Kidney360

Association of Baseline Urinary Metabolic Biomarkers with ADPKD Severity in TAME-PKD Clinical Trial Participants

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Key Points

- Urine excretion of two key glycolytic enzymes correlated with autosomal dominant polycystic kidney disease (ADPKD) severity (height-adjusted total kidney volume and eGFR) at baseline in the TAME-PKD study population.
- These findings are the first to provide evidence in human urine samples that upregulated glycolytic flux is a feature of ADPKD severity.
- Future analyses will test whether metformin affects ADPKD disease progression and urinary metabolic biomarkers in patients during the study.

Abstract

Background Recent work suggests that dysregulated cellular metabolism may play a key role in the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD). The TAME-PKD clinical trial is testing the safety, tolerability, and efficacy of metformin, a regulator of cell metabolism, in patients with ADPKD. This study investigates the cross-sectional association of urinary metabolic biomarkers with ADPKD severity among TAME-PKD trial participants at baseline.

Methods Concentrations of total protein, targeted metabolites (lactate, pyruvate, succinate, and cAMP), and key glycolytic enzymes (pyruvate kinase M2 [PKM2], lactate dehydrogenase A [LDHA], and pyruvate dehydrogenase kinase 1 [PDK1]) were measured by ELISA, enzymatic assays, and immunoblotting in baseline urine specimens of 95 TAME-PKD participants. These analytes, normalized by urinary creatinine or osmolality to estimate excretion, were correlated with patients' baseline height-adjusted total kidney volumes (htTKVs) by MRI and eGFR. Additional analyses were performed, adjusting for participants' age and sex, using multivariable linear regression.

Results Greater htTKV correlated with lower eGFR ($r=-0.39$; $P=0.0001$). Urinary protein excretion modestly correlated with eGFR (negatively) and htTKV (positively). Urinary cAMP normalized to creatinine positively correlated with eGFR. Among glycolytic enzymes, PKM2 and LDHA excretion positively correlated with htTKV, whereas PKM2 excretion negatively correlated with eGFR. These associations remained significant after adjustments for age and sex. Moreover, in adjusted models, succinate excretion was positively associated with eGFR, and protein excretion was more strongly associated with both eGFR and htTKV in patients <43 years old.

Conclusions Proteinuria correlated with ADPKD severity, and urinary excretion of PKM2 and LDHA correlated with ADPKD severity at baseline in the TAME-PKD study population. These findings are the first to provide evidence in human urine samples that upregulated glycolytic flux is a feature of ADPKD severity. Future analysis may reveal if metformin treatment affects both disease progression and the various urinary metabolic biomarkers in patients throughout the study.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenetic, life-threatening, genetic disorder, affecting approximately 1 in 500–1000 individuals, and is caused by mutations in the genes encoding the proteins polycystin 1 and polycystin 2.

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ADPKD is characterized by a slow, continuous development and enlargement of cysts that compromise normal kidney parenchyma and function, eventually resulting in kidney failure in approximately 50% of all patients by the age of 50–60 (1). Although the only currently approved ADPKD therapy is tolvaptan (2), there is a growing list of potential therapeutic targets that are under various stages of investigation in preclinical and clinical studies (3).

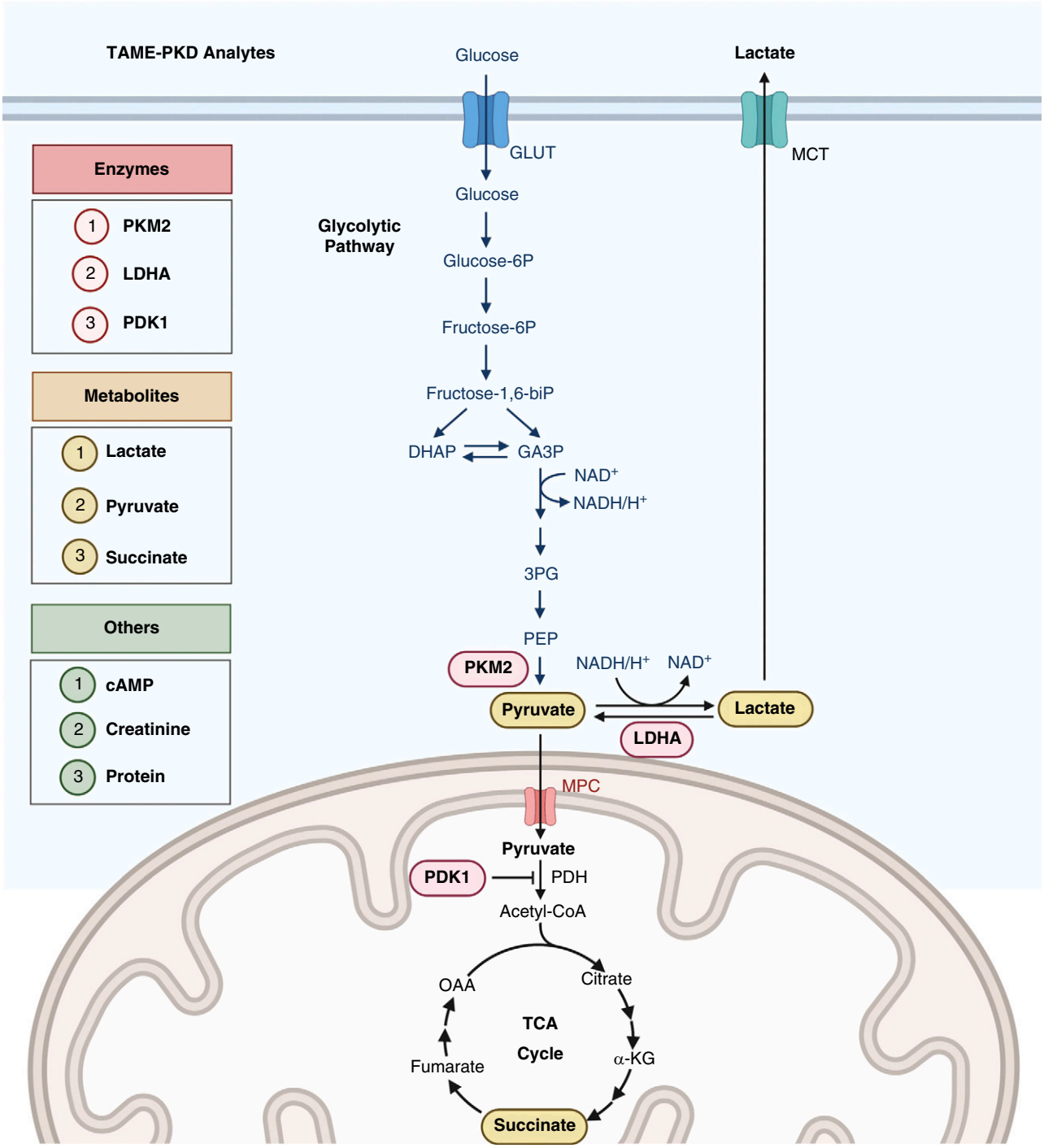

Figure 1. | Schematic diagram illustrating the glycolytic pathway and the TCA-cycle mitochondrial oxidative metabolic pathway, highlighting the key urinary analytes measured in the TAME-PKD study. Glycolytic enzymes are shown in red. Metabolites are shown in yellow. Other analytes are shown in green. DHAP, dihydroxyacetone phosphate; fructose-1,6-biP, fructose-1,6-bisphosphate; fructose-6P, fructose-6-phosphate; GA3P, glyceraldehyde-3-phosphate; glucose-6P, glucose-6-phosphate; GLUT, glucose transporter; α-KG, α-ketoglutarate; LDHA, lactate dehydrogenase A; MCT, monocarboxylate transporter; MPC, mitochondrial pyruvate carrier; NADH/H^+, oxidized and reduced forms of NAD; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; PKM2, pyruvate kinase M2.

