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Renal Tubular Dysfunction in Pediatric Urolithiasis: Proteomic Evidence

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Keywords

Urolithiasis; Proteomics; Tubular dysfunction; Children

Introduction

Experimental and animal studies have shown crystal-induced oxidative stress and tubular injury in kidney cells (¹, ²). Crystals interact with renal epithelial tubular cells causing oxidative stress and inflammation. Indeed, stone patients have significantly higher urinary concentration of malondialdehyde, a product of lipid peroxidation (³). Total oxidant and antioxidant status is elevated in children with stones, and the former correlates with urinary N-acetyl-beta-D-glucosaminidase, a marker of renal tubular injury (⁴). Oxidative stress causes release of various cytokines and growth factors maintaining inflammation (⁵). This is followed by fibrosis and collagen deposition (⁵). Crystal-induced tubular injury leads to crystal attachment and further renal epithelial damage (⁶). Markers of inflammation and tubulointerstitial damage were found in adults with calcium oxalate (CaOx), calcium phosphate (CaP), and uric acid stones (⁷).

Impaired tubular cells alter reabsorption of low molecular weight (LMW) proteins. Higher urinary excretion of mucoproteins including lysozyme and beta2 microglobulin, and enzymes such as beta-galactosidase and gamma-glutamyl transpeptidase were found in patients with stones (⁸). However, there is little evidence of tubular dysfunction in pediatric nephrolithiasis. We aimed to investigate whether inflammation, oxidation and tubular injury is present in children with stones (RS) compared to healthy controls (HC) by measuring urinary proteins involved in these processes. We hypothesized that children with kidney stones have a higher urinary excretion of inflammatory, oxidative and tubular proteins compared to controls.

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Conflict of Interest: None

Methods

We prospectively collected the second morning mid-stream fresh urine samples obtained from children with renal calculi and healthy controls between April 2011-December 2014. None of the patients were on treatment for urolithiasis at the time of urine collection. We performed quantitative proteomic comparison of pooled urine from RS versus age- and gender-matched HC, using liquid chromatography-mass spectrometry (LC-MS/MS).

This study was IRB approved (IRB number: 075511MP4E)

Patient selection

Patient inclusion criteria consisted of age 5-18 years, history of clinically (typical renal colic) and radiographically (ultrasound or CT) proven renal stone, at least two 24 hour satisfactory urine collections (urinary creatinine more than 15 mg/kg/day), absence of hematuria or pyuria, and normal renal function. The criterion of two 24 hour urine collection was chosen to increase the diagnostic yield since significant variation in the urinary parameters between urine collections was reported in the literature (^{9, 10}). At the time of urine collection, these children were free of symptoms (no flank or abdominal pain, no urinary symptoms), and were on no medication for renal stones. Thirteen of 30 patients (43%) had renal ultrasound at the time of urine collection, and urolithiasis was noted in three. Hypercalciuria was defined as excretion of calcium greater than 4 mg/kg/day (¹¹) and hypocitraturia was diagnosed when urinary citrate was less than 310 mg/1.73 m²/day in girls and 365 mg/1.73 m²/day in boys (¹²). Children who were not toilet trained, or those with bladder stones, nephrocalcinosis, neuropathic bladder, major congenital bladder abnormality, active urinary tract infection, presence of blood in urine, chronic kidney disease, previous major reconstructive bladder surgery requiring catheterization, and significant cardiac, pulmonary, gastro-intestinal, and neurological problems were excluded. Control group consisted of age- and gender-matched healthy children seen in our clinic for bedwetting that resolved at the time of urine collection.

Sample collection and preparation

Second morning mid-stream fresh urine samples from both patients and controls were obtained in sterile cups, prepared within 3 hours of collection (centrifuged at 2500 rpm for 15 minutes) and stored as recommended by standardized protocols (developed by the Human Urine and Kidney Proteome Project, HUKPP, and the European Urine and Kidney Proteomics, EuroKUP Initiatives) until use (¹³).

Proteins in each sample were concentrated in a Centricon-type filter. Albumin and IgG were removed by anti-HAS/IgG resin (Sartorius). Protein concentration in each sample was measured by the BCA protein assay (Pierce).

2D LC-MS/MS and Protein quantitation

Pooled samples were used in patients and controls. 10 individual samples of 10 µg each were pooled for a total of 100 µg protein per group. Pooled samples were digested with trypsin and analyzed by two dimensional liquid chromatography – mass spectrometry (2D LC-MS/

MS). Full details of proteomic methods can be found in the Supplemental section. When comparing control groups to patient groups, proteins were specified as differentially abundant if they met the following criteria: 1) 5 spectral counts; 2) 0.05 *p*-value for the Fisher's Exact Test; and 3) 2-fold difference in spectral counts. These criteria were chosen to be at or above the threshold used for most label free proteomic analyses (¹⁴).

