Assessment of Urine Proteomics in Type 1 Primary Hyperoxaluria

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Key Words

Primary hyperoxaluria type 1 · Urine proteomics · Urine oxalate · Idiopathic hypercalciuria · Fetuin A · Calcium oxalate crystals · Tam Horsfall protein · Osteopontin · Epidermal growth factor

Abstract

Background: Primary hyperoxaluria type 1 (PH1) and idiopathic hypercalciuria (IHC) are stone-forming diseases that may result in the formation of calcium (Ca) oxalate (Ox) stones, nephrocalcinosis, and progressive chronic kidney disease (CKD). Poorer clinical outcome in PH1 is segregated by the highest urine (Ur)-Ox (UrOx), while IHC outcomes are not predictable by UrCa. We hypothesized that differences would be found in selected Ur-protein (PRO) patterns in PH1 and IHC, compared to healthy intra-familial sibling controls (C) of PH1 patients. We also hypothesized that the PRO patterns associated with higher UrOx levels would reflect injury, inflammation, biomineralization, and abnormal tissue repair processes in PH1. **Methods:** Twenty four-hour Ur samples were obtained from 3 cohorts: PH1 (n = 47); IHC (n = 35) and C (n = 13) and were analyzed using targeted platform-based multi-analyte profile immunoassays and for UrOx and UrCa

by biochemical measurements. **Results:** Known stone matrix constituents, osteopontin, calbindin, and vitronectin were lowest in PH1 (C > IHC > PH1; p < 0.05). Ur-interleukin-10; chromogranin A; epidermal growth factor (EGF); insulin-like growth factor-1 (IGF-1), and macrophage inflammatory PRO-1α (MIP-1α) were higher in PH1 > C (p = 0.03 to p < 0.05). Fetuin A; IGF-1, MIP-1α, and vascular cell adhesion molecule-1 were highest in PH1 > IHC (p < 0.001 to p = 0.005). **Conclusion:** PH1 Ur-PROs reflected overt inflammation, chemotaxis, oxidative stress, growth factors (including EGF), and pro-angiogenic and calcification regulation/inhibition compared to the C and IHC cohorts. Many of the up- and down-regulated PH1-PROs found in this study are also found in CKD, acute kidney injury, stone formers, and/or stone matrices. Further data analyses may provide evidence for PH1 unique PROs or demonstrate a poorer clinical outcome.

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Introduction

Primary hyperoxaluria type 1 (PH1: OMIM #259900) is a rare autosomal recessive disorder that has more than 100 identified mutations in the hepatic-specific peroxi-

somal enzyme alanine:glyoxylate aminotransferase (AGXT) gene, which is located on chromosome 2q37.3 [1]. Reduced hepatic AGXT-1 (AGT1) peroxisomal enzymatic activity results in a severe, inexorable hepatic overproduction of oxalate (Ox), high urine (Ur)-Ox (UrOx) concentrations, calcium Ox (CaOx) monohydrate (COM) nephrolithiasis, nephrocalcinosis, and chronic kidney disease (CKD) [2]. In a majority of patients, end-stage renal disease (ESRD) develops and kidney and liver transplantation is the only answer for long-term survival.

To date a modest genotype-phenotype correlation has been demonstrated in PH1 between patients and within families, resulting in a wide spectrum of heterogeneity of PH1 phenotypic disease expression. One exception may be the PH1-homozygous p.Gly170Arg AGXT mutation that is responsive to pharmacologic doses of vitamin B6 with UrOx substantially reduced or normalized, depending on whether 1 or 2 copies of the mutation exist [3]. However, for most PH1 mutations, the age of symptom onset and progression of CKD are highly variable. Thus, with incomplete insight into how rapidly the disease will manifest itself, along with a prevalence of 1:151,887, it is not surprising that this rare orphan disease is often not diagnosed until ESRD and systemic oxalosis develop [4, 5]. Longitudinal research reports of PH1-CKD patterns are limited since late diagnosis has historically prohibited the study of early clinical patterns of disease progression. Only in recent years have PH1 biobanks been formed to archive and study biospecimens for research on this rare disease. Two such registries with biobanks are the Rare Kidney Stone Consortium (RKSC) with its International Registry and Biobank for Hereditary Calcium Stone Diseases, at Mayo Clinic, Rochester, Minn., USA; and the European Hyperoxaluria Consortium (OXALEUROPE), a PH registry and biobank at University Children's Hospital, University of Bonn, Germany.

