Combined proteomic and metabonomic studies in three genetic forms of the renal Fanconi syndrome


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Vilasi A, Cutillas PR, Maher AD, Zirah SF, Capasso G, Norden AW, Holmes E, Nicholson JK, Unwin RJ. Combined proteomic and metabolomic studies in three genetic forms of the renal Fanconi syndrome. Am J Physiol Renal Physiol 293: F456–F467, 2007. First published May 9, 2007; doi:10.1152/ajprenal.00095.2007.—The renal Fanconi syndrome is a defect of proximal tubular function causing aminoaciduria and low-molecular-weight proteinuria. Dent’s disease and Lowe syndrome are defined X-linked forms of Fanconi syndrome; there is also an autosomal dominant idiopathic form (ADIF), phenotypically similar to Dent’s disease though its gene defect is still unknown. To assess whether their respective gene products are ultimately involved in a common reabsorptive pathway for proteins and low-molecular-mass endogenous metabolites, we compared renal Fanconi urinary proteomes and metabolomes with normal (control) urine using mass spectrometry and 1H-NMR spectroscopy, respectively. Urine from patients with low-molecular-weight proteinuria secondary to ifosfamide treatment (tubular proteinuria; TP) was also analyzed for comparison. All four of the disorders studied had characteristic proteomic and metabolomic profiles. Uromodulin was the most abundant protein in normal urine, whereas Fanconi urine was dominated by albumin. 1H-NMR spectroscopic data showed differences in the metabolic profiles of Fanconi urine vs. normal urine, due mainly to aminoaciduria. There were differences in the uric acid metabolism and protein compositions between the three genetic forms of Fanconi syndrome: cluster analysis grouped the Lowe and Dent’s urinary proteomes and metabolomes together, whereas ADIF and TP clustered together separately. Our findings demonstrate a distinctive “polypeptide and metabolite fingerprint” that can characterize the renal Fanconi syndrome; they also suggest that more subtle and cause-specific differences may exist between the different forms of Fanconi syndrome that might provide novel insights into the underlying mechanisms and cellular pathways affected.

kidney; urine; mass spectrometry; NMR

There is increasing interest in applying “-omics” technologies to discover new biomarkers and to explore pathophysiological processes (39). There is also greater use of these methods in combination to obtain a multilayered description of diseases at different levels of biomarker organization (17, 46). In the present study, we have combined proteomic and metabolomic methods to try and obtain deeper insights into the molecular processes underlying the renal Fanconi syndrome (FS), a disorder that can exist in several forms and can arise from different genetic mutations.

Dent’s disease and Lowe syndrome are X-linked genetic forms of the renal FS with impaired proximal tubular function. An autosomal dominant idiopathic form (ADIF) has also been described (5). The defect in the proximal tubule results in a failure to reabsorb a variety of filtered substances, particularly low-molecular-weight proteins (LMWP) and peptides, leading to their increased excretion in urine with other substances like glucose, uric acid, phosphate, and amino acids. Dent’s disease typically presents with renal stones, nephrocalcinosis, and reduced renal function; it is due to mutations in CLCN5 on the X chromosome, which encodes the chloride channel CIC-5. This channel is expressed mainly in kidney proximal tubular cells, which can endocytose filtered polypeptides and proteins (13, 45, 47, 57). This endocytic process is receptor mediated (12, 19, 41) and involves the multiligand receptor proteins megalin and cubulin. The role of CIC-5, together with a V-ATPase proton pump, is thought to be to acidify the early endosomes, which is necessary to dissociate the receptor-ligand complex as part of the normal reabsorptive uptake of proteins and polypeptides from tubular fluid, as well as the regulated endocytosis of some plasma membrane transport proteins (2). Disturbed endosomal acidification in proximal tubular cells may underlie the impaired reabsorption and increased urinary losses of solutes and LMWP that occur in FS (32, 37). Lowe syndrome (also known as oculocerebrorenal syndrome) is a rarer X-linked multisystem disorder that is associated with mental retardation, lens cataracts, glaucoma, muscular hypotonia, growth defects, and renal failure (with LMWP); it is due to a mutation in OCRL1 (8), which encodes