ELISA validation

According to the findings from MS, we selected two proteins, retinol-binding protein 4 (RET4) and liver fatty acid-binding protein (FABPL) for validation using ELISA analysis in individual samples (Human FABPL ELISA kit from R&D System, catalog No. Z-001 and Human retinol binding protein 4 ELISA kit from Sigma-Aldrich, catalog No. RAB0551-1KT).

Statistical analysis

The *p* value of each category included in the GO annotation was calculated using an EASE score, a modified Fisher's Exact test. Statistical analyses were conducted with IBM SPSS® version 20.

Results

Demographics and characteristics of the groups

Stone group consisted of 30 children (8 males), mean age 12.95±4.03 years (5-18 years). Of these, 10 had hypercalciuria, 10 had hypocitraturia, and 10 had normal metabolic work-up. All had normal urinary dipstick at the time of urine collection. Control group included 30 age- and gender-matched children (mean age 13.04±4.04 years, range 5.5-17.4 years). The urine dipstick at the time of urine collection was normal in all children.

Overall we identified 1813 proteins, with 1639 proteins found in children with stones, and 1396 proteins found in controls. Of those, 417 were found only in patients and 174 only in controls. Using the above mentioned criteria, 163 proteins were upregulated and 67 were down-regulated in stone patients. These significantly differentially abundant proteins were used to analyze the group differences for biological processes, cellular components, and molecular function, using Gene Ontology (GO) mapping (Figures 1A and 1B). GO is a bioinformatics platform which provides a statistical likelihood that a group of proteins are enriched within a specific biological classification. The top 3 biological processes over-represented in stone patients were response to wounding, proteolysis, and inflammation/immune response. Functional analysis revealed 19 inflammatory proteins, 5 proteins involved in oxidative stress, and 5 involved in tubular injury (Table 1). Subgroup analysis revealed more pronounced changes in the hypercalciuria and hypocitraturia groups compared to normal metabolic group and controls (Table 2). The cellular distribution analyses showed that most proteins overexpressed in stone patients were localized in the extracellular region. Moreover, most proteins that were upregulated in stone children were involved in endopeptidase activity, and calcium ion binding.

Inflammation—Out of 163 proteins that were upregulated in stone patients, 19 were related to inflammation, and mainly consisted of classical (C1q, C3, C4, C9) and alternative (C-H and C-D) complement proteins (Table 1). C1q and C-H were uniquely seen in stone patients. Additionally, 2 acute inflammatory proteins, ferritin and fibrinogen, were found to be at higher levels in stone children compared to healthy controls.

Oxidative stress—Five proteins involved in oxidative stress were over-represented in stone children, and 2 of these (NADPH and catalase, CATA) were found only in the stone group (Table 1). Glutathione S- transferase A2 (GSTA2), an important antioxidant enzyme, was more abundant in controls.

Tubular dysfunction—Levels of five proteins that are known markers of proximal and distal tubular injury were higher in children with kidney stones (Table 1). Of those, retinol-binding protein 4 (RET4) and beta-2-microglobulin (B2MG) showed the highest ratio between patients and controls. ELISA analysis confirmed statistically significant difference in the urinary excretion of RET4 (Figure 2) and nearly significant difference in the urinary excretion of FABPL between stone patients with hypercalciuria and controls.

Discussion

Using a proteomic approach, we demonstrated the presence of several markers of oxidative stress, inflammation, and tubular dysfunction in the urine of children with kidney stones. Markers indicative of both proximal (RET4, B2MG, LYSC, CYTC) and distal (FABPL) tubular dysfunction were found. These findings were more pronounced in children with hypercalciuria, hypocitraturia and renal stones. Indeed, experimental and animal studies have shown that calcium oxalate crystals interact with renal tubular epithelial cells and initiate oxidative stress and inflammation that may cause tubular cell dysfunction⁽⁵⁾. Both calcium and oxalate crystals are injurious to cells⁽¹⁵⁾. Calcium is not simply the cationic component of the crystals, but is also a second messenger and is involved in redox signaling⁽¹⁵⁾. However, there is scarce evidence of tubular dysfunction/injury in patients with urolithiasis.