With these resources available, we examined 24-hour PH1-Ur proteomic patterns and compared them to patients with a history of a common stone-forming disease, idiopathic hypercalciuria (IHC). These cohorts were compared to healthy siblings of PH1 patients, which served as intra-familial controls (C).

We hypothesized Ur-PH1 and -IHC would exhibit similar protein (PRO) patterns typical of stone formers and posited that PH1 would demonstrate divergent profiles of cellular injury, immune response, inflammation, biomineralization, oxidative stress, and abnormal tissue repair associated with higher UrOx levels. Our study aims included (1) demonstrate and characterize cross-

sectional differences in Ur-PRO patterns in PH1, IHC, and C cohorts; and (2) identify, quantitate, and correlate unique PH1 biomarkers with clinical and other laboratory data.

Methods

Human Subjects Research Approval, Enrollment, and Biobank Specimens

The study protocol and consent documents were reviewed and approved by the Ann & Robert H. Lurie Children's Hospital of Chicago Institutional Review Board (IRB), Mayo Clinic's IRB, and ethics boards at the participating institutions in Europe. Subjects seen clinically, as well as those who contacted us were enrolled in Chicago (n = 33) and the University La Laguna, Hospital Universitario de Canarias, Tenerife, Spain (n = 2), with specimens and data de-identified, after they gave their consent to be part of this study. Patients enrolled in Chicago also gave written permission to utilize their clinical data. Anonymized 24-hour Ur aliquots and data were obtained from: OXALEUROPE (n = 23) and the RKSC at Mayo Clinic (n = 37).

Study Cohorts Defined

Three cohorts were defined for this study. PH1 patients (cohort 1), were diagnosed by molecular genetic confirmation of homozygous or compound heterozygous mutations of the AGXT gene and/or by renal or hepatic biopsy. IHC patients (cohort 2) were diagnosed by 2 24-hour Ur collections with UrCa >4 mg/kg/24 h and/or an UrCa/creatinine (Cr) ratio >0.21 mg/mg [6], and had a history of nephrolithiasis. Controls 'C' (cohort 3) were healthy siblings of PH1 patients studied.

Biochemical Measurements

Twenty four-hour Ur samples were collected by IHC and PH1 subjects and instructed to continue their medications/supplements. Following specimen preparation in Chicago, de-identified specimens were sent to Litholink Corporation (LabCorp, Chicago, Ill., USA) for biochemical measurements of Ur-Ox, -Ca, -citrate, and -Cr concentrations unless biochemical data were provided by the biobank or clinical site. Aliquots were stored at -80°C until all Ur collections and aliquots were received.

Targeted Proteomics

Proteomic analyses of the Ur specimens were completed using a proprietary Luminex platform-based immunoassay to perform established multiplexed microsphere assays for measurement of 192 targeted PROs (Myriad Rules Based Medicine, Austin Tex., USA) as previously described [7–10]. Table 1 identifies targeted Ur PROs in this investigation that were significantly different between the cohorts and/or within the PH1 cohort, and data from the literature regarding Ur-PROs from healthy individuals, CKD, acute kidney injury (AKI), stone formers, and in calculi matrices.

Calculation of eGFR

Estimated glomerular filtration rate (eGFR) values for those less than 18 years were calculated using the Schwartz formula, while adult eGFRs (≥18 years) were calculated using the Modification of Diet in Renal Disease study formula [11, 12].

Table 1. Ur proteomics found in health, injury, disease, stone formers and stone matrix

