Change in Urinary Myoinositol/Citrate Ratio Associates with Progressive Loss of Renal Function in ADPKD Patients

Shosha E.I. Dekker\textsuperscript{a} Aswin Verhoeven\textsuperscript{b} Daria Frey\textsuperscript{c,d} Darius Soonawala\textsuperscript{a,e} Dorien J.M. Peters\textsuperscript{f} Oleg A. Mayboroda\textsuperscript{b} Johan W. de Fijter\textsuperscript{a} on behalf of the DIPAK Consortium

\textsuperscript{a}Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands; \textsuperscript{b}Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands; \textsuperscript{c}Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands; \textsuperscript{d}Laboratory of Clinical Metabolomics, Tomsk State University, Tomsk, Russia; \textsuperscript{f}Department of Internal Medicine, Haga Teaching Hospital, The Hague, The Netherlands; \textsuperscript{f}Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

Keywords
Urine metabolites · Autosomal dominant polycystic kidney disease · Progression · Estimated glomerular filtration rate slope · Biomarker

Abstract
Introduction: In autosomal dominant polycystic kidney disease (ADPKD) patients, predicting renal disease progression is important to make a prognosis and to support the clinical decision whether to initiate renoprotective therapy. Conventional markers all have their limitations. Metabolic profiling is a promising strategy for risk stratification. We determined the prognostic performance to identify patients with a fast progressive disease course and evaluated time-dependent changes in urinary metabolites. Methods: Targeted, quantitative metabolomics analysis ($^1$H NMR-spectroscopy) was performed on spot urine samples at two time points, baseline ($n = 324$, 61% female; mean age 45 years, SD 11; median eGFR 61 mL/min/1.73 m$^2$, IQR 42–88; mean years of creatinine follow-up 3.7, SD 1.3) and a sample obtained after 3 years of follow-up ($n = 112$). Patients were stratified by their eGFR slope into fast and slow progressors based on an annualized change of $> -3.0$ or $\leq -3.0$ mL/min/1.73 m$^2$/year, respectively. Fifty-five urinary metabolites and ratios were quantified, and the significant ones were selected. Logistic regression was used to determine prognostic performance in identifying those with a fast progressive course using baseline urine samples. Repeated-measures ANOVA was used to analyze whether changes in urinary metabolites over a 3-year follow-up period differed between fast and slow progressors. Results: In a single urinary sample, the prognostic performance of urinary metabolites was comparable to that of a model including height-adjusted total kidney volume (htTKV, AUC = 0.67). Combined with htTKV, the predictive value of the metabolite model increased (AUC = 0.75). Longitudinal analyses showed an increase in the myo-
in patients with fast progressing disease course. © 2022 The Author(s). Published by S. Karger AG, Basel

**Introduction**

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited renal disease [1, 2]. The disease is characterized by progressive growth of multiple bilateral renal cysts over time, resulting in loss of functioning nephrons and decline in glomerular filtration rate. ADPKD frequently leads to end-stage renal disease (ESRD), but progression is associated with high inter- and intrafamilial variability in disease course [3, 4]. The advent of a disease-modifying treatment for ADPKD [5] has emphasized the need for early risk stratification. Identifying patients at high risk of fast progression is important because these patients may benefit most from treatment [6]. Conventional risk assessment strategies currently used to predict disease progression all have their limitations. Estimated glomerular filtration rate (eGFR) indexed for age is less sensitive in early-stage disease, when renal function remains preserved, because progressive cyst formation and loss of nephrons precede kidney function decline [7]. Gene type mutation (PKD1 vs. PKD2) [4, 8] and total kidney volume (TKV) measurement [9, 10] are more reliable, but assessment is laborious and expensive. Therefore, there is an unmet clinical need for alternative markers that associate with the rate of progression and that could eventually be used either alone or in combination with the conventional markers to support risk assessment in patients with ADPKD.

Cross-sectional analyses of urinary metabolites have been reported previously [11]. In the current study in patients with ADPKD (PKD1), the aim was twofold; first, to validate the prognostic performance of urinary metabolites measured at a single time point to identify patients with a fast progressing disease course and second, to assess whether there is a difference over time in the change in urinary metabolites between fast and slow progressors.

**Materials and Methods**

**Study Cohort**

Patients with chronic kidney disease (all ADPKD-related) were included from the DIPAK observational study. For the current longitudinal cohort study, we included patients with reliable sequential eGFR data, defined as having a follow-up time of at least 2 years with at least three sequential serum creatinine measurements. Furthermore, only patients with a confirmed PKD1 mutation were included to increase homogeneity. Subjects were excluded if the baseline (BL) urine sample was not available and/or if they used a vasopressin V2R-antagonist or somatostatin analog at BL or during follow-up.

