarding the training parameters are shown in Appendix A. A representative performance of this training with 12 hidden neurons is shown in Figure 5.

Results of this analysis are shown in Table 3. As is shown, both the linear regression and neural network strategies led to 65% accuracy on the testing set.

Interestingly, 3 out of 17 patients with pre-renal azotemia had an FENa > 1% whereas 8 of the 29 subjects with ATN had an FENa < 1% (Tables 1 and 2, respectively). The accuracy of the FENa on this population (assuming the clinical assignment was accurate) was 76%.

Table 3:

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Training</th>
<th>Testing</th>
<th>%Correct Testing</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Set</td>
<td>Set</td>
<td></td>
</tr>
<tr>
<td>Pre ATN</td>
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<td>ATN</td>
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<td>14</td>
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</tr>
<tr>
<td>14</td>
<td>11</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Linear Regression True=6 False=3 True=13 False=1 True=14 False=1</td>
<td>65%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFNN True=8 False=1 True=13 False=1 True=3 False=5</td>
<td>65%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5: Graph illustrating neural network training with a training subset (blue), testing subset (red) and validation subset (green) all drawn randomly from the 23 testing subjects as per Matlab™ software, see Appendix 2. Network established by this method then applied to 23 testing subjects described in methods with results reported in Table 1.

DISCUSSION

There are two points which our experience has taught us. First and probably foremost, Tamm Horsfall was quite prominent in all control subjects. Rather than looking like control urine, we noted that the pre-renal subjects’ urine had markedly less of the peak attributed to Tamm Horsfall protein and a pattern quite distinct from normal urine specimens. This should, perhaps not be surprising. It demonstrates that renal ischemia sufficient to impair renal function has consequences on urine protein excretion, a phenomenon noted by other workers in this area.3,6,7

In addition, our data suggest that screening urine proteins with SELDI-TOF could potentially assist clinicians in the differential diagnosis of acute kidney injury. Although our study was quite preliminary, we were able to identify candidate peaks that had some discriminatory possibility. Although it is premature to comment, it is certainly possible that some of our candidate peaks correspond to previously described urinary biomarkers.3 We emphasize however that masses measured with the SELDI-TOF method may not correspond directly to actual masses and therefore identification of specific proteins will require more detailed analysis.8-11 It is possible that with more detailed analyses, other candidate peaks offering discriminatory power might be identified. Perhaps most importantly, larger sample sizes will be necessary to develop and test the utility of urine SELDI-TOF as a clinical tool in the differential diagnosis of acute kidney injury.
REFERENCES:


Appendix 1:

Surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry was used to generate protein profile spectra from the urine specimens. In order to determine the optimal conditions and array surface for subsequent pattern profiling, urine specimens from 4 different conditions (control, proteinuria, acute tubular necrosis, interstitial nephritis) were applied under varied conditions to 5 different ProteinChip array (Ciphergen Biosystems, Palo Alto, CA) surfaces. The different array surfaces, which employ varied means of binding and retaining proteins and peptides based on physical interactions, included:

1) The SAX ProteinChip array (strong anion exchanger) consisting of a cationic surface of ammonium groups which binds negatively charged molecules.
2) The WCX2 ProteinChip array (weak cation exchanger) consisting of an anionic surface of carboxylate groups which binds positively charged molecules.
3) The IMAC ProteinChip array (immobilized metal affinity capture) consisting of metal chelating surface which binds molecules based on affinity of amino acid residues for various metal ions.
4) The NP1 ProteinChip array (normal phase) consisting of silicon dioxide surface which binds molecules based on electrostatic and dipole-dipole interactions.
5) The H4 ProteinChip array (hydrophobic) consisting of 16 methylene groups which bind molecules through interactions with hydrophobic amino acids (alanine, valine, phenyalanine, tryptophan, tyrosine, leucine, isoleucine).

All experiments were performed according to standard protocols supplied by the manufacturer. Experiments carried out on the IMAC ProteinChip array used 100 mM solutions of nickel sulfate and zinc sulfate as the source of nickel and zinc ions responsible for binding molecules with histidine, tryptophan, and cysteine residues. For experiments carried out on the SAX and WCX2 ProteinChip arrays, binding and washing stringency was conferred using .05 M solutions of sodium acetate (pH 4), sodium phosphate (pH 6), Tris buffer (pH 8) and sodium carbonate (pH 10). For experiments carried out on NP1 and H4 ProteinChip arrays, binding and washing stringency was conferred using 10%, 25%, 50%, and 100% acetonitrile. Additionally, all chips were analyzed under low (250) and high (300) laser intensity in order to increase the sensitivity of peak detection to ionize and desorb molecules with both low (≤ 20 KDa) and high (>20 KDa) molecular weights.