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a phosphatidylinositol (PtdIns)-(4,5)-bisphosphate (P₂) 5-phosphatase present in the trans-Golgi network and in early endosomes (48). This enzyme belongs to the α PIP₂ 5-phosphatase type II group and hydrolyzes PtdIns(4,5)P₂ to PtdIns(4)P (60). Both its substrate and product are associated with clathrin-mediated trafficking: the former predominantly at the plasma membrane and the latter at the Golgi (11). PtdIns(4,5)-bisphosphate (P₂) 5-phosphatase is the major hydrolyzing enzyme in normal human kidney proximal tubule cells and is absent in cells grown from Lowe syndrome patients, in which PtdIns(4,5)P₂ accumulates (43, 59). How loss of PtdIns(4,5)-bisphosphate (P₂) 5-phosphatase activity in Lowe syndrome causes the eye, brain, and kidney defects that occur is still unclear. As in Dent’s disease, receptor-mediated endocytosis is affected, although a direct link between OCRL and defective endocytosis has not so far been demonstrated. The gene defect in ADIF, which is phenotypically very similar to Dent’s disease, has not been defined so far, although it has been mapped to a large segment of chromosome 15 (33). If the final common pathway for these genetic forms of FS is receptor-mediated endocytosis, then their gene products should interact in some way and this might be reflected in their respective urinary proteomes and/or metabolomes, which was the reasoning behind this exploratory study.

Thus we describe a qualitative and quantitative comparison of the urinary proteomes and metabolomes of Lowe syndrome, Dent’s disease, ADIF, tubular proteinuria (TP), and normal subjects (with normal renal function) (35). Two different comparative proteomic strategies using mass spectrometry (MS) were adopted to enhance the number and confidence of comparative proteomic strategies using MS were adopted (23, 42).

MATERIALS AND METHODS

Subjects. We studied eight male patients with X-linked Dent’s disease, all with previously characterized mutations of CLCN-5; seven male patients and one female with Lowe syndrome, all characterized by mutations in OCRL; and eight normal subjects (41). In addition, three ADIF male patients and two male subjects with ifosfamide-induced TP, but without full-blown FS, were also studied. All patients were between 35 and 50 yr old.

Control urine designated as “normal” was collected from eight male subjects with no history of renal disease and aged between 40 and 50 yr. Patients and normal volunteers gave informed consent for the collection and analysis of their urine; 50 ml of early morning urine were collected, rapidly frozen in liquid nitrogen, and stored at ~80°C until analyzed.

Urine samples were concentrated and separated from organic salts by solid-phase extraction (SPE) using a reverse-phase (RP) C₁₈ POROS R2 20 (Applied Biosystem, Warrington, UK) as the stationary phase. The protein concentration of extracted proteins was determined using the standard Bradford assay (4). To determine both qualitative and quantitative differences, two different analytic proteomic strategies using MS were adopted (23, 42).

2D-gel separation of Lowe syndrome and Dent’s disease urine proteins followed by peptide mass fingerprinting. Equal amounts (150 μg) of normal, Lowe, and Dent’s urinary proteins were loaded on Immobiline DryStrip (pH = 3–10, 18 cm; Amersham GE Healthcare Life Sciences) and focused for 24 h. The second dimension was 12.5% of acrylamide gels, which were subsequently stained using Coomassie brilliant blue (CBB) G-250. Gel runs were in triplicate, and individual (that is, not pooled) urine samples were analyzed. Gels were scanned using a densitometer (Bio-Rad 800), and gel images were analyzed using Melanie software, which allows spot matching and quantitation. To compensate for image differences caused by variations in experimental conditions, spot intensity was expressed as % optical density (OD), defined as

\[
%OD = \left( \frac{OD}{\sum_{i=1}^{N} OD_i} \right) \times 100
\]

where \( N \) is the total spot number. To assess the correlation between %OD values of two protein patterns, Student’s t-test was used to determine whether the value of their Pearson correlation coefficient \( R \) was statistically different from 0 at \( P < 0.05 \). All the spots that could be visualized were excised from the gels, in-gel digested with trypsin (Sequencing Grade Modified Trypsin, Porcine, Promega, Madison, WI), and analyzed by MALDI-MS. MALDI mass spectra of 2DE spots were acquired using an Ultraflex time-of-flight (TOF)/TOF instrument (Bruker Daltonics, Bremen, Germany).