The DIPAK observational study is an ongoing longitudinal multicenter study of more than 600 patients with ADPKD (Ravine criteria [12]) in the Netherlands, aged ≥18 years. Inclusion started in 2012 and patients will be followed for >6 years. Exclusion criteria included ESRD, renal replacement therapy, and medical conditions, other than ADPKD that negatively affect the natural course of ADPKD. Blood and urine samples were collected annually. Serum creatinine was measured with an enzymatic assay (IDMS-traceable method; Modular, Roche Diagnostics) at the relevant study center. GFR was estimated for each time point using the 2009 Chronic Kidney Disease Epidemiology equation [13]. Abdominal magnetic resonance imaging was performed at BL and every 3 years using a standardized protocol without the use of intravenous contrast. TKV was measured on magnetic resonance imaging-derived T2-weighted coronal images by an artificial multi-observer deep neural network model for fully automated segmentation [14]. TKV was adjusted for patients’ height (height-adjusted TKV [htTKV]). Genetic analysis was performed at BL using combined protocols [15–17].

**Renal Outcomes**

Individual eGFR slopes were calculated as absolute progression rate in mL/min/1.73 m²/year using linear regression slopes through serial eGFR measurements. These were expressed as annualized changes in eGFR. Patients were stratified into fast (eGFR slope greater than −3.0 mL/min/1.73 m²/year) and slow progressors (eGFR slope less than or equal to −3.0 mL/min/1.73 m²/year). This is in-line with the established cutoff to define fast progressing disease in ADPKD, although the field lacks a conclusive definition [18–26]. The ERA-EDTA risk assessment algorithm includes a definition for fast progressing disease based on a documented decline in eGFR of ≥5 mL/min/1.73 m² in 1 year (adopted from the KDIGO CKD Guideline [27]) and/or ≥2.5 mL/min/1.73 m²/year over a period of 5 years [6], which is comparable to the eGFR decline in class 1C patients of the Mayo classification [10].

**Urine Sample Collection and NMR Analysis**

We used spot urine samples that were obtained at BL and at year three for our analyses. Sampling, processing, and analysis using nuclear magnetic resonance (NMR) spectrometry were performed as previously reported [11]. In short, early morning-voided fasting urine samples were centrifuged directly after collection, and the aliquoted supernatant was stored at −80°C until analysis took place. Sampling of fresh urine to measure pH was not part of the protocol. For NMR analysis, urine samples were manually ordered in random order (study number and time point), thawed, transferred into 96 deep-well plates, and centrifuged at 1,550 g for
we were log transformed to meet the assumption of normality. The efficiency of the transformation was assessed by the Shapiro-Wilk test. For imputation of the missing htTKV values, a multivariate imputation by chained equations algorithm was used (R package mice 3.14.0).

For cross-sectional analyses, logistic regression analysis was performed with fast versus slow progressors as the dependent variable. The reported model description included the model coefficients and their standard errors, odds ratios, area under the curve (AUC), χ² statistics (Chisq), and its probability (Pr > Chisq). To address a possible confounding effect due to gender, all the models that are included in this report were compared with a gender corrected model using an ANOVA test. For all these models the H0 could not be rejected. Therefore, the simplest model (without gender) was used. Relevant clinical variables that were significantly different in univariate analysis were included as independent variables (BL eGFR, BL log htTKV) in multifactorial models. In addition, age was also included, because in combination with BL eGFR and/or htTKV it is an important risk factor for future disease progression; although in our cohort it was not found to be significantly associated with the univariate analysis.

For longitudinal analyses, to determine whether changes in urinary metabolites between a urine sample at BL and a subsequent sample after 3 years differed between fast and slow progressors, we used a repeated measure ANOVA analysis (a base R aov function). An individual model was built for each metabolite or metabolite ratio.

The quantification of each metabolite was performed by integration of its proton peaks in the NMR spectrum using the deconvolution fitting algorithm of Chenomx NMR Suite (9.0). The fitting was performed in semiautomatic mode, and the output was curated manually. To compensate for urine dilution differences, the data were normalized using probabilistic quotient normalization and/or htTKV it is an important risk factor for future disease progression; although in our cohort it was not found to be significantly associated with the univariate analysis.

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Results

Study Cohort and Descriptive Data of the Metabolites

BL characteristics are summarized in Table 1. We included 324 ADPKD patients, 61% female. The mean age was 45 years (SD 11) and the median eGFR was 61 mL/min/1.73 m² (IQR 42–88). The mean follow-up time was 3.7 years (SD 1.3) in which patients had a mean annual change in eGFR of −3.5 mL/min/1.73 m²/year (SD 3.0). Measurement of htTKV at BL was available for 283 patients. Median htTKV was 822 mL/m (IQR 512–1,305). The cohort was stratified into 193 fast progressors and 131 slow progressors as defined by the cutoff value for the eGFR slope of at least −3 mL/min/1.73 m²/year. As can be expected in fast progressors, their htTKV, urinary albumin-creatinine ratio, and blood pressure were higher, use of antihypertensive treatment more frequent, and eGFR lower as compared to those with a slow progressive course.