Because there is considerable variability in disease severity among affected individuals, it is important to identify patients at high risk for disease progression for prognostic reasons, and to determine a patient's potential eligibility to receive new therapeutics and participation in clinical trials. Current tools to assess disease severity and predict progression include measuring height-adjusted total kidney volume (htTKV) relative to the patient's age and genotype (1,4), but these assessments may be time consuming, expensive, and/or inconvenient. There has been significant recent interest in identifying more convenient biomarkers that may reliably associate with disease severity, progression, and the potential clinical response to new ADPKD therapies (5).

Although many cellular signaling pathways are dysregulated in ADPKD, critical pathophysiologic details are still unclear (6). Metabolic derangements have been detected in ADPKD cells derived from animal ADPKD models with loss of polycystin 1 function and in ADPKD kidney tissue, which may contribute to cyst formation and expansion, including increased aerobic glycolysis (the Warburg effect), impaired fatty-acid oxidation, and reduced AMP-activated protein kinase (AMPK) activity (7,8). In preclinical studies, the AMPK activator metformin was shown to inhibit ADPKD kidney cyst growth and cell proliferation *in vitro* and in mouse models with rapid ADPKD progression (9). Metformin promotes cellular AMPK activation (10), which may confer beneficial effects in the treatment of diseases such as the metabolic syndrome, diabetes, and polycystic ovary syndrome (11). However, it is uncertain the extent to which metabolic changes that occur in the ADPKD kidney are reflected in changes in measured urinary metabolites and metabolic enzymes, and whether they play a causative role in disease pathogenesis and progression in patients. The potential role of urinary metabolic biomarkers in monitoring response to therapies that target dysregulated metabolism is also unclear.

The TAME-PKD study is a multicenter, double-blind, placebo-controlled, phase 2 clinical trial (NCT02656017) that is testing the hypothesis that metformin treatment will be safe and tolerable and ameliorate ADPKD progression in patients with an eGFR of ≥50 ml/min per 1.73 m^2, over a 2-year treatment period (12). Boletta and colleagues (7) identified a Warburg effect–like shift to excessive aerobic glycolysis—as evidenced by increased levels of certain key glycolytic enzymes (*e.g.*, lactate dehydrogenase A [LDHA], pyruvate dehydrogenase kinase 1 [PDK1], and the pyruvate kinase M2 [PKM2] isoform), along with a decrease in signaling through the AMPK pathway—occur in ADPKD cystic epithelial cells as compared with normal kidney epithelial cells in ADPKD mouse models and in patient kidney tissue. In this study, we examined the levels of these key urinary biomarkers and metabolites indicative of changes in glycolytic versus oxidative fluxes (see Figure 1), in relation to htTKV and eGFR, to assess the extent to which any of these markers correlate with disease severity at study enrollment in the TAME-PKD study population. We hypothesized that increasing levels of glycolytic pathway enzymes and metabolites would correlate with greater ADPKD severity, as indicated by surrogate disease markers (*i.e.*, higher htTKV or lower eGFR), in our patient population.

Materials and Methods

Urine Specimen Collection and Preparation

This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All study subjects have given their written informed consent, and the TAME-PKD study protocol was approved by the institutional review boards at each of the study sites. Eligible patients in the TAME-PKD study population included adults without diabetes (N=95) aged 18–60 years, with an eGFR of ≥50 ml/minute per 1.73 m^2, and ADPKD (12). Baseline clean-catch spot urine samples were obtained in the morning under overnight-fasting conditions, by standard methods, in sterile containers and then processed into 1.8-ml aliquots before storing within 3 hours at −80°C. They were then shipped on dry ice to the University of Southern California for biomarker analyses. In preparation for analyses, samples were thawed and centrifuged at 1000 × g for 20 minutes at 4°C. The supernatant was then used to perform biomarker assays.

Urinary Biomarker Analyses

For each baseline urine sample, measurements of urine osmolality and the concentrations of total protein, key metabolites (lactate, pyruvate, succinate, and cAMP), and the candidate glycolytic enzymes (PKM2, LDHA, and PDK1) were performed in triplicate (for creatinine, lactate, succinate, and PKM2), duplicate (for pyruvate, cAMP, and osmolality), or singly (total protein, LDHA and PDK1).

All enzymatic assay measurements were fit to standard curves, at appropriate dilutions, so that the levels fell within a linear range of the standard-fit curve. The measured absorbance or fluorescence was used to calculate the concentration of the above biomarkers in the urine samples by subtracting the sample background control readings from the sample readings and then comparing the sample signals to those of a standard curve. Reported concentrations were then normalized by urinary creatinine and/or osmolality. If the coefficient of variation of measurements in triplicate samples was >25%, or if the two measured values in duplicate samples differed by >25%, the samples were rerun and/or the outlier was discarded in the subsequent analysis. The mean value of replicate measurements for each sample was then normalized, as indicated, to the urine creatinine and to the urine osmolality measured for the same sample.

Creatinine, Lactate, Pyruvate, and Succinate Measurements

These analytes were measured using colorimetric or fluorometric assay kits (BioVision, Milpitas, CA) following the manufacturer's instructions. Duplicate or triplicate 50-μl samples of the thawed urine specimens, either undiluted or diluted, were added into a 96-well, flat-bottomed plate.

For creatinine measurements, a creatinine colorimetric/fluorometric assay kit (#K625-100) was used. The thawed urine specimens were diluted by 1:4, 1:10, and 1:25 to ensure an optimal measurement concentration. Standard and diluted urine aliquots (50 μl) and 50 μl of reaction mix were added to each well. Reaction mixes without creatininase were used as background controls. During the reaction, creatinine is converted to creatine by creatininase, and the creatine is then converted to sarcosine, which is specifically oxidized to produce a product that reacts with a probe to generate a red color. OD was measured at 570 nm on a Synergy HTX multiwell microplate reader (BioTek Instruments, Inc., Winooski, VT).