Our results indicate the presence of oxidative stress in children with renal stones. High urinary concentration of NADPH exclusively seen in stone patients indicates excessive reactive oxygen species (ROS) generation. The production of antioxidant CATA was also uniquely seen in children with stones, while urinary concentrations of SODC and PERM were higher in patients than controls. Additionally, poor oxidant defense was reflected by decreased GSTA production that we found in children with stones compared to controls. Altogether, this indicates a defective balance between oxidative- antioxidative processes in pediatric urolithiasis. ROS are cytotoxic free radicals with one or more unpaired electrons that are produced by enzymes including NADPH oxidase. The injured epithelium promotes crystal formation and retention within the kidneys. It has also been suggested that ROS may damage the inhibitory molecules reducing their capacity to inhibit crystallization and/or their retention within the kidney. ROS are cleaved by antioxidants including CATA, SODC, PERM and GST, and antioxidant therapy prevents calcium oxalate nucleation and retention in the renal tubules⁽¹⁶⁾.

We found a high number of immune-response and inflammatory proteins in children with renal stones. These may initiate crystal induced tubular dysfunction/injury that may favor crystal attachment and further renal epithelial damage. Defective tubular cells alter reabsorption of LMW proteins causing their increased urine excretion. We found elevated urinary concentration of several LMW proteins, with RET4 levels showing the highest ratio between patients and controls. These changes were more pronounced in children with hypercalciuria and hypocitraturia. For this reason, we chose to perform ELISA measurements to determine urinary RET4 concentration in individual samples obtained from children with hypercalciuria and renal stones and their matched healthy controls. ELISA confirmed statistically significant increased RET4 excretion, and nearly significant FABPL excretion in children with hypercalciuria and stones compared to healthy controls.

Hypercalciuria is the result of impaired renal tubular reabsorption of calcium. Children with hypercalciuria were found to have increased urinary concentration of N-acetyl-beta-D-glucosaminidase (NAG), a lysosomal enzyme considered to be a sensitive marker of proximal tubular injury (¹⁷). Calcium crystals may damage tubular epithelial cells affecting their function. Idiopathic hypocitraturia has been linked to intrinsic renal disease including impairment of the sodium-citrate transport in the proximal tubule (¹⁸). Detection of LMW proteins in urine has been shown to indicate tubular dysfunction in various diseases, including diabetic and inherited nephropathies (¹⁹). RET4 is a carrier for lipophilic vitamin A (retinol) in the blood. This LMW protein (21 kDa) is easily filtered in the renal glomerulus and reabsorbed by the proximal convoluted tubules. RET4 measurement does not require urine alkalization because it remains stable in acidic urine. For this reason, RET4 was found to be a more sensitive marker of tubular damage than B2MG and beta-N-acetyl-D-glucosaminidase (^{20, 21}). B2MG is a 12–14 kDa immunoglobulin-like protein, and is synthesized in the lymphatic system. It is filtered in the glomerulus and is also reabsorbed in the proximal tubule. Unlike RET4, B2MG is unstable in acidic urine, and therefore, more difficult to measure. FABPL is a 14kDa protein found in the cytoplasm of human renal proximal tubules. It appears to be an early diagnostic and prognostic marker of kidney disease, and it accurately predicts the degree of tubulointerstitial damage (²²). A recent trial found that LFABP is more sensitive than urinary protein in predicting the progression of chronic kidney disease (²²). Recently, it was approved as a new distal tubular biomarker in Japan (²³).

To our knowledge, this is the first proteomic analysis in children with renal stones. We found that inflammation, oxidative stress and tubular dysfunction are present in these children, and are more pronounced in those with hypercalciuria and hypocitraturia. Early tubular involvement is indicated by the detection of several tubular proteins in the urine, whose significance is yet to be determined. One major limitation of this study is the inability to establish causal-effect relationship between these processes, due to the cross-sectional study design. To address this issue, we will perform a follow-up study in which we will compare patient's urinary protein profile before and after stone passage (spontaneous or surgical removal). Another limitation includes the initial use of pooled samples. The use of pooled samples was intended to reduce the sample intra- and inter-variability, and the cost. However, this is an acceptable screening method in proteomics for the aforementioned reasons. The results should be confirmed by more sensitive tests (ELISA, Western blot, etc)

performed in individual samples. In our study, ELISA confirmatory test done in individual samples in RET4 further supported these results.