Protein biomarkers	Role	Healthy	CKD	AKI	Stone formers Ur	Identified in stone matrix	PH1 24-hour Ur
α-1 antitrypsin	Inflammation: (cytokine; chemokine; complement pathways/cell injury/defense/immune response)	X	X	X	X	X	X
CD40; CD40 ligand							X
Complement factor H		X	X		X	X	X
IgA			X			X	X
IFN-γ							X
IL-1α; IL-1β; IL-1 receptor α; IL-6 ^Δ receptor (IL-6r) [#] ; IL-8 ^Δ ; IL-10; IL-15; IL-18*		X*, #		X ^Δ , *	X*, Δ		X
MIP-1α							X
MCP-1					X		X
Myeloperoxidase					X	X	X
RANTES							X
SAP		X				X	X
TNFr ₂		X					X
SOD-1	Oxidative stress inhibition	X			X		X
Albumin (serum)	Protein binding/transport (plasma, cell, cell membrane)	X	X	X	X		X
Apo A-I, II ^Δ ; C-III; and D		X	X		X ^Δ	X	X
α-1 microglobulin					X	X	X
α-2 macroglobulin		X	X		X	X	X
FABP 1 (liver)		X	X				X
Haptoglobin		X	X		X	X	X
Microalbumin			X		X		X
NGAL		X		X	X		X
S-100 calcium binding protein B		X	X				X
Transferrin		X			X	X	X
Fetuin A	Calcification inhibition/promoter						X
OPN*		X				X	X
Uromodulin (THP)*		X	X	X	X	X	X
Lectin-like oxidized low density lipoprotein-1	Extracellular matrix development/degradation/remodeling						X
MMP 9		X	X				X
TIMP 1		X		X			X
ICAM 1	Endothelial injury/adhesion	X					X
VCAM-1				X		X	X

Table 1. (continued)

Protein biomarkers	Role	Healthy	CKD	AKI	Stone formers	Ur	Identified in stone matrix	PH1 24-hour Ur
EGF	Growth factor/cell biogenesis/regeneration/fibrosis	X	X	X				X
FGF-4		X						X
HGF								X
IGF-1 and IGFBP-2		X	X				X	X
TFF 3		X	X		X			X
VEGF		X						X
CgA	Pro/anti-angiogenic protease: protein catabolism		X					X
Cystatin C		X	X	X				X

Ur data from random and 24-hour specimens [25–27, 29, 36, 42–45].

α = Alpha; β = beta; γ = gamma; RANTES = regulated on activation normal T cell expressed and secreted; Apo = apolipoprotein; FABP = fatty acid binding protein 1; NGAL = neutrophil gelatinase associated lipocalin; MMP 9 = matrix metalloproteinase 9; TIMP 1 = tissue inhibitor of metalloproteinases-1; ICAM 1 = intracellular adhesion molecule 1; VCAM-1 = vascular cell adhesion molecule-1; FGF-4 = fibroblast growth factor-4; TFF 3 = trefoil factor 3; VEGF = vascular endothelial growth factor.

The following symbols represent biomarkers in row 6: $\wedge$, *, #, $\dagger$ for interleukin-6 (IL-6 $\wedge$), IL-6 receptor (IL-6r $\#$), IL-8 $\dagger$, and IL-18*.

Statistical Analyses

Physiologic data were log transformed and statistical comparisons were made between the 3 cohorts using one-way analysis of variance (ANOVA) or ANOVA by Ranks for data that were not normally distributed. Student's t test or non-parametric Wilcoxon rank sum tests were used to compare between 2 cohorts. The Spearman correlation test and linear and forward stepwise regression analyses were used. All work was done using SigmaPlot 12.5® (Systat Software; ©2003–2013; Chicago, Ill., USA). Gene Ontology® was used to determine relevant pathways for PH1 (GO, Gene Ontology Consortium, St. Joseph, Mich., USA).

Results

Subject Characteristics

Ninety five 24-hour Ur samples and corresponding data were studied (PH1 = 47; IHC = 35; C = 13). Of 192 targeted PROs measured, 152 (79%) were statistically analyzed. There were 2 reasons for excluding 21% of the markers from the statistical analyses: (1) marker levels below the detectable range and (2) some subjects had insufficient specimen volume for analyses of all markers.

Subject characteristics and their medications are in table 2. There was a significant difference in age between the 3 cohorts, with the controls being older ($p < 0.05$). UrCa was greater in C vs. PH1 ($p < 0.05$) and higher in IHC compared to PH1 ($p < 0.001$). UrOx concentration

was highest in PH1 compared to C and IHC ($p < 0.05$). Also, the IHC-eGFR was higher than that of PH1 = 155.7 ± 49.6 ; 77.4 ± 34.5 ml/min/1.73 m² ($p < 0.001$).