For lactate measurements, a lactate colorimetric/fluorometric assay kit (#K607-100) was used for aliquoted samples. The specimen specifically reacts with an enzyme mix to generate a product, which interacts with lactate in the sample to produce a red color. OD was measured at 570 nm, as above.

For succinate measurements, a succinate colorimetric assay kit (#K649-100) was used for aliquoted samples. In this assay, succinate present in the samples is used by succinyl-CoA synthetase to form an intermediate that undergoes a series of reactions, which reduces a colorless probe to a colored product with strong absorbance at 450 nm, measured as above.

For pyruvate measurements, a pyruvate colorimetric/fluorometric assay kit (#K609-100) was used for aliquoted samples. Pyruvate present in samples is oxidized by pyruvate oxidase *via* enzyme reactions to generate fluorescence, and measurements were performed at excitation and emission wavelengths of 535 and 587 nm, respectively.

Urinary cAMP Measurements

cAMP was measured with a colorimetric cAMP direct immunoassay kit (#K371-100; BioVision), following the manufacturer's instructions. Thawed urine samples were diluted 1:20 into 0.1% hydrogen chloride, and then 100 μl of the standard and diluted samples were acetylated following the manufacturer's protocol. Fifty microliters of the acetylated, standard cAMP and test samples were then added into a recombinant protein G–coated, 96-well plate, followed first by cAMP polyclonal antibody. Then cAMP–horseradish peroxidase (cAMP-HRP) conjugate was added, which directly competes with cAMP from the sample binding to the cAMP antibody on the plate. After incubation and washing, the amount of cAMP-HRP bound to the plate was then determined by measuring absorbance at 450 nm. These 450-nm absorbance measurements are inversely proportional to the cAMP concentrations in the urine samples.

Urinary PKM2 Measurements

Human PKM2 in the urine was measured with an ELISA kit (#MBS2505089; MyBiosource Inc., San Diego, CA), following the manufacturer's instructions. Briefly, duplicate standards or triplicate 100-μl aliquots of diluted (1:4) urine samples were added into human PKM2 antibody–precoated ELISA microplate wells. A biotinylated detection antibody specific for human PKM2, and then avidin-HRP conjugate, were added successively to each well and incubated. After washing away the free components, a substrate solution was added to each well, causing a blue color change proportional to the concentration of PKM2 in the sample. OD at a wavelength of 450 nm was measured, as above, for each sample.

Urinary Osmolality Measurements

Urine osmolality of each sample was measured in duplicate with a Wescor Vapro 5600 vapor pressure osmometer (Wescor, Logan, UT) in "normal mode," following the manufacturer's instructions.

Urinary Total Protein, LDHA, and PDK1 Measurements

Urinary total protein was isolated by the methanol-chloroform precipitation method. Briefly, 1000 μl of thawed urine sample was added to 810 μl of methanol and 190 μl of chloroform. Samples were then vortexed briefly, centrifuged at 12,000 $\times$ *g* for 1 minute, and then the upper phase was carefully removed. Methanol (300 μl) was then added again before vortexing briefly, centrifuging again to pellet the proteins, removing the liquid, allowing the pellets to dry, and then resuspending in 1$\times$ sample buffer for SDS-PAGE.

Electrophoresis and Immunoblotting Analysis

LDHA and PDK1 standards and total protein from normal control urine and patient samples were loaded into individual wells and then separated by a 4%–15% Criterion TGX Precast Midi Protein Gel (Bio-Rad, Hercules, CA) before electrical transfer to a nitrocellulose membrane (Bio-Rad). The membrane was stained with Revert 700 Total Protein Stain kit (LI-COR, Lincoln, NE), and then total protein in each lane was detected and quantified by densitometry using an Odyssey Fc Imaging System (LI-COR), with analysis using Image Studio Lite version 5.2 software (LI-COR). After destaining, the membrane was blocked in LI-COR Odyssey Blocking Buffer for 1 hour, and then incubated in 1:1000 diluted LDHA mouse mAb (#MAB9158; R&D Systems, Minneapolis, MN) and PDK1 rabbit polyclonal antibody (#NB100-2383; Novus Biologicals, Centennial, CO) in blocking buffer with 0.2% Tween 20 overnight at 4°C. After washing in TBS with 0.1% Tween 20, the membrane was incubated for 1 hour with secondary antibody in blocking buffer with 0.2% Tween 20. After another wash, LDHA and PDK1 protein levels were detected and quantified by densitometry of the relevant bands. LDHA and PDK1 concentrations were calculated by comparison to densitometric quantitation of standards run on the same gel.

Statistical Analyses

Descriptive statistics are presented as mean$\pm$SD or median (range) for continuous variables and frequency (percentage) for categoric variables. The Pearson correlation coefficient is reported as a measure of the strength of linear association between eGFR and htTKV, and as a measure of each of the respective biomarkers and eGFR/htTKV. Multivariable linear regression analyses were used to test whether the associations between the respective biomarkers and eGFR/htTKV remain consistent when controlling for patient sex and age. Tests of statistical interaction were also performed, using multivariable linear regression models, to test whether the relationship between each biomarker and dependent variable (eGFR/htTKV) remained consistent by sex and age. For each biomarker and dependent-variable combination, a multivariable regression model was constructed, including the biomarker, sex, and the biomarker*-sex interaction; similarly, another multivariable regression model was constructed including the biomarker, age, and biomarker*age interaction. In these models, a statistically significant result for the interaction term would suggest the strength of the relationship between the biomarker and eGFR/htTKV varies according to sex or age, respectively. We acknowledge that the sample size is relatively small for testing statistical interactions. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). *P* values <0.05 were considered statistically significant.

Because there may be differences in the association between individual biomarkers and disease severity as a function of specific patient characteristics, such as sex and age, regression analyses were performed on the overall data to adjust for, and test interactions by, sex and age.

Results

Demographics and Clinical Characteristics

The demographic and clinical characteristics of the TAME-PKD study participants at the baseline visit are shown in Table 1. As expected on the basis of the eligibility criteria for the study participation (12), these patients were relatively young, with a median age of approximately 42 years (range, 20–60 years), and they had preserved eGFR (median, 87 ml/min per 1.73 m^2; range, 45–127 ml/min per 1.73 m^2). The TAME-PKD population was mostly female (73%), predominantly White (95%), and 92% were not Hispanic or Latino. Other clinical characteristics of the cross-section of patients with ADPKD and CKD stages 1–3a enrolled in the TAME-PKD study are shown in Table 1 (12).