Conclusion

We provide proteomic evidence of oxidative stress, inflammation, and tubular dysfunction in children with renal stones. We speculate that inflammation and changes in the oxidant-antioxidant balance may cause tubular damage in these patients. Larger studies to investigate these proteins using more sensitive tests such as ELISA are imperative. Targeting these proteins may have therapeutic benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 0001 **Figure 0002****Figures 1A and 1B.**

Gene ontology (GO) analysis of urinary proteins with relative decreased or increased abundance in children with kidney stones compared to healthy children. Proteins which were either 1) only identified in either group, or 2) estimated to have at least a 2-fold difference in relative abundance were mapped against GO term lists to determine which biological processes were enriched. *, p -value < 0.05

Figure 2. Urinary retinol-binding protein 4 (RET4) concentration in children with hypercalciuria and kidney stones compared to healthy children as assessed by ELISA.

Table 1

Tubular, oxidants/ antioxidants and inflammatory urinary proteins identified in children with kidney stones with at least 2-fold increased or decreased abundance relative to healthy controls.

Accession Number ¹	Assigned peptides ² [Patient - Control]	Ratio (Patient/Control)	Fisher's Exact Test ³ (P-Value)
BLVRB	5 - 0	Unique	0.0630
CATA	13 - 0	Unique	0.0003
SODC	35 - 13	2.69	0.0034
PERM	95 - 43	2.21	< 0.0001
GSTA2	19 - 38	0.59	0.0076
RET4	520 - 70	4.89	< 0.0001
B2MG	156 - 27	6.54	< 0.0001
LYSC	109 - 19	4.68	< 0.0001
CYTC	146 - 34	3.87	< 0.0001
FABPL	14 - 5	2.8	0.0655
KLKB1	11 - 2	5.5	0.0231
C1S	12 - 2	6	0.0134
FHR1	13 - 0	unique	0.0003
CO9	24 - 10	2.4	0.0262
A1AT	377 - 186	2.01	< 0.0001
C1QC	5 - 0	unique	0.0630
APOA2	57 - 7	8	< 0.0001
CO4A	182 - 76	2.39	< 0.0001
CFAH	98 - 26	3.76	< 0.0001
REG3A	8 - 0	unique	0.0080
CO3	312 - 64	4.8	< 0.0001
LBP	5 - 0	unique	0.0630
A1AG1	599 - 293	2	< 0.0001
CFAD	330 - 2	165	< 0.0001
TRFE	1506 - 695	2.15	< 0.0001
A2MG	238 - 51	4.5	< 0.0001
IBP4	17 - 4	4.25	0.0077
FIBA	125 - 64	2	< 0.0001
TRY2	6 - 0	unique	0.03162

Abbreviations: BLVRB- flavin reductase; CATA- catalase, SODC- Cu/Zn superoxide dismutase; PERM- myeloperoxidase, GSTA2- glutathione S-transferase A2; RET4- retinol-binding protein 4; B2MG- beta-2-microglobulin; LYSC- lysozyme C; CYTC- cystatin-C; FABPL- fatty acid-binding protein, liver; KLKB1- kallikrein B, plasma (Fletcher factor) 1; C1S- complement component 1, s subcomponent; FHR1- complement factor H-related 1; CO9- complement component 9; A1AT- serpin peptidase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 1; C1QC- complement component 1, q subcomponent, C chain; APOA2- apolipoprotein A-II; CO4A- complement component 4A (Rodgers blood group); CFAH- complement factor H; REG3A- regenerating islet-derived 3 alpha; CO3- Complement C3 precursor; complement component 3; LBP- lipopolysaccharide binding protein; A1AG1- orosomucoid 1; CFAD- complement factor D (adipsin); TRFE- transferrin; A2MG- alpha-2-macroglobulin; IBP4- insulin-like growth factor binding protein 4; FIBA- fibrinogen alpha chain; TRY2- trypsin-2.

¹ UniProt accession number

²Represents the spectral count, or total number of MS/MS spectra assigned to each protein

³Significant values with $p < 0.05$ are shown in bold.

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Table 2

Tubular, oxidants/ antioxidants and inflammatory urinary proteins in children with kidney stones and hypercalciuria, hypocitraturia, and normal metabolic work-up, with at least 2-fold increased or decreased abundance relative to healthy controls.