The PH1 cohort was 45% heterozygous for p.Gly170Arg and 11.4% homozygous, which is greater than expected in the PH1 population based on the literature but as most specimens were from biobanks, the prevalence in this study may be skewed [3, 13]. Two of the p.Gly170Arg heterozygous patients also had the minor allele mutant Gly41Arg replacement, and might be expected to show partial response to pyridoxine [14]. AGT mutants on the minor allele Phe152Ile were expressed in 15.9%, while 2.3% exhibited Ile244Thr, and may have been partially responsive to pyridoxine [14]. Many more patients were taking pyridoxine than those with a documented p.Gly170Arg mutation (almost 80% were on B6 (table 2) vs. 56.8% with the p.Gly170Arg mutation with/without minor allele mutant replacement), yet all receiving B6 were reported to be 'therapy responsive,' albeit we did not receive data on their degrees of responsiveness. Table 2 includes supplement and medication information regarding K-phosphate, K-citrate, Mg, pyridoxine, hydrochlorothiazide, chlorathiodone and other therapies taken by PH1 and IHC cohorts [15, 16].

Table 3 includes 24-hour Ur-PRO cohort differences we observed with 6 PROs different between PH1 and C, and 11 PROs higher in PH1 vs. IHC. Insulin-like growth

Table 2. Characteristics of subjects (n = 95; mean ± SD)

Variable	Cohorts			p value
	PH1 (n = 47)	IHC (n = 35)	C (n = 13)	
Gender, M/F	23/24	17/18	6/7	–
Age, years	21.4±16.3*	12.1±5.2*	35.2±17.8*	<0.001
UrCa, mg/kg/24 h	1.59±1.51*	4.23±3.78	2.55±1.09*	<0.001
UrOx, mmol/l/1.73 m ²	1.24±0.74*	0.53±0.45	0.40±0.22	<0.001
Ur citrate/Cr	1.28±3.50*	0.52±0.31*	1.34±1.23	<0.05
<i>Supplements and medications, %</i>				
Pyridoxine	78.7	3.0	0.0	
Potassium citrate	29.7	47.0	0.0	
Potassium phosphate	38.3	0.0	0.0	
Sodium bicarbonate/sodium citrate	10.6	3.0	0.0	
Magnesium	6.3	3.0	0.0	
Phosphate (elemental)	2.0	0.0	0.0	
Hydrochlorothiazide	2.0	17.6	0.0	
Chlorathiodone	0.0	44.0	0.0	

Table 3. Protein biomarkers highest in PH1 compared to C and IHC

Biomarker	Role	p value
<i>PH1 > C</i>		
CgA	Pro- or anti-angiogenic	<0.05
EGF	Cell biogenesis/growth and proliferation	<0.05
FGF-4		
IGF-1*		
IL-10	Anti-inflammatory	0.03
MIP-1α*	Chemotaxis	<0.05
<i>PH1 > IHC</i>		
Apo A-II and C-III	Transport/lipid metabolism	0.002 to 0.004
IGF-1*	Cell biogenesis/growth and proliferation	<0.001
Fetuin A	Inhibition of crystallization/extracellular calcification	0.002
IL-1α, IL-8, IL-15	Inflammation; chemotaxis; remodeling	<0.001 to 0.049
MIP-1α*		
Microalbumin	Transport/loss of GBM integrity	0.02
Transferrin	Transport/iron binding/transport/acute phase reactant/loss of GBM integrity/inflammation	0.015
VCAM-1	Endothelial injury/adhesion	0.0007

α = Alpha; FGF-4 = fibroblast growth factor-4; Apo = apolipoprotein; VCAM-1 = vascular cell adhesion molecule-1.

* PROs increased in PH1 vs. C and IHC cohorts.

Fig. 1. Median IGF-1 in the Ur of control, IHC and PH1 cohorts. PH1 > C; PH1 > IHC. Data are log transformed.

Fig. 2. a, b Known stone matrix components are lower in PH1-Ur. Data are log transformed and expressed as mean $\pm$ SD. **a** OPN (C > IHC > PH1; * $p < 0.05$); **b** calbindin (C > IHC > PH1; * $p < 0.05$).

factor-1 (IGF-1; fig. 1) and macrophage inflammatory PRO-1 α (MIP-1 α) were consistently higher in PH1 vs. C ($p < 0.05$) and IHC ($p < 0.001$). Additionally, concentrations of osteopontin (OPN) and calbindin (known COM stone matrix components) were different between the cohorts, with C > IHC > PH1 ($p < 0.05$; fig. 2). Other known stone components that were higher in C vs. PH1 were serum amyloid P (SAP; $p = 0.035$), immunoglobulin A (IgA; $p = 0.048$), and clusterin ($p = 0.029$).