Distribution of Baseline Disease Parameters and Urinary Biomarker Measurements

To compare with eGFR, as calculated by the Chronic Kidney Disease Epidemiology Collaboration equation (13), and htTKV (14) (Table 1), the distributions of mean and median values for the various baseline urinary biomarkers measured in this study are shown in Table 2. These biomarkers include the urine creatinine concentration and osmolality for each sample, used for normalization of the other urinary biomarkers across different samples, along with the concentrations of total protein, key metabolites (lactate, pyruvate, succinate, and cAMP), and key glycolytic enzymes (PKM2, PDK1, and LDHA) (7), which were assayed as described in the *Materials and Methods*. In general, there is a high degree of variability in these parameters across the patient samples in this study population (Table 2). There was a strong, albeit scattered, negative association between htTKV and eGFR for the patients at baseline in this study ($r=-0.39$, $P=0.0001$) (Figure 2), consistent with what has been previously reported in larger study populations and thus suggests similarity of our patient population to those previously reported (15).

Normalization Considerations

Regarding the use of urinary creatinine and osmolality as normalization factors for the measured biomarkers, we observed a strong, positive correlation between these measured parameters ($r=0.78$, $P<0.0001$), as shown in Figure 3. This robust correlation suggests that both the measured osmolality and the creatinine concentration may reasonably serve as normalization factors to apply to these spot urine sample measurements to facilitate comparisons across

Table 1. Demographic and clinical characteristics of TAME-PKD participants (*N*=95)

Characteristics	Median (range) or *N* (%)
Demographics	
Age at screening (yr)	43 (20–60)
Sex	
Male	26 (27)
Female	69 (73)
Race	
American Indian/Alaska Native	1 (1)
Asian	3 (3)
Black	1 (1)
White	90 (95)
Ethnicity	
Hispanic or Latino	8 (8)
Not Hispanic or Latino	87 (92)
Clinical characteristics	
eGFR calculated per CKD-EPI (ml/min per 1.73 m^2)	87.3 (44.7–126.9)
Height-adjusted total kidney volume (ml/m^2)	609.9 (197.9–2506.0)
Age at time of PKD diagnosis (yr)	29 (0–57)
Vitamin B12 (pg/ml)	432 (251–2000)
Glucose (mg/dl)	89 (66–123)
Hemoglobin A1C (%)	5.2 (4.5–6.2)
Serum creatinine (mg/dl)	0.85 (0.56–1.55)
Systolic BP (mm Hg) (at screening)	122 (81–167)
Diastolic BP (mm Hg) (at screening)	76 (55–97)
BMI (at screening)	25.8 (18.5–44.7)
Diagnosis due to	
Screening (family history)	26 (27)
Incidental Imaging	21 (22)
Pain	22 (23)
Hypertension	9 (10)
Routine Physical	6 (6)
Hematuria	5 (5)
UTI	6 (6)
Family history of ADPKD	71 (75)

Baseline data for one patient was missing for height-adjusted total kidney volume. CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; PKD, polycystic kidney disease; BMI, body mass index; UTI, urinary tract infection; ADPKD, autosomal dominant polycystic kidney disease.

Table 2. ADPKD severity markers and distribution of urinary biomarkers

Analytes	*N*	Mean±SD	Median (range)
Creatinine (μM)	95	1086.5±844.0	818.2 (141.8–3771.1)
Osmolality (mmol/kg)	95	399.0±219.7	374 (91–1098)
Protein (mg/dl)	95	1.65±1.48	1.19 (0.09–7.87)
Protein/creatinine ratio	95	0.0018±0.0015	0.0014 (0.0001–0.0118)
Protein/osmolality ratio	95	0.0045±0.0046	0.0034 (0.0007–0.0366)
Lactate (μM)	95	13.3±12.3	10.4 (0.4–67.9)
Lactate/creatinine ratio	95	0.018±0.022	0.011 (0.001–0.133)
Lactate/osmolality ratio	95	0.039±0.038	0.029 (0.002–0.177)
Pyruvate (μM)	95	9.32±4.85	8.56 (2.04–27.17)
Pyruvate/creatinine ratio	95	0.013±0.011	0.012 (0.001–0.071)
Pyruvate/osmolality ratio	95	0.029±0.016	0.025 (0.004–0.096)
Lactate/pyruvate ratio	95	1.64±1.80	1.19 (0.04–11.10)
Succinate (μM)[a]	94	41.8±24.5	36.6 (8.8–128.9)
Succinate/creatinine ratio	94	0.062±0.060	0.042 (0.008–0.376)
Succinate/osmolality ratio	94	0.131±0.099	0.103 (0.031–0.656)
cAMP (μM)	95	1.67±0.77	1.57 (0.02–3.99)
cAMP/creatinine ratio	95	0.0022±0.0013	0.0017 (0.0000–0.0071)
cAMP/osmolality ratio	95	0.0048±0.0024	0.0043 (0.0001–0.0138)
PKM2 (pg/ml)	95	7819±6435	5795 (6–27,542)
PKM2/creatinine ratio	95	8.39±7.33	6.47 (0.01–43.30)
PKM2/osmolality ratio	95	20.47±18.33	15.87 (0.01–134.50)
PDK1 (pg/ml)	95	178.0±97.8	152.2 (120.8–851.3)
PDK1/creatinine ratio	95	0.271±0.210	0.208 (0.043–1.177)
PDK1/osmolality ratio	95	0.621±0.552	0.474 (0.149–4.627)
LDHA (pg/ml)[a]	94	3230±4416	1661 (13–28,763)
LDHA/creatinine ratio	94	3.26±3.42	2.22 (0.03–24.21)
LDHA/osmolality ratio	94	8.23±9.55	5.16 (0.06–55.31)

ADPKD, autosomal dominant polycystic kidney disease; PKM2, pyruvate kinase M2; PDK1, pyruvate dehydrogenase kinase 1; LDHA, lactate dehydrogenase A.
[a]Succinate and LDHA values were below the lower limit of detection for one patient each.

samples from different patients, and, in the future, across longitudinal samples obtained from the same patient. The purpose of this study was to identify potential biomarker associations with disease severity and generate additional hypotheses to be tested. Accordingly, because it is unclear whether creatinine concentration versus osmolality should