LC-ESI-MS/MS analysis of tryptic peptides of different forms of FS urine proteins. Desalted proteins were also separated by 1D SDS-PAGE. Forty micrograms of extracted proteins from individual samples of each subtype of FS and control were pooled. Pooled samples were representative for each class. Gel lanes corresponding to total urinary proteomes were cut into five sections of approximately equal molecular weight range. Proteins present in these sections were in-gel digested using standard protocols (18). Peptides derived from trypsin digestion of normal, Dent’s, Lowe, ADIF, and TP urine proteins were spiked with 2 pmol of a protein internal standard (IS; carbonic anhydrase, which was added to the gel pieces just before in-gel digestion), and separated by RP-HPLC. Peptides eluted from the C₁₈ RP column were mass analyzed and sequenced online by a hybrid quadrupole-time of flight (Q-Tof) mass spectrometer (Micromass) equipped with a nanoelectrospray (nanoESI) ion source.

Ions were automatically selected for MS/MS by the automatic switching program in MassLynx 4.0 when their intensity in the MS survey scan was >25 ion counts/s. MS/MS spectra were smoothed...
Mascot used uninterpreted MS/MS data to interrogate the NCBI protein database restricted to mammalian entries. Allowed mass accuracies were 100 ppm and 150 millimass units for parent and fragment ions, respectively. Protein hits were accepted when they had statistically significant Mascot scores (P < 0.05) and at least two peptides matched the protein entry.

Quantification was derived by chromatographic peak integration as described previously (1, 10, 20, 25). We used an in-house computer program to extract peak areas and heights, which were taken as peptide ion intensity readings. Ion intensities were normalized for each analyte peptide ion to those of the IS; final values were normalized to the addition of the total peak heights for each particular gel fraction.

Hierarchical “cluster analysis” of quantitative LC-MS/MS data was performed using Cluster and Treeview (24) by complete linkage clustering and the Pearson correlation (uncentered) similarity metric method for both genes and arrays. Data were log2-converted before cluster analysis.

Table 1. Proteins identified by peptide mass fingerprinting that returned significant “hits” and their corresponding spot number reported in Fig. 1

<table>
<thead>
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<th>NCBI Accession No.</th>
<th>Protein Name</th>
<th>Lowe Spot No.</th>
<th>Dent Spot No.</th>
<th>Control Spot No.</th>
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<td>330b</td>
<td>374b</td>
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<td>gi 4502005</td>
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<td>208b</td>
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<td>Anti-TNF-α antibody light-chain FAB fragment</td>
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<tr>
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<td>Mitochondrial ATP synthase, H+ transporting F1 β-subunit</td>
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<td>419b</td>
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<td>gi 758073</td>
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<td>368b</td>
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<tr>
<td>gi 59850812</td>
<td>Uromodulin</td>
<td></td>
<td></td>
<td>81</td>
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%Optical density (OD) values of the proteins in bold are correlated in Fig. 2.
The pulse sequence of the form $d_1$ is a short delay ($4\tau$-m), where $\tau$ is the mixing time ($100\,\text{ms}$). For each sample, 128 transients were collected into 32k data points. Before Fourier transformation, the free-induction decays (FID) were multiplied by an exponential line-broadening factor of 1 Hz. Spectra were phased in XWINNMR (Bruker) and referenced to the TSP resonance ($0.00\,\text{ppm}$) using in-house software (MetaSpectra v3.0, O Cloarec, Imperial College London, London, UK).

$\text{H-NMR spectroscopy}$.

Data acquired from complex biofluids like urine (and especially from human blood plasma) are information rich. Each metabolite gives rise to several peaks in the spectra, with each peak varying in multiplicity (singlets, doublets, triplets, etc.), and some peaks overlapping with neighboring peaks. Analysis of such complex data sets is made possible by the application of unbiased statistical (chemometric) techniques, such as principal components analysis (PCA) and orthogonal projections to latent structures by partial least squares discrimination analysis (O-PLS-DA). An NMR spectrum is a plot of intensities (y-axis) over frequency (x-axis), as a variable having a value defined by the coordinates of each frequency coordinate (each proportional to their respective concentrations in the sample. For multivariate analysis, we consider each frequency coordinate (each peak) as a variable having a value defined by the coordinates of each frequency coordinate (each proportional to their respective concentrations in the sample). For each sample, 128 transients were collected into 32k data points. Before Fourier transformation, the free-induction decays (FID) were multiplied by an exponential line-broadening factor of 1 Hz. Spectra were phased in XWINNMR (Bruker) and referenced to the TSP resonance ($0.00\,\text{ppm}$) using in-house software (MetaSpectra v3.0, O Cloarec, Imperial College London, London, UK).