Clean-catch urine specimens frozen at –20°C were thawed. All samples were diluted 1:5 and deposited directly onto a 2 mm spot of the respective ProteinChip arrays (final volume of 1 μL sample and 4 μL of the respective binding solution) which had been pre-incubated for 10 minutes with 50% acetonitrile. The arrays were allowed to incubate in a humidity chamber for the various times specified in the manufacturer’s protocol. The spots were then washed with the appropriate washing solution. Twice, one half microliter of energy absorbing matrix (EAM, a saturated solution of 3,5-Dimethoxy-4- hydroxycinnamic acid in aqueous 50% acetonitrile and .5% trifluoroacetic acid) was applied to the sample and allowed to air dry.

The ProteinChip array was transferred to a ProteinChip reader and a laser (N2 320 nm- UV) was focused on the sample in a vacuum chamber. After 2 warming laser shots,
proteins absorbed to the matrix were ionized and desorbed from the array surface. Ionized proteins were detected and molecular masses were determined using TOF analysis. The TOF mass spectra were collected in the positive ion mode with a ProteinChip System (PBSII series, Ciphergen) using Ciphergen Peaks (version 2.1b) software. Real-time signal averages of 65 laser shots were averaged to generate each spectrum. Data were collected without filters and were later used for analyses.
Appendix 2:

% Neural Net program
% put all training and testing sources into trainv and testv
% put respective targets into t and tt. Alternatively, just
% define total set of source as pl and target as tl and eliminate the code on
% lines 9 and 10

% CODE TO DIVIDE THE DATA INTO TRAINING & TESTING SETS
pl=cat(2,trainv,testv);
t1=cat(2,t,tt);

% CODE TO DIVIDE THE DATA INTO TRAINING & TESTING SETS
% uses 1/2 testing and 1/2 training
[TrainV, ValV, TestV]=dividevec(pl,t1,0,0.5);

%******************************************************************************************
Epochs=100; %Set the Number of Epochs to Run in the Neural
Network Goal=0.0; %Set the Performance Goal of the Neural
Network S_Hidden1=10; %Set the Number of Neurons in the Hidden
Layer S=S_Hidden1; clear S_Hidden1

% CODE TO SETUP THE NEURAL NETWORK
net=newff(TrainV.P, TrainV.T, S, {'tansig',
'purelin'},'trainlm'); net=init(net); % Initialize network

    net.trainParam.epochs =
    Epochs; net.trainParam.goal =
    Goal; net.trainParam.min_grad
    = 1e-12;
    net.trainParam.max_fail = 10;

% Train the neural network
[net,tr]=train(net,TrainV.P,TrainV.T) ;

% Simulate the neural network
Ytrain=sim(net,TrainV.P) ;
Ytest=sim(net,TestV.P);
Yval=sim(net,ValV.P);

%******************************************************************************************
% CALCULATE SOME STATISTICAL MEASUREMENTS TO EVALUATE THE ACCURACY OF THE
% NETWORK SIMULATION OUTPUTS (PREDICTIONS)

%******************************************************************************************
% CALCULATE SOME STATISTICAL MEASUREMENTS TO EVALUATE THE ACCURACY OF THE
% NETWORK SIMULATION OUTPUTS (PREDICTIONS)

% Calculate the Error
YError_train=round(TrainV.T-Ytrain)       %Error in the Training
Set
YError_test=round(TestV.T-Ytest)      %Error in the Testing Set

[k h]=size(TrainV.P);
m=cat(2,ones(h,1),TrainV.P');

[b, bint, r, rint, stats] = regress(TrainV.T', m);
c=zeros(h,1);
for i=1:h
    for j=1:k+1
        c(i)=c(i)+b(j)*m(i,j);
    end
end

fprintf('Results of "regress" are:
');
fprintf(' R^2   F-Statistic   p   error
');
disp(stats)

[k h]=size(TestV.P);
zz=cat(2, ones(h,1), TestV.P');
cc=zeros(h,1);
for i=1:h
    for j=1:k+1
        cc(i)=cc(i)+b(j)*zz(i,j);
    end
end

diary foo.out
Train_RES=round(c-TrainV.T');

Test_RES=round(cc-TestV.T');

A=cat(2,TrainV.T', round(Ytrain)', round(c))
AA=cat(2, TestV.T', round(Ytest)', round(cc))
diary