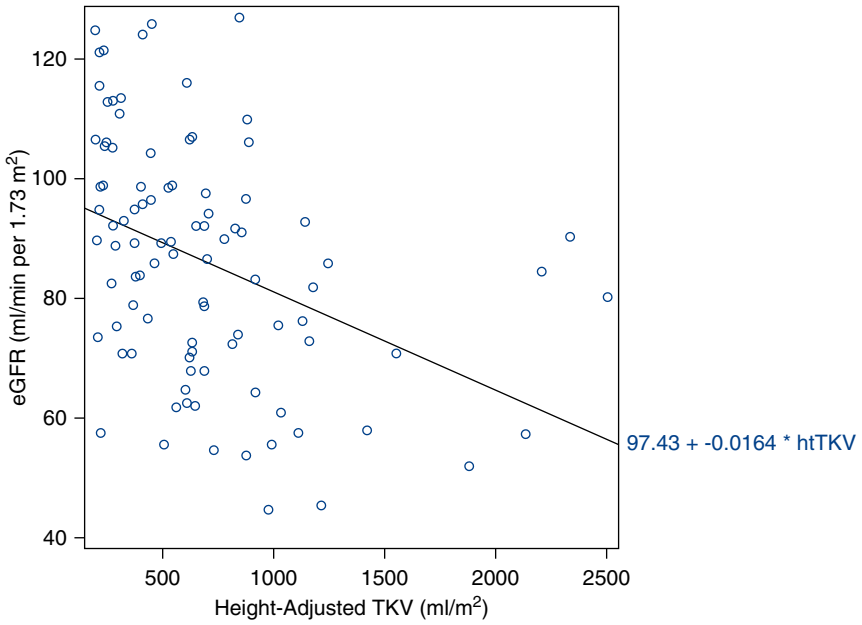

Figure 2. | Scatterplot of height-adjusted total kidney volume (htTKV) versus eGFR shows negative correlation in the baseline TAME-PKD study population. Least-squares linear regression fit of the data (solid line) and statistical parameters are shown, with Pearson correlation. $r=-0.39$; $P=0.0001$.

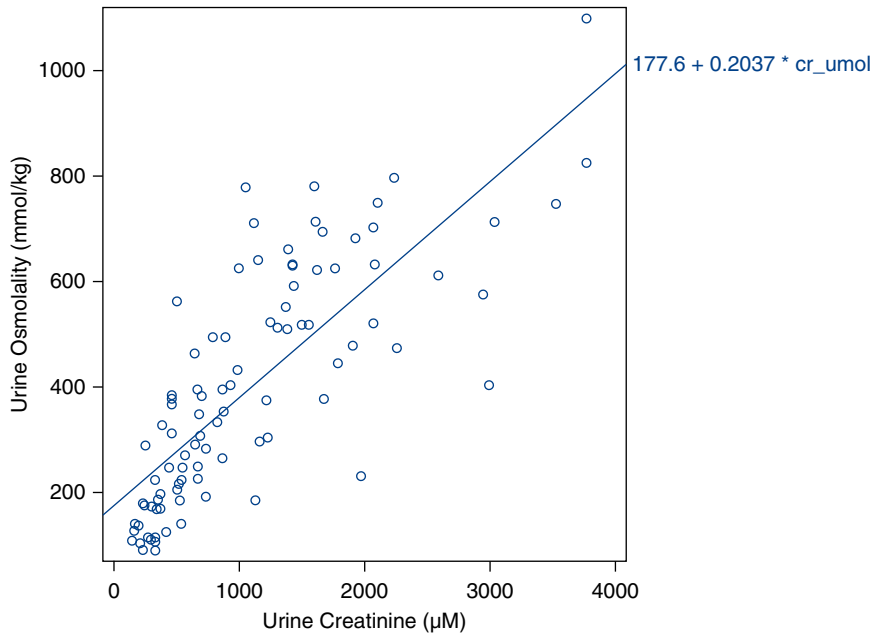

Figure 3. | Scatterplot of urine osmolality (U_{osm}) versus urine creatinine concentration (U_{Cr}) demonstrates strong positive correlation. Least-squares linear regression fit of the data (solid line) and statistical parameters are shown, with Pearson correlation. $r=0.78$; $P<0.0001$.

be favored, we have reported values normalized by both parameters in our analysis.

Association of Urinary Biomarkers with eGFR

Tables 3 and 4 show the relationship between normalized urinary biomarkers and eGFR in the baseline samples. Urinary total protein excretion negatively correlated with eGFR, both when normalized to urine osmolality ($r=-0.30$, $P=0.004$) and when normalized to urine creatinine ($r=-0.21$, $P=0.04$). Urinary excretion of the glycolytic enzyme PKM2 normalized to osmolality also negatively correlated with eGFR ($r=-0.26$, $P=0.009$). Of note, there was also a positive correlation between eGFR and urinary cAMP normalized to creatinine ($r=0.25$, $P=0.02$).

Association of Urinary Biomarkers with htTKV

Tables 5 and 6 show the relationship between normalized urinary biomarkers and htTKV in the TAME-PKD baseline samples. Consistent with the above described relationship with eGFR, there was a trend toward a positive correlation between htTKV and urinary total protein excretion normalized by either creatinine ($r=0.20$, $P=0.06$) or osmolality ($r=0.19$, $P=0.07$). Although none of the measured metabolites correlated significantly with htTKV, urinary PKM2 and LDHA enzyme levels, normalized by either creatinine or osmolality, positively correlated with htTKV in the baseline samples. Specifically, significant Pearson correlations were observed for the PKM2/creatinine ratio ($r=0.28$, $P=0.006$), the PKM2/osmolality ratio ($r=0.22$, $P=0.03$), the LDHA/creatinine ratio ($r=0.23$, $P=0.03$), and the LDHA/osmolality ratio ($r=0.22$, $P=0.04$).

Regression Analyses to Adjust for, and Test Interactions by, Sex and Age

Tables 3 and 4 present relationships for the samples between eGFR and each biomarker of interest while adjusting for patient sex and age, respectively. The overall significance of the above described correlations in normalized total protein, PKM2, and cAMP levels with eGFR remained similar in linear regression analyses adjusted for sex (Table 3). Of note, the metabolites pyruvate and succinate, normalized to creatinine, had significant positive correlations with eGFR in males only ($r=0.50$, $P=0.009$ and $r=0.48$, $P=0.01$, respectively). Conversely, the glycolytic enzyme LDHA, normalized to osmolality, had a significant negative correlation with eGFR in males only ($r=-0.53$, $P=0.006$). Correspondingly, tests of interaction suggest that the strength of the relationships between pyruvate, succinate, and LDHA with eGFR differ significantly for male versus female patients, with stronger associations in males (Table 3, right). In addition, after adjustment for age, there was a significant negative correlation between urinary LDHA/osmolality and eGFR ($P=0.04$; Table 4, right), which was apparent only in older patients (≥43 years; $r=-0.36$, $P=0.01$). Of note, testing for interaction by age, there is evidence that the strength of the association between succinate excretion and eGFR differs significantly depending on age, with a stronger association in younger versus older patients with ADPKD ($P=0.01$ and 0.03 when normalized by creatinine and osmolality, respectively; Table 4, right). Finally, there was a greater correlation between protein/osmolality ratio and eGFR in patients <43 years old ($r=-0.43$, $P=0.003$); testing for interaction between protein excretion and eGFR suggested this relationship also differs significantly depending on the patient's age ($P=0.006$; Table 4, right).