$\text{H-NMR spectral processing and multivariate analysis}$.

Data acquired from complex biofluids like urine (and especially from human blood plasma) are information rich. Each metabolite gives rise to several peaks in the spectra, with each peak varying in multiplicity (singlets, doublets, triplets, etc.), and some peaks overlapping with neighboring peaks. Analysis of such complex data sets is made possible by the application of unbiased statistical (chemometric) techniques, such as principal components analysis (PCA) and orthogonal projections to latent structures by partial least squares discrimination analysis (O-PLS-DA). An NMR spectrum is a plot of intensities (y-axis) over frequency (x-axis), as a variable having a value defined by the coordinates of each frequency coordinate (each proportional to their respective concentrations in the sample). For multivariate analysis, we consider each frequency coordinate (each peak) as a variable having a value defined by the coordinates of each frequency coordinate (each proportional to their respective concentrations in the sample). PCA and O-PLS-DA (14, 50) models were constructed using MetaSpectra. Statistical TOtal Correlation Spectroscopy Y (STOCSY) was performed on the data set to detect statistical correlations between resonances within the spectra (15) and to facilitate assignment. Visualizing these correlated resonances was facilitated by back projecting the unit variance as a colored heat map on the O-PLS coefficient plot.

Database searches. Raw data from mass spectra were submitted to Mascot, a software search engine designed for high-throughput and automated interpretation of mass spectrometric data derived from polypeptides (44). This software was used to interrogate the NCBI database, as described in Supplemental Information S1 (all supplementary material is available in the online version of this article on the AJP-Renal Physiology web site).

RESULTS

Analysis of urinary polypeptides of Dent’s disease and Lowe syndrome by 2D gel electrophoresis. We compared the protein patterns present in the urine of Lowe and Dent’s patients with normal individuals by 2DE. Equal amounts (150 $\mu\text{g}$) of normal, Lowe, and Dent’s urinary proteins were loaded on IPG strips. After electrophoretic separation and CBB G-250 staining, all visualized spots were excised from each of the three gels displayed in Fig. 1, and the identities of these spots were determined by MALDI-MS. Labeled spots shown in Fig. 1 correspond to those proteins identified that returned significant “hits,” as listed in Table 1. Identification details and spectra are described in more detail in Supplemental Information S2. Most of the proteins identified are of plasma origin and are normally reabsorbed by receptor-mediated endocytosis in the proximal tubule.

Several proteins, such as complement factor D, zinc-$\alpha_2$-glycoprotein, apolipoprotein IV, vitamin and prosthetic group carriers like vitamin D binding protein, retinol binding protein, transferrin hemopexin, and growth factors such as pigment epithelium-derived factor, were found in significantly higher amounts in Lowe and Dent’s urine; they represented a larger proportion of the urinary proteome compared with normal urine. In contrast, uromodulin and kininogen, proteins of renal origin that are not involved in receptor-mediated endocytosis, were reduced or absent in FS urine. The findings in Dent’s urine confirm our earlier reported observations (19).

Just on visual inspection of the 2D gels, it is apparent that the proteomic patterns of the Dent’s and Lowe FS urine are very similar (Fig. 1). The actual values of the gel spots %ODs (of the proteins shown in bold and in the same order as listed in Table 1) for Dent’s vs. Lowe correlate significantly ($P < 0.0001$).
0.05) (see Fig. 2B) in contrast to their correlation with normal urine ($P > 0.05$) (see Fig. 2, A and C).

Analysis of urinary polypeptides in four different forms of FS by LC-ESI-MS/MS. To determine whether other forms of the FS also have similar patterns of urinary proteins, we used a label-free quantitative proteomics approach (peak area with internal standard) that allows exhaustive quantitative cross-comparison of an unlimited number of proteomes (20). This strategy (Fig. 3A) involves separating pooled protein mixtures in parallel by 1D-SDS-PAGE. After staining, gel lanes were cut into five sections (fractions) of equal molecular weight, regardless of stain intensity. Proteins present in these gel sections were digested using trypsin, and the peptides produced were sequenced and quantified by LC-MS/MS. To illustrate how this analysis was done, see Fig. 3B, which illustrates some examples of the peptides present in fraction 4 derived from hemopexin, uromodulin, and carbonic anhydrase (which is the IS added to gel pieces before in-gel digestion). Peptides were selected for MS/MS and quantified as described in MATERIALS AND METHODS. Figure 3B shows that hemopexin was more
abundant in FS urine, whereas uromodulin was more abundant in control urine. Nine peptide ions of the IS were identified. By normalizing all protein-derived peptides to the IS peptide ions, it was possible to use the signal intensities in LC-MS to derive quantitative information from MS across samples (20). Figure 3C shows some examples of proteins that were quantified in this way in fraction 4. Quantification details of proteins identified are listed in Supplementary Table A.