Correlations and Regressions

PH1 correlations demonstrated tumor necrosis factor receptor 2 (TNFR₂) to be associated with chromogranin A (CgA; $r = 0.65$; $p = 0.00005$), while extracellular newly identified RAGE (receptor for advanced glycation end products)-binding PRO (ENRAGE) was correlated with interleukin-8 (IL-8); $r = 0.80$; $p = 2.0E-07$ and myeloperoxidase ($r = 0.86$; $p = 2.0E-07$). Superoxide dismutase-1 (SOD-1) was associated with cystatin C ($r = 0.77$; $p =$

2.0E-07), IL-1 α and IL-6 r ($r = 0.61$; $p = 9.7E-06$ and $r = 0.61$; $p = 8.6E-06$).

We explored relationships between PH1-fetuin A and PROs associated with pathologic extraosseous mineralization. Fetuin A was best predicted by: SOD-1, IL-8, MIP-1 α , and monocyte chemoattractant PRO-1 (MCP-1; $R^2 = 0.75$; $p < 0.001$) and correlated with IL-8 ($p = 1.75E-08$).

PH1-Tam Horsfall PRO (THP) was an independent predictor of OPN ($R^2 = 0.14$; $p = 0.003$). THP was strongly correlated with the epidermal growth factor (EGF; $r = 0.81$; $p = 2.0E-07$).

MIP-1 α was highest in PH1 and best explained by CgA, with eGFR and cystatin C held constant in the model ($R^2 = 0.93$; $p = 0.035$). PH1-CgA was also inversely correlated with eGFR ($r = -0.35$; $p = 0.04$).

PH1 Data Dichotomized for 24-Hour UrOx

We dichotomized PH1-UrOx and eGFR data by their median (Md) value, creating 2 sub-groups (table 4). The PH1-Md UrOx concentration = 1.045 (IQR 0.256–3.046) mmol/l/1.73 m². No significant difference was found between the UrOx sub-groups for eGFR (mean $\pm$ SD 80.7 $\pm$ 30.8; 74.7 $\pm$ 39.0 ml/min/1.73 m²; $p = 0.56$). The Md PH1-eGFR was 77.8 (IQR 8.0–164.1) ml/min/1.73 m². Similarly, no significant difference existed for 24-hour UrOx (mean $\pm$ SD 1.35 $\pm$ 0.82; 1.13 $\pm$ 0.67 mmol/l/1.73 m²; $p = 0.45$) between the eGFR sub-groups.

OPN was lowest when UrOx was highest, while OPN and THP were observed to be lower in PH1 when eGFR was low (eGFR < Md) and higher when eGFR > Md. In contrast, PH1-fetuin A, a potent calcification inhibitor, was higher than in IHC ($p < 0.05$) and in the low eGFR and UrOx sub-groups, respectively. Cytokines, chemokines, and antioxidant defense were also higher when eGFR was low but was not observed when UrOx < Md. Additionally, Ur-IGF-1 was highly expressed in PH1 vs. IHC and C ($p < 0.05$) and in both PH1-UrOx sub-groups. IGF-1 was also higher in the PH1 low eGFR sub-group, denoting more advanced CKD.

Using Gene Ontology[®], p38 mitogen-activated PRO kinase (MapK) was recognized as a focal pathway element and was supported by our findings of the genes that were elevated in PH1-Ur, including cell proliferation ($p = 5.78E-06$); regulation of ERK1 and ERK2 cascade ($p = 5.23E-05$); and stem cell development and proliferation ($p = 4.23E-05$ and $p = 9.15E-05$). We further noted that interferon- γ (IFN- γ), an immunomodulator, was significantly lower in the PH1-UrOx > Md and eGFR < Md sub-groups. Also, IL-15, which is expressed in renal cells and a modulator of immune response [17], was higher in PH1-UrOx > Md.