Table 3. Relationship between normalized biomarkers and eGFR, overall and by sex

Analytes	Pearson Correlation			*P* Value Adjusted for Sex[a]	*P* Value Testing for Interaction[b]
	All Patients (*N*=95)	Males Only (*N*=26)	Females Only (*N*=69)		
Creatinine (μM)	−0.07	−0.40[c,d]	0.08	0.49	0.04[d]
Osmolality (mmol/kg)	0.05	−0.10	0.10	0.64	0.40
Protein/creatinine ratio	−0.21[c,d]	−0.04	−0.25[c,d]	0.04[d]	0.65
Protein/osmolality ratio	−0.30[d,e]	−0.51[d,e]	−0.25[c,d]	0.004[d]	0.08
Lactate/creatinine ratio	0.12	0.14	0.13	0.22	0.69
Lactate/osmolality ratio	0.07	−0.01	0.10	0.46	0.70
Pyruvate/creatinine ratio	0.17	0.50[d,e]	0.10	0.10	0.03[d]
Pyruvate/osmolality ratio	0.14	0.26	0.12	0.18	0.34
Lactate/pyruvate ratio	0.03	0.05	0.02	0.79	0.91
Succinate/creatinine ratio	0.12	0.48[c,d]	0.08	0.23	0.03[d]
Succinate/osmolality ratio	0.11	0.09	0.14	0.23	0.84
cAMP/creatinine ratio	0.25[c,d]	0.43[c,d]	0.19	0.01[d]	0.31
cAMP/osmolality ratio	0.16	0.10	0.18	0.12	0.96
PKM2/creatinine ratio	−0.15	−0.10	−0.16	0.16	0.84
PKM2/osmolality ratio	−0.26[c,d]	−0.46[c,d]	−0.24	0.01[d]	0.09
PDK1/creatinine ratio	0.12	0.24	0.09	0.22	0.45
PDK1/osmolality ratio	−0.08	−0.23	−0.05	0.47	0.33
LDHA/creatinine ratio	−0.09	−0.12	−0.09	0.39	0.65
LDHA/osmolality ratio	−0.14	−0.53[d,e]	−0.09	0.21	0.01[d]

PKM2, pyruvate kinase M2; PDK1, pyruvate dehydrogenase kinase 1; LDHA, lactate dehydrogenase A.
[a]*P* value from multivariable regression analysis testing association between each biomarker and eGFR while adjusting for sex.
[b]*P* value formally testing interaction by sex: significant *P* value indicates there is evidence that the strength of the relationship between a biomarker and eGFR differs significantly for male versus female patients.
[c]Denotes Pearson correlation with $P<0.05$.
[d]Denotes significant *P* value.
[e]Denotes Pearson correlation with $P<0.01$.

Table 4. Relationship between normalized biomarkers and eGFR, overall and by age

Analytes	Pearson Correlation			*P* Value Adjusted for Age[a]	*P* Value Testing for Interaction[b]
	All Patients (*N*=95)	Age <43 (*N*=48)	Age ≥43 (*N*=47)		
Creatinine (μM)	−0.07	−0.12	−0.03	0.20	0.99
Osmolality (mmol/kg)	0.05	0.09	0.02	0.73	0.98
Protein/creatinine ratio	−0.21[c,d]	−0.21	−0.25	0.04[d]	0.15
Protein/osmolality ratio	−0.30[d,e]	−0.43[d,e]	−0.27	0.001[d]	0.006[d]
Lactate/creatinine ratio	0.12	0.16	0.05	0.25	0.48
Lactate/osmolality ratio	0.07	0.13	−0.009	0.58	0.33
Pyruvate/creatinine ratio	0.17	0.20	0.13	0.07	0.36
Pyruvate/osmolality ratio	0.14	0.11	0.10	0.35	0.51
Lactate/pyruvate ratio	0.03	0.07	0.05	0.63	0.92
Succinate/creatinine ratio	0.12	0.29	0.10	0.08	0.01[d]
Succinate/osmolality ratio	0.11	0.16	0.09	0.21	0.03[d]
cAMP/creatinine ratio	0.25[c,d]	0.33[c,d]	0.24	0.002[d]	0.17
cAMP/osmolality ratio	0.16	0.17	0.13	0.14	0.36
PKM2/creatinine ratio	−0.15	−0.08	−0.23	0.29	0.85
PKM2/osmolality ratio	−0.26[c,d]	−0.29	−0.26	0.02[d]	0.15
PDK1/creatinine ratio	0.12	0.21	0.06	0.12	0.47
PDK1/osmolality ratio	−0.08	−0.04	−0.11	0.54	0.97
LDHA/creatinine ratio	−0.09	0.003	−0.31[c,d]	0.17	0.09
LDHA/osmolality ratio	−0.14	−0.04	−0.36[c,d]	0.04[d]	0.09

PKM2, pyruvate kinase M2; PDK1, pyruvate dehydrogenase kinase 1; LDHA, lactate dehydrogenase A.
[a]*P* value from multivariable regression analysis testing association between each biomarker and eGFR while adjusting for age.
[b]*P* value formally testing interaction by age: significant *P* value indicates there is evidence that the strength of the relationship between a biomarker and eGFR differs significantly depending on the patient's age.
[c]Denotes Pearson correlation with $P<0.05$.
[d]Denotes significant *P* value.
[e]Denotes Pearson correlation with $P<0.01$.

Table 5. Relationship between normalized biomarkers and htTKV, overall and by sex

Analytes	Pearson Correlation			*P* Value Adjusted for Sex[a]	*P* Value Testing for Interaction[b]
	All Patients (*N*=95)	Males Only (*N*=26)	Females Only (*N*=69)		
Creatinine (μM)	0.12	0.24	0.04	0.35	0.49
Osmolality (mmol/kg)	0.06	0.05	0.03	0.73	0.93
Protein/creatinine ratio	0.20	0.04	0.24[c,d]	0.05[d]	0.66
Protein/osmolality ratio	0.19	0.33	0.16	0.07	0.33
Lactate/creatinine ratio	0.13	0.03	0.18	0.14	0.78
Lactate/osmolality ratio	0.11	0.12	0.15	0.18	>0.99
Pyruvate/creatinine ratio	−0.01	−0.38	0.09	0.97	0.05
Pyruvate/osmolality ratio	−0.01	−0.05	0.02	0.96	0.81
Lactate/pyruvate ratio	0.08	0.09	0.10	0.38	0.88
Succinate/creatinine ratio	−0.08	−0.32	−0.02	0.62	0.13
Succinate/osmolality ratio	0.01	0.08	0.05	0.62	0.77
cAMP/creatinine ratio	−0.11	−0.41[c,d]	0.02	0.38	0.09
cAMP/osmolality ratio	−0.08	−0.05	−0.07	0.53	0.97
PKM2/creatinine ratio	0.28[d,e]	0.06	0.36[d,e]	0.002[d]	0.66
PKM2/osmolality ratio	0.22[c,d]	0.37	0.22	0.02[d]	0.27
PDK1/creatinine ratio	−0.14	−0.33	−0.06	0.25	0.26
PDK1/osmolality ratio	−0.05	−0.04	−0.04	0.71	0.93
LDHA/creatinine ratio	0.23[c,d]	0.09	0.29[c,d]	0.01[d]	0.97
LDHA/osmolality ratio	0.22[c,d]	0.39[c,d]	0.22	0.03[d]	0.16

htTKV, height-adjusted total kidney volume; PKM2, pyruvate kinase M2; PDK1, pyruvate dehydrogenase kinase 1; LDHA, lactate dehydrogenase A.
[a]*P* value from multivariable regression analysis testing association between each biomarker and htTKV while adjusting for sex.
[b]*P* value formally testing interaction by sex: significant *P* value indicates there is evidence that the strength of the relationship between a biomarker and htTKV differs significantly for male versus female patients.
[c]Denotes Pearson correlation with $P<0.05$.
[d]Denotes significant *P* value.
[e]Denotes Pearson correlation with $P<0.01$.