Comparative analysis of three forms of the FS, two cases of TP, and control urinary proteomes confirmed our previous findings (19, 21, 22) and the data presented before (Figs. 1 and 2), indicating that the proteomes in FS are quantitatively and qualitatively different from control. A good example is shown in Fig. 4, in which uromodulin is seen to be the most abundant protein in control urine, whereas it is albumin in FS urine. Other quantitative differences between control and FS urine are apparent in Fig. 4.

The ratios of the amounts of FS urinary proteins relative to the same protein in normal urine were similar among the different forms of the FS, with no obvious differences in the overall patterns by visual inspection (Fig. 4). However, an unbiased and unsupervised (i.e., assumes no a priori knowledge about the origin of the sample) analysis of the data (using the “clustering” tools designed for analysis of gene microarray data) (see above and legend to Fig. 5) clustered Lowe and Dent’s urinary proteomes together, whereas ADIF and TP were clustered separately and together (Fig. 5). All FS proteomes clustered separately from control. These results suggest (and confirm) that the composition of the Lowe and Dent’s proteomes is very similar, yet subtly different from ADIF and TP urinary proteomes, which are more similar to each other.

**NMR analysis of FS and control urine.** 1H-NMR analysis of FS and control urine by NMR spectroscopy can detect and analyze the chemical fraction in urine that cannot be detected by standard MS -based proteomics. To complement the proteomics data, we looked for differences among the metabolite compositions in the different forms of FS and made comparisons with control. Nineteen urine samples in total were analyzed by 1H-NMR spectroscopy. The samples were obtained from four patients with Dent’s disease, five with Lowe syndrome, three with ADIF, two with isolated TP, and five normal subjects. A representative 1H-NMR spectrum from each of the five subclasses is shown in Fig. 6. The analysis of such complicated spectra is greatly facilitated by chemometric methods, such as PCA and O-PLS-DA (51). All spectra were initially subject to PCA, an unsupervised pattern recognition method that considers each data point in the frequency dimension as an independent variable. PCA presents an overview of the data that can reveal grouping of observations, trends, and outliers in a data set. A PCA model was constructed on 18 samples from this data set (one was removed due to contamination). Figure 7A shows that normal subjects were clearly separated from FS patients. There was also clustering of the metabolic profiles of urine from patients with Dent’s disease and Lowe syndrome and patients with TP and ADIF. Within the first three PCs (comprising 69.8% of the total variance in the dataset), all five subclasses were mutually exclusive. To eliminate the possibility that paracetamol metabolites were...
Fig. 5. Patterns of FS and normal urinary proteins. LC-MS/MS analysis led to the identification of 130 proteins in 5 fractions. Some of these proteins were present in more than one fraction. The data were analyzed by average hierarchical cluster analysis: complete linkage clustering was performed, as for genes and arrays, using a Pearson correlation (uncentered) similarity metric method (see text); green for downregulation, red for upregulation, and black for no change. Note that the control urinary proteome clustered separately from the four FS proteomes; that Dent’s and Lowe clustered together, and separated from ADIF and TP, which clustered together.
influencing the separation, PCA models were also constructed for restricted regions of the spectra known predominantly to contain resonances from amino acids (i.e., 8.0 to 8.2). Again, control subjects were clearly separated from FS patients and, within FS subtypes, Lowe and Dent’s patients separated from ADIF and TP (data not shown).

Further chemometric analysis was carried out on the 1H-NMR spectra using O-PLS-DA (50). This is a supervised statistical approach that takes into account the class of sample and referenced to an internal standard (TSP, 80). Units on the ordinate are arbitrary.

Fig. 6. Representative 1H-NMR spectra from each subtype of FS and controls. Spectra were Fourier transformed with line broadening corresponding to 1 Hz and referenced to an internal standard (TSP, 80). Units on the ordinate are arbitrary.