Table 4. Proteomic marker differences by PH1 sub-group (dichotomized by Md-UrOx concentration and eGFR[†]); $p < 0.05$

Variables	UrOx		eGFR	
	UrOx < Md	UrOx > Md	eGFR < Md	eGFR > Md
OPN	↑	↓	↓	↑
HGF				
ICAM-1				
IFN- γ	↑	↓	(-)	(-)
IL-6 r				
LOX-1				
RANTES				
EGF	(-)	(-)	↓	↑
Uromodulin (THP)				
CD40				
CD40L				
IL-1 α				
NGAL				
SOD-1	↑	↓	↑	↓
TFF-3				
TNFr ₂				
VCAM-1				
VEGF				
IL-15	↓	↑	(-)	(-)
IGF-1	↓	↑	↑	↓
Angiotensinogen				
Fetuin A	(-)	(-)	↑	↓
IL-1 β , IL-8				

[†] Md-PH1 UrOx = 1.045 (IQR 0.256–3.046) mmol/l/1.73 m²/24 h; Md-PH1-eGFR = 77.8 (IQR 8.0–164.1) ml/min/1.73 m².

α = Alpha; β = beta; γ = gamma; ICAM-1 = intracellular adhesion molecule-1; LOX-1 = lectin-like oxidized LDL receptor 1; RANTES = regulated on activation normal T cell expressed and secreted; CD40 = cluster of differentiation-40 antigen; CD40L = CD40 ligand; NGAL = neutrophil gelatinase-associated lipocalin; TFF-3 = trefoil factor-3; VCAM-1 = vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor.

Discussion

Renal injury and cell death in PH1 are directly related to Ox exposure, its poor solubility and tendency for renal tubular crystallization. Consistent with this, AGXT knock out $-/-$ (KO) mice demonstrate cell injury and death linked to plasma Ox in a dose-dependent manner, and OPN and THP exhibit an affinity for intra-tubular epithelial binding to CaOx crystals in the UF [18]. Moreover, when OPN and THP are not expressed in double KO

mice with experimentally induced hyperoxaluria, extensive crystal and stone formation occur. Thus, OPN and THP play important roles for the inhibition of extraosseous calcification in renal tubular epithelial cells [18].

K citrate and K Phos treatments are used to reduce or eliminate CaOx crystals, stone formation and resulting tubular nephrotoxicity. While almost half of the IHC cohort was taking K-citrate, the mean $\pm$ SD of IHC-UrCa = 4.23 ± 3.78 , which is 'persistent IHC' [15]. Conversely, 38.3% of PH1 were taking K Phos with fewer on K citrate, yet had the highest UrOx and lowest UrCa levels, the latter most likely from Ca retention for stone formation and nephrocalcinosis.

The mechanisms of Ox-associated nephrotoxicity exhibited by PH1 proteomics encompasses p38 MAPK pathway upregulation, phosphorylation, and activation of transcription factors that are critical for eliciting inflammation, oxidative stress, PT cell injury, necrosis and apoptosis. However, this pathway does not appear to be unique to PH1, as similar PROs are found in CKD, AKI and stone formers [19–23].

Clearly, many of the PH1 markers we found to be up- or downregulated are important mediators (or receptors) of pro-inflammatory cytokines, adaptive immune responses, and compensatory responses to inhibit inflammation. Five such examples include MIP-1 α , IL-8, IL-15, IL-10, and TNF α ; a chemokine; neutrophil chemotactic factor; pro-inflammatory cytokine that upregulates macrophages; other inflammatory cytokines and immune responses; and a cytokine receptor, respectively, that are directly associated with NF- κ B pathway upregulation. Other upregulated PH1 biomarkers were vascular cell adhesion molecule-1, IL-1 β , TNF- α , MCP-1, and IFN- γ , which are associated with inflammatory cell migration as is IHC-MCP-1 [15]. Higher PT injury marker levels were observed when PH1-eGFR < Md: neutrophil gelatinase-associated lipocalin, trefoil factor-3, and SOD-1.

Our Gene Ontology results were consistent with inflammation in PH1 occurring due to NF- κ B pathway upregulation, providing important credence to our conclusions. Expression of some PH1 biomarkers that reflect MAPK pathway activation also change in CKD and AKI [24–29]. In cell culture, this pathway is upregulated due to CaOx crystal deposition within the renal tubular epithelium creating crystal-induced disruption of DT epithelial cell tight junctions and loss of tubular paracellular barrier integrity [30]. These findings soundly emphasize the consequences of chronic tubular over exposure to CaOx crystals and the mechanisms by which PH1-CKD progresses over time and tubular injury is demonstrated in CaOx stone disease (IHC).