Table 6. Relationship between normalized biomarkers and htTKV, overall and by age

Analytes	Pearson Correlation			*P* Value Adjusted for Age[a]	*P* Value Testing for Interaction[b]
	All Patients (*N*=95)	Age <43 (*N*=48)	Age ≥43 (*N*=47)		
Creatinine (μM)	0.12	0.09	0.15	0.25	0.74
Osmolality (mmol/kg)	0.06	0.10	0.005	0.59	0.49
Protein/creatinine ratio	0.20	0.43[d,e]	0.05	0.06	0.03[d]
Protein/osmolality ratio	0.19	0.32[c,d]	0.12	0.07	0.11
Lactate/creatinine ratio	0.13	0.10	0.16	0.23	0.41
Lactate/osmolality ratio	0.11	−0.05	0.27	0.27	0.14
Pyruvate/creatinine ratio	−0.01	0.17	−0.19	0.96	0.98
Pyruvate/osmolality ratio	−0.01	0.06	−0.07	0.99	0.20
Lactate/pyruvate ratio	0.08	−0.02	0.17	0.45	0.99
Succinate/creatinine ratio	−0.08	−0.25	−0.02	0.41	0.68
Succinate/osmolality ratio	0.01	−0.24	0.18	0.92	0.13
cAMP/creatinine ratio	−0.11	−0.03	−0.17	0.29	0.87
cAMP/osmolality ratio	−0.08	−0.17	−0.004	0.43	0.22
PKM2/creatinine ratio	0.28[d,e]	0.46[d,e]	0.11	0.006[d]	0.10
PKM2/osmolality ratio	0.22[c,d]	0.41[d,e]	0.12	0.03[d]	0.10
PDK1/creatinine ratio	−0.14	−0.12	−0.15	0.19	0.98
PDK1/osmolality ratio	−0.05	−0.11	−0.03	0.61	0.56
LDHA/creatinine ratio	0.23[c,d]	0.23	0.24	0.02[d]	0.32
LDHA/osmolality ratio	0.22[c,d]	0.16	0.30[c,d]	0.04[d]	0.13

htTKV, height-adjusted total kidney volume; PKM2, pyruvate kinase M2; PDK1, pyruvate dehydrogenase kinase 1; LDHA, lactate dehydrogenase A.
[a]*P* value from multivariable regression analysis testing association between each biomarker and htTKV while adjusting for age.
[b]*P* value formally testing interaction by age: significant *P* value indicates there is evidence that the strength of the relationship between a biomarker and htTKV differs significantly depending on the patient's age.
[c]Denotes Pearson correlation with $P<0.05$.
[d]Denotes significant *P* value.
[e]Denotes Pearson correlation with $P<0.01$.

Tables 5 and 6 present relationships for the samples between each biomarker of interest and htTKV while adjusting for patient sex and age, respectively. The overall significance of the above-described correlations in normalized total protein, PKM2, and LDHA levels with htTKV remained after adjusting for sex (Table 5). Of note, adjustments for age strengthened the positive correlations between the protein/creatinine and protein/osmolality ratios and htTKV in patients <43 years old ($r=0.43$, $P=0.004$ and $r=0.32$, $P=0.03$, respectively) (Table 6). Correspondingly, there was a strengthening of the relationship between the protein/creatinine ratio and htTKV depending on patient age (P testing for interaction=0.03; Table 6, right).

Additional Regression and Subgroup Analyses

Adjusting the results obtained for both patient age and sex simultaneously in a multivariable analysis did not qualitatively affect the above conclusions obtained when analyzing results adjusted for age and sex separately (see Supplemental Table 1).

The two surrogates used in this study for ADPKD disease severity, eGFR and htTKV, represent two distinct facets of disease progression and severity, with cyst development and overall increases in kidney size generally preceding losses of eGFR early in the disease course (1). Thus, an additional analysis of the baseline biomarkers of TAME-PKD patients that had relatively preserved eGFR (≥90 ml/min; $n=41$) was performed to determine potential associations with changes in htTKV in this subgroup. We reasoned that this analysis might elucidate biomarkers that are more sensitive in identifying patients with cystic kidney enlargement in early disease. First, we found the positive correlation of protein/creatinine ratio with htTKV was strengthened in patients with preserved eGFR ($r=0.41$, $P=0.007$), an effect that was pronounced in female patients ($r=0.56$, $P=0.003$; see Supplemental Table 2). Second, lactate and pyruvate excretion normalized to creatinine positively correlated with htTKV ($r=0.36$, $P=0.02$ and $r=0.32$, $P=0.04$, respectively) in patients with preserved eGFR. The positive lactate/creatinine correlation was significant when adjusted independently for sex ($P=0.007$) and age ($P=0.02$) (Supplemental Tables 2 and 3). Third, and most notably, PKM2 excretion had a greatly strengthened positive correlation with htTKV in patients with preserved eGFR when normalized to either creatinine ($r=0.60$, $P<0.001$) or osmolality ($r=0.42$, $P=0.006$), and the PKM2 excretion correlation with htTKV in this subgroup had more significant P values when adjusted independently for both sex and patient age.

Discussion

The variable course of ADPKD and the emerging availability of therapies highlight the importance of risk stratification and the identification of new biomarkers that may inform disease severity and the risk of disease progression. In this study, we analyzed the extent to which targeted urinary biomarkers correlate with eGFR and htTKV in the TAME-PKD patient population with relatively early ADPKD at baseline. In general, we consider a biomarker to be associated with ADPKD disease severity if it positively correlates with htTKV and negatively correlates with eGFR, our surrogate disease markers. Our goal was to help define additional surrogate markers for disease severity on the basis of the emerging notion that metabolic dysregulation is an underlying driver of disease severity and progression (16). This report will serve as the basis for a subsequent longitudinal analysis of how these biomarkers may change over time with disease progression and in response to metformin treatment.