	Assigned peptides ² [Patient - Control]			Ratio (Patient/Control)			Fisher's Exact Test ³ (p-Value)		
Acc. Number ¹	CAL	CIT	NM	CAL	CIT	NM	CAL	CIT	NM
BLVRB	4 - 0	1 - 0	0 - 0	unique	unique	0	0.0600	0.6200	NA
CATA	1 - 0	9 - 0	3 - 0	unique	unique	unique	0.4900	0.0130	0.0470
SODC	15 - 6	18 - 3	2 - 4	2.5	6	0.5	0.0350	0.0170	0.6200
PERM	9 - 10	79 - 7	7 - 26	0.9	11.29	0.27	0.5200	< 0.0001	0.0500
GSTA2	8 - 16	5 - 11	6 - 11	0.5	0.45	0.55	0.0830	0.0130	0.5800
RET4	405 - 28	105 - 25	10 - 17	14.46	4.2	0.59	< 0.0001	< 0.0001	0.5300
B2MG	120 - 9	36 - 13	0 - 5	13.33	2.77	0	< 0.0001	0.0590	NA
LYSC	81 - 5	28 - 4	0 - 10	16.2	7	0	< 0.0001	0.0013	NA
CYTC	127 - 17	18 - 8	1 - 9	7.47	2.25	0.11	< 0.0001	0.2800	0.0750
FABPL	9 - 1	4 - 1	1 - 3	9	4	0.33	0.0098	0.3700	0.5400
KLKB1	3 - 2	8 - 0	0 - 0	1.5	unique	0	0.4900	0.0210	NA
C1S	7 - 1	4 - 1	1 - 0	7	4	unique	0.0330	0.3700	0.3600
FHR1	10 - 0	3 - 0	0 - 0	unique	unique	0	0.0009	0.2400	NA
CO9	16 - 6	7 - 0	1 - 4	2.6	unique	0.25	0.0230	0.0340	0.4100
A1AT	153 - 91	201 - 55	23 - 40	1.68	3.65	0.57	< 0.0001	< 0.0001	0.5200
C1QC	4 - 0	1 - 0	0 - 0	unique	unique	0	0.0600	0.62	NA
APOA2	27 - 6	28 - 0	2 - 1	4.5	unique	2	0.0001	< 0.0001	0.3000
CO4A	66 - 47	100 - 29	16 - 0	1.40	3.45	unique	0.0350	0.0001	< 0.0001
CFAH	56 - 10	41 - 8	1 - 8	5.6	5.13	0.13	< 0.0001	0.0008	0.1100
REG3A	8 - 0	0 - 0	0 - 0	unique	0	0	0.0036	NA	NA
CO3	161 - 50	128 - 9	23 - 5	3.2	14.2	4.6	< 0.0001	< 0.0001	< 0.0001
LBP	4 - 0	1 - 0	0 - 0	unique	unique	0	0.0600	0.6200	NA
A1AG1	271 - 101	231 - 109	97 - 83	2.7	2.1	1.16	< 0.0001	0.0100	< 0.0001
CFAD	287 - 1	43 - 0	0 - 1	287	unique	0	< 0.0001	< 0.0001	NA
TRFE	467 - 436	929 - 127	110 - 132	1	7.3	0.83	0.0920	< 0.0001	0.0018
A2MG	86 - 39	135 - 7	17 - 5	2.2	19.2	3.4	< 0.0001	< 0.0001	0.0001
IBP4	14 - 2	3 - 2	0 - 0	7	1.5	0	0.0018	0.6300	NA
FIBA	60 - 27	61 - 27	4 - 10	2.22	2.25	0.4	0.00018	0.088	0.39
TRY2	6 - 0	0 - 0	0 - 0	unique	0	0	0.015	N/A	N/A

Abbreviations: CAL-hypercalciuria; CIT-hypocitraturia; NM- normal metabolic; BLVRB-flavin reductase; CATA- catalase; SODC-Cu/Zn superoxide dismutase; PERM- myeloperoxidase; GSTA2- glutathione S-transferase A2; RET4- retinol-binding protein 4; B2MG- beta-2-microglobulin; LYSC- lysozyme C; CYTC-cystatin-C; FABPL- fatty acid-binding protein, liver; KLKB1- kallikrein B, plasma (Fletcher factor) 1; C1S-complement component 1, s subcomponent; FHR1- complement factor H-related 1; CO9- complement component 9; A1AT- serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; C1QC- complement component 1, q subcomponent, C chain ; APOA2- apolipoprotein A-II; CO4A-complement component 4A (Rodgers blood group); CFAH- complement factor H; REG3A- regenerating islet-derived

3 alpha; CO3- Complement C3 precursor, complement component 3; LBP-lipopolysaccharide binding protein; A1AG1- orosomucoid 1; CFAD- complement factor D (adipsin); TRFE- transferrin; A2MG- alpha-2-macroglobulin; IBP4- insulin-like growth factor binding protein 4; FIBA- fibrinogen alpha chain; TRY2- trypsin-2.

¹UniProt accession number

²Represents the spectral count, or total number of MS/MS spectra assigned to each protein

³Significant values with $p < 0.05$ are shown in bold; NA-Not applicable, P value could not be calculated because the peptide was not found.