PH1-MIP-1 α , which was higher vs. IHC and C may serve as a renal injury marker associated with CaOx crystal-related nephrotoxicity. CgA was also an independent predictor of MIP-1 α , correlated with TNF α , and increases in adults with impaired renal function and ESRD [24, 31]. Thus, CgA may play a role in renal fibrosis development via endothelin-1 in PH1-CKD progression since the renal tubulointerstitium contains dendritic cells and envelop the nephron [32].

Our results also confirmed upregulation of a number of growth factors in PH1-CKD. Results from a large international study of 3 independent adult CKD cohorts identified Ur-EGF/Cr as a significant predictor of interstitial fibrosis, tubular atrophy, eGFR, and CKD progression. A one unit increase in Ur-EGF/Cr (log) was associated with a hazard ratio for CKD progression = 3.73 (1.85–7.69)-fold increased risk [26]. Our findings of upregulated Ur-EGF may constitute relevant and promising data. Other work in adults with more advanced CKD than seen in our study subjects demonstrates some overlap with our Ur-PRO findings, including lower THP and higher apolipoprotein A-1 and α -1 antitrypsin associated with rapid kidney function decline [22]. Perhaps PH1 leads to more rapid changes in PRO regulation despite having a higher eGFR.

Another growth factor, IGF-1, which is highly expressed in the kidneys was consistently higher in PH1 vs. IHC, and when UrOx > Md. It has been positively associated with CKD in adult gender- and multivariate-adjusted models [25]. Thus, IGF-1 upregulation in PH1-CKD is plausible and may provide a protective role of cell proliferation, differentiation, growth, and repair when Ox crystal related renal tubular injury occurs [33].

Alternatively, hepatocyte growth factor (HGF), which has anti-inflammatory properties was increased when PH1-UrOx < Md but reduced when UrOx was highest, possibly reflecting a protective response. HGF suppresses macrophage infiltration and abrogates chemokine expression via disruption of NF- κ B in human kidney epithelial cell culture and downregulates TGF- β -induced renal fibrosis in nephrotic mice [34, 35]. However, it is possible that protective responses by HGF may be downregulated (lost) when PH1-UrOx is highest.

Using Gene Ontology, IGF-1 theoretically serves as an upregulator of the NF- κ B pathway and fetuin A upregulation ($p = 0.04$). Fetuin A was higher when eGFR was low, suggesting it may inhibit pathologic biomineralization in the PT when GFR is low. It is a potent calcification inhibitor and was higher in PH1 > IHC. However, we could not establish whether PH1-Ur-fetuin A, -Ur-Ox or -Ur-Ca

were different in the presence or absence of nephrolithiasis/uroolithiasis (94% had a positive history of calculi) or nephrocalcinosis (15% had documented nephrocalcinosis) because the sub-groups were small and unequally divided, with insufficient power to make any determinations.

PH1 also exhibited lower concentrations of PROs that are known to be found within the organic matrix of COM calculi (OPN and calbindin) [36]. SAP, IgA and clusterin were downregulated in Ur-PH1 < C. Similarly, OPN and THP were lower when PH1-eGFR < Md, which may also reflect calculi organic matrix macromolecule retention. OPN is upregulated in renal tubules following ethylene glycol and vitamin D₃ administration in THP-/- (KO) mice with development of CaOx crystals, while THP [37] directly correlates with CKD-eGFR [27]. We similarly demonstrated THP to be lower when PH1-eGFR < Md and conversely THP was higher when eGFR > Md. Thus, it is plausible that OPN and THP may exhibit a diminished ability to inhibit extrasosseous mineralization or are downregulated as GFR declines and UrOx rises.