Consistent with previous studies derived from the CRISP cohort (15,17), there was a highly significant, albeit scattered, negative correlation between eGFR and htTKV (Figure 2). Another confirmatory finding of this study is that proteinuria in this population correlates with ADPKD severity, as assessed by decreasing eGFR and increasing htTKV in this population with mild-to-moderate disease (Tables 3–6), as previously reported (2,17,18). Of note, the association between protein excretion and htTKV was strengthened in the subgroup of patients with preserved eGFR (≥90 ml/min; Supplemental Tables 2 and 3). Although severe proteinuria is relatively uncommon in ADPKD, it is a significant predictor of disease progression (19), so these findings reinforce the notion that proteinuria, along with htTKV, is a useful predictive biomarker in early ADPKD.

Secondly, urinary excretion of the key glycolytic enzyme PKM2, a marker for the excessive aerobic glycolysis previously shown to be elevated in ADPKD preclinical models and in human ADPKD cystic kidney tissue (7), was both negatively correlated with eGFR and positively correlated with htTKV in our study. This positive correlation of PKM2 excretion with htTKV was strengthened in patients with preserved eGFR (Supplemental Tables 2 and 3), suggesting PKM2 is an informative biomarker for kidney size/growth in patients with ADPKD before any perceptible loss of kidney function. Similarly, urinary excretion of LDHA, another key enzyme marker for excessive glycolytic flux, was positively associated with htTKV in the overall analysis. However, unlike PKM2, the association of LDHA with htTKV was not observed in the subgroup of patients with preserved eGFR. Overall, these findings support the notion that increasing ADPKD severity in the TAME-PKD population is associated with a metabolic shift toward increased aerobic glycolysis, the so-called Warburg effect. An alternative interpretation is suggested by data reporting that PKM2 can be a biomarker for AKI (see Cheon *et al.* [20]). Urinary LDH has also been used as a marker of kidney injury and may differentiate between upper- and lower-tract urinary infection (see Sun *et al.* [21]). It is thus conceivable that the correlations found here may represent evidence of cellular injury, more pronounced in patients with rapid cyst growth, and not evidence of increased reliance on aerobic glycolysis by cystic cells.

Although none of the measured metabolites were significantly correlated with either eGFR or htTKV in the overall analysis, lactate and pyruvate excretion normalized to creatinine did positively correlate with htTKV in patients with preserved eGFR (Supplemental Tables 2 and 3), suggesting that larger kidneys in early disease may have greater glycolytic flux and thus production of these glycolytic end products. Moreover, excretion of succinate, a TCA cycle intermediate important for oxidative metabolism, was positively correlated with eGFR in male patients and in younger patients (Tables 3 and 4). These findings are consistent with

the idea that greater oxidative metabolic flux may be present in kidney epithelial cells from younger male patients with ADPKD who have preserved renal function. However, because of the relatively small sample size for males (N=26) in this population, these findings should be verified in a larger study population. Because male sex is generally associated with more severe ADPKD and because significant reductions in eGFR, especially when seen in younger patients, are indicative of a more rapidly progressive disease phenotype (19), these findings raise the possibility that reductions in urinary excretion of the TCA cycle intermediate succinate might be associated with more rapidly progressive disease. The above hypotheses may be further tested once acquisition and analysis of the longitudinal data is complete for the TAME-PKD study.

It may appear surprising, at first glance, that a modest positive correlation was detected between cAMP excretion and eGFR, because excessive cAMP generation in ADPKD cystic epithelial cells is a principal feature underlying ADPKD disease pathogenesis (6). One conceivable explanation for the observed correlation between eGFR and urinary cAMP may relate to the concept that, although cAMP production is expected to increase as ADPKD progresses, patients with more advanced disease (and thus lower eGFR) may also have a higher proportion of cysts that are walled off and no longer communicate with the sampled urine. If this notion is correct, one would expect to observe that fluid sampled directly from cysts would contain higher cAMP levels, and potentially other disease-associated biomarkers, in patients with more severe disease/lower eGFR. This seemingly paradoxic result thus highlights a caveat for sampling urine for biomarkers in ADPKD. Although easy to obtain and convenient, sampled urine from patients with ADPKD may predominantly reflect biomarkers produced from areas of the kidneys that are relatively less affected (or cystic) and may, therefore, be more informative for biomarkers that are important earlier in the underlying disease process.

The power to detect or discern effects on the basis of sex was limited in this study because of the relatively small proportion of males in the TAME-PKD population. The ability to predict disease severity (eGFR or htTKV) on the basis of the measured value of any of our baseline biomarkers is limited due to scatter in the data. The extent to which this variability in urinary biomarkers represents intrinsic differences across patients in the study population versus other temporal or environmental factors or differences (*e.g.*, diet) is unclear. Future longitudinal analyses of the biomarkers in this study population, over 24 months of the trial, should help to address this question. Of note, a recent study by Dekker *et al.* (22) investigating targeted urinary metabolites in patients with ADPKD reported that longitudinal changes in alanine/citrate ratio associated significantly with future annual change in eGFR. However, this association was weak (R^2=0.15), thus limiting its predictive utility. Moreover, Dekker *et al.* did not measure levels of any urinary metabolic enzymes, which, as shown herein, appear to associate more robustly with disease severity among metabolic biomarkers.

Follow-up analyses will be performed, when the TAME-PKD study data collection is complete, to determine whether and the extent to which longitudinal values of any of these biomarkers may track with treatment status (metformin versus placebo), disease progression, and the potential response to therapy with metformin. Apart from the ability to see whether a particular biomarker at baseline is related to disease severity and progression, we will also be able to estimate cross-sectional and longitudinal effects on htTKV and eGFR among untreated participants.

In summary, our findings at baseline in the TAME-PKD study population support the idea that metabolic changes involving increased glycolytic flux may be characteristic of ADPKD and may track with disease severity in patients with mild-to-moderate disease.

Disclosures

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Author Contributions

K.Z. Abebe and A.D. Althouse were responsible for formal analysis; K.Z. Abebe, A.D. Althouse, H. Li, and B. Saitta were responsible for data curation; K.Z. Abebe, K.T. Bae, K.R. Hallows, R.D. Perrone, S.L. Seliger, and T.J. Watnick conceptualized the study and were responsible for project administration; K.T. Bae was responsible for funding acquisition; K.T. Bae, K.R. Hallows, H. Li, D.C. Miskulin, R.D. Perrone, B. Saitta, S.L. Seliger, and T.J. Watnick were responsible for investigation; K.R. Hallows wrote the original draft; K.R. Hallows, H. Li, R.D. Perrone, B. Saitta, S.L. Seliger, and T.J. Watnick were responsible for methodology; H. Li was responsible for visualization; and all authors reviewed and edited the manuscript.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0005962020/-/DCSupplemental.

Supplemental Table 1. Relationship between biomarkers and eGFR/htTKV adjusted for both age and sex.

Supplemental Table 2. Relationship between normalized biomarkers and htTKV in participants with eGFR ≥90: Overall and by sex.

Supplemental Table 3. Relationship between normalized biomarkers and htTKV in participants with eGFR ≥90: Overall and by age.

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