DISCUSSION

Applying -omics science to physiology and clinical medicine faces several practical problems, some of which are still technical, but a critical one (similar to that in molecular genetics) is how well defined or characterized (“phenotyped”) are the animal models or patient groups under study. Only if robust subclasses are defined can valid comparisons be made between normal, disease and disease variants, and potential biomarkers identified. Moreover, data analysis strategies are also needed that emphasize differences in patterns, rather than technical, but a critical one (similar to that in molecular genetics). The O-PLS coefficient plot for this model, showing many highly significant (red) positive regions. STOCSY analysis and assignment tables showed these belong mainly to glucose, along with neutral branched-chain amino acids valine and leucine compared with controls. Visual inspection of the data also showed ADIF patients had considerably lower levels of citrate in their urine compared with controls but this was not detected by O-PLS analysis due to the significant peak-positional variation in the citrate resonances. Finally, ADIF patients were directly compared with the grouped Dent’s and Lowe patients’ urine. The O-PLS coefficient plot, shown in Fig. 7E, showed specific and significant differences in the metabolic profiles: urine spectra from ADIF patients were significantly higher in glucose, valine and, to a lesser extent, leucine, while lysine was more significant in discriminating Dent’s and Lowe patients as a group.

Several qualitative and quantitative differences in the proteomic and metabolomic profiles of the different genetic forms of FS patients compared with normal subjects were found using MS and NMR. A preliminary study was conducted on Dent’s disease and Lowe syndrome urinary proteins with two different proteomic methods, 2DE and LC-MS/MS. This was done because the physiochemical heterogeneity of proteins (which is the basis of their diverse biological functions) makes a single analytic approach inadequate for a comprehensive analysis of all proteins in a given sample (28). As an alternative to 2DE, we used liquid chromatography for protein or proteo-
lytic product separation of FS and normal urine. With this strategy, polypeptide detection is less discriminatory of the physicochemical properties of the analyte. For example, proteins such as megalin, apolipoprotein A-II, apolipoprotein C-III, β-2-microglobulin precursor, cystatin C, cystatin M precursor, immunoglobulins, insulin-like growth factor binding protein 6, pro-epidermal growth factor precursor, and osteopontin were not detected by 2DE.

Fig. 7. Principal components analysis (PCA) and orthogonal projections to latent structures by partial least squares discriminant analysis (O-PLS-DA) output from 1H-NMR data from FS patients’ urine. A: 3D PCA scores plot for the first 3 principal components. Each data point represents 1 NMR spectrum (hence 1 patient) of reduced dimensionality, and the closer 2 data points are to each other in PC “space,” the more similar the corresponding metabolic profile. Numbers in brackets represent the percentage of the variance explained in each component; key = blue for Dent’s; red for TP; green for ADIF; magenta for Lowe; black for control. B–E: O-PLS coefficients plotted as a function of chemical shift color-coded according to significance in differentiating between pairs of groups. Numbers in brackets are the cross-validation parameter $Q^2_{Yhat}$. B: Dent’s and Lowe (as a group) vs. normal ($Q^2_{Yhat}$ = 0.74). C: Dent’s vs. Lowe ($Q^2_{Yhat}$ = 0.11). D: ADIF vs. normal ($Q^2_{Yhat}$ = 0.91). E: ADIF vs. Dent’s and Lowe (as a group) ($Q^2_{Yhat}$ = 0.87).
Our results show that the urine protein patterns of patients with Dent’s disease and Lowe syndrome are very similar. We found that the plasma proteins normally reabsorbed from the glomerular filtrate along the first part of the proximal tubule by receptor-mediated endocytosis represent a larger proportion of the FS urinary proteome compared with normal urine. These results indicate that tubular reabsorption of plasma-derived proteins is similarly reduced in Dent’s disease and Lowe syndrome. In contrast to normal urine, proteins of renal origin are decreased in the urine of patients with these forms of FS. Thus the FS urinary proteome is dominated by proteins of plasma origin, and proteins derived from the kidney itself (and are present in normal urine) make up a smaller proportion of the FS urinary proteome. These findings confirm our original observations in Dent’s disease only (19) and indicate that in this respect Lowe syndrome and Dent’s disease are indistinguishable.