The PH1 Ur-PROs we found to be upregulated suggest that chemotaxis, oxidative stress, inflammation, cell growth and proliferation, are reflective of compensatory responses to control tubular injury associated with chronic CaOx crystal exposure. We also found evidence suggesting PH1-CKD glomerular injury, including upregulated transferrin and microalbumin [28]. Although, we found some similar Ur-PRO patterns in PH1 and IHC for PROs known to be incorporated into COM stone organic matrix, we also observed divergent profiles between IHC and PH1 that we had hypothesized. Hence, an increased presence of Ur-PROs indicative of toxic injury and cell death added relevant data regarding patterns of compensatory and protective mechanisms that are driven by co-existing high UrOx and declining eGFR as PH1-CKD progresses. Consistent with our results, our RKSC colleagues recently published data regarding higher UrOx being linked with a rapid GFR decline [38].

Study Limitations

Our work discussed here was limited to cross-sectional 24-hour Ur specimens. In a separate study, we are currently examining relationships between cross-sectional and prospective PH1 Ur-PROs, which will permit a more rigorous examination of longitudinal changes in inflammatory and protective tissue responses over the course of PH1-CKD progression. A majority of the PH1 patients who provided anonymized Ur samples for this study (78.7%) were taking daily pyridoxine and noted to be 'responsive to therapy,' such that UrOx levels had declined with clinical treat-

ment. However, the mean UrOx concentration = 1.24 ± 74 mmol/l/1.73 m², which was more than 3 times higher than that of controls, even with B6 treatment. As this was a cross-sectional investigation, we do not have data on prospective treatment effects on PH1-Ur proteomic patterns. We can only suggest that higher UrOx is associated with a more robust inflammatory proteomic response and likely contributes to PH1-CKD progression, which might stabilize with UrOx reduction from B6 therapy.

We are aware that the age difference of the control subjects (35.2 ± 17.8 years) vs. PH1 (21.4 ± 16.3 years) and IHC (12.1 ± 5.2 years) may present some confounding of our results. It is possible that the older control subjects may have had other age-related sub-clinical conditions resulting in low-grade inflammation, modest immune system disturbances, or moderate age-related decline in GFR [39]. However, we did not observe a skewed proteome profile in the cohort when compared to PH1 or IHC. The controls were known to be otherwise healthy and were screened to eliminate individuals taking prescription medicine or over-the-counter medications on a regular basis, since their medical records were released for our review.

One other consideration is that although we statistically examined whether eGFR differences existed for PH1 patients with high vs. low UrOx levels and found no differences, in reality, higher circulating levels of Ox are found in the plasma (oxalosis), with systemic CaOx crystal deposition when GFR is low. Conversely, it is possible that we may have found differences if we had included a control cohort with reduced eGFR that was not associated with PH1 or other stone diseases.

Our study methodology utilized targeted proteomic platform-based immunoassays. Therefore, other biomarkers that play a critical role in PH1-CKD may not have been identified and could have provided pertinent data on pathophysiologic mechanisms in PH1 not described here.

The strength of this study is centered on examination of 3 distinct cohorts, with 2 of them representing CaOx stone-forming diseases. The third cohort, controls, was comprised of first-degree relatives of PH1 patients. Comparison of healthy controls against stone-forming groups supported our identification of up- and downregulation of Ur biomarkers and possible pathologic mechanisms involved in CaOx crystal-related nephrotoxicity, calculi formation, and renal tissue injury and repair. In PH1, the renal tubules are reactive to much larger concentrations of Ox and CaOx crystals compared to other 'traditional' stone formers and exact an overwhelming inflammatory response leading to

downregulation of cell repair [20, 30, 40, 41]. Our findings clearly demonstrate a picture of inflammatory and oxidative upregulation, and growth factors-associated cell proliferation in PH1-Ur. Our future investigative findings from prospective study of PH1 will likely add further credence to understanding PH1-CKD disease progression and perhaps also identify targets for treatment.

Acknowledgments

We thank the patients and their family members who made this study possible.

Support and Financial Declaration

This work was supported by the National Institutes of Health grant #1R44DK084634-01 and co-supported with de-identified specimens and data by the Rare Kidney Stone Consortium (RKSC) and the European Hyperoxaluria Consortium (OXALEUROPE). The RKSC, Mayo Clinic, Rochester, Minn., USA (U54DK083908) is a member of the Rare Diseases Clinical Research Network (RDCRN), an initiative of the Office of Rare Diseases Research (ORDR) and NCATS. This consortium is funded through collaboration between NCATS and the NIDDK.

The authors have no personal or financial conflict of interests regarding any organizations or individuals that could be perceived as influencing this research, its design, implementation, or results published.

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