$^1$H-NMR offers a robust and information-rich analytic approach that can complement proteomic data (38). By combining this highly reproducible form of spectroscopy with the chemometric methods of PCA and O-PLS-DA, a more general comparison of the different sources of FS urine and normal urine can be made, and potential biomarkers for each type of FS identified. Consistent with the proteomic findings, the metabonomic analysis showed that the metabolic profiles of urine from Dent’s and Lowe patients were very similar and quite different from normal urine. The differences were due mainly to increased amounts of the amino acids lysine (basic) and alanine (neutral) in the urine of FS patients, and decreased amounts of p-cresol sulfate and N-methyl nicotinic acid. This is in keeping with early descriptions of Lowe syndrome, in which basic and neutral (but not branch-chained) aminoaciduria was a recognized feature (9). The decrease in N-methyl nicotinic acid excretion suggests cation transporter dysfunction in the proximal tubule (7, 52), while the decreased p-cresol sulfate, a clostridial metabolite, could be due to altered metabolism by gut microflora (56). It is perhaps relevant here that some forms of FS are associated with different effects on amino acid transport in the kidney and intestine (3), which may influence gut microflora metabolism. Indeed, CIC-5 has been detected in the rat intestine (53), but its function there, or its presence in humans, is unknown. Alternatively, it may be that altered renal function in FS patients results in inefficient clearing of p-cresol sulfate, a well-known uremic toxin (54, 55). However, whether this is a nonspecific reflection of impaired renal function or is more specific to tubular dysfunction in FS is unclear at present.

In both Dent’s disease and Lowe syndrome, urine megalin, the receptor thought to be largely responsible for protein and polypeptide reabsorption in the proximal tubule, is reduced or absent in urine (41), which is again in keeping with our finding of almost identical urinary proteomes and metabonomes, and is thus a common underlying defect in receptor-mediated endocytosis. The products of the gene defects in Dent’s disease and Lowe syndrome could be closely involved in the same reabsorptive pathway, and one that may be common to other forms of FS. To explore this concept, we analyzed the urine composition of a phenotypically similar autosomal dominant idiopathic form of FS (ADIF) and compared it with Dent’s disease, Lowe syndrome, ifosfamide-induced TP, and normal subjects.

ADIF is another form of inherited FS, clinically similar to Dent’s disease (5), but its underlying gene defect is autosomal and still unknown (33). Although proteomic analysis, as for Dent’s disease and Lowe syndrome, confirmed that the ADIF urinary proteome is different from control and broadly similar to Dent’s and Lowe proteomes (with no clearly distinct proteins or peptides), the urinary metabonome of ADIF showed several qualitative differences in composition, with glucose and valine (neutral) identified as potentially distinguishing biomarkers for this form of FS (Fig. 7D). By cluster analysis the ADIF and TP urinary proteomes appeared to be quite similar, yet different from Dent’s and Lowe proteomes (Fig. 5). An earlier $^1$H-NMR study of urine from patients treated with ifosfamide reported glycosuria and amino aciduria, with concomitant decreases in hippurate and citrate (26, 27). We observed similar metabolite excretion patterns for ifosfamide-induced TP, except there was no apparent increase in histidine in the urine of our patients. In grouping ADIF and TP together, and Dent’s and Lowe together, both proteomic and metabonomic analyses were consistent. The discriminators in ADIF were glucose and valine, while in Dent’s and Lowe syndrome it was lysine.

Although no specific mechanism(s) has been be identified by this combined analysis, and our interpretation is both limited and somewhat speculative, taking the proteomic and metabonomic data together, the results do suggest that the molecular defects and pathways affected in ADIF may be different from those shared by Dent’s disease and Lowe syndrome, and more similar to those occurring in ifosfamide-induced TP. Moreover, our results do confirm our original proteomic findings in Dent’s disease and show that the urinary protein and metabolite patterns in Dent’s disease and Lowe syndrome are almost identical, qualitatively and quantitatively, and it is likely that their respective gene products are closely involved in the same proximal tubular reabsorptive pathways. Indeed, the recent and confirmed report that a significant number of adult patients with a Dent’s-like (predominantly renal) phenotype had mutations of OCRL1, and not CLCN5 (30), is consistent with our findings and this interpretation.

Finally, in this small study we have demonstrated how urinary proteomics and metabonomics can be combined effectively to provide a more complete analysis of urine and that these analytic methods are consonant. By extending urinaryomics in this way (and with the development of high-throughput sampling methods), especially in well-defined and characterized renal disorders, or by comparing clinically similar disorders (as in FS), potential biomarkers and novel mechanistic insights may be revealed.

REFERENCES


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ANALYSIS OF RENAL FANCONI URINE BY MS AND NMR