Thirty-eight urinary metabolites and seventeen physiologically relevant metabolite ratios were analyzed in this study (in total 55 variables). Descriptive data of the metabolite concentrations are summarized in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000524851).

Part 1: Urinary Metabolites Showed Prognostic Value for Distinguishing Fast from Slow Progressors Based on a Single Sample

Cross-sectional analyses were performed on BL samples from all patients included in this study (n = 324) to select the best predictors of progression when evaluated in a single urine sample. First, we made a univariate overview of the differences in the measured metabolites and metabolite ratios between fast (n = 193) and slow (n = 131) progressors. Figure 1 shows that, in agreement with our previous report [11], the alanine/citrate ratio is significantly higher in the group with fast progressive disease. Next, a logistic regression model was built for each variable separately, with fast and slow progressors as the dependent variable. Figure 2 summarizes the odds ratios and confidence intervals for all variables. To find the best subset of metabolite predictors for distinguishing fast from slow progressors, we used a model optimization routine and found that an optimal combination consists of two metabolites (betaine and phenylacetylglycine) and one metabolite ratio (alanine/citrate). Table 2 shows a numeric summary for the metabolite model built on the best subset of urinary metabolites, and models including conventional clinical predictors (age, BL eGFR, log HtTKV) to identify patients with fast progressive disease course. The metabolite model (AUC = 0.72, Chisq = 49.19, Pr > Chisq = <0.001) clearly outperformed the models built on age and BL eGFR in distinguishing fast from slow progressors. It showed better prognostic performance as compared with an imaging-based model (log htTKV, AUC = 0.67, Chisq = 26.37, Pr > Chisq = <0.001). When the urinary metabolite subset was combined with the imaging-based model, the fit of the model improved (AUC = 0.75, Chisq = 62.29, Pr > Chisq = <0.001). The model fit did not improve when BL eGFR (Table 2) and age were added. Nor did albuminuria perform better than a random model (data not shown). Figure 3 shows the receiver operating characteristic curves for the models presented in Table 2. In online supplementary Figure S1 summarizes the dif-
Fig. 2. Forest plot of all quantified metabolites and metabolite ratios ($n = 55$). This plot represents a summary of the logistic regression models for each BL metabolite and metabolite ratio. In the models, fast ($n = 193$) and slow ($n = 131$) progressors were the dependent variables. The side color bar shows the degree of significance. Note: Fast and slow progressors were stratified based on an annualized change in eGFR of $>1.73$ m$^2$/year, respectively.
ferences in individual predictors between fast and slow progressors for the most optimal composite (metabolites and the imaging data) model.

Part 2: Changes in Urinary Metabolites over Time Associated with Fast Progressing Disease

For the longitudinal analysis, we included patients who supplied both a BL and subsequent urine sample after 3 years of follow-up: \( n = 112 \) (fast progressors, \( n = 56 \); slow progressors \( n = 56 \)). In this cohort 55% were female, mean age 44 years (SD 13), median BL eGFR 71 mL/min/1.73 m\(^2\) (IQR 44–93), median BL htTKV 744 mL/m\(^2\) (480–1,176). To evaluate whether there is a difference over time in the change in urinary metabolites between the fast and slow progressors over a 3-year follow-up period, we used, for every metabolite and metabolite ratio, an individual two factor (time and progression) repeated measure ANOVA model. Values were log transformed to comply with the modeling method assumptions. Using \( F \) factor and \( p \) value of the ANOVA models as selection criteria, we found that only a single metabolite ratio namely myoinositol/citrate passed a significance cutoff on the interaction term (\( F = 3.6, p = 0.05 \)). Figure 4 gives a visual summary of the difference in this urinary metabolite ratio between the two time points at the individual level within each progressor group. It clearly demonstrates that in fast progressors, after 3 years of follow-up, the urinary myoinositol/citrate ratio increased significantly as compared to the BL value (relative rise in the ratio of 68%, \( p < 0.001 \), Fig. 4a), whereas in slow progressors the relative rise in the myoinositol/citrate ratio was 6% (\( p = 0.38 \); Fig. 4b). In fast progressors, this phenomenon was driven by an increase in myoinositol, combined with a decrease in citrate over time (online suppl. Fig. S2). We also calculated the change (slope, \( \Delta \)) in the myoinositol/citrate ratio between the two time points (BL and year 3). In online supplementary Figure S3 shows that fast progressors have a significantly higher change in metabolite ratio over time than slow progressors (\( p = 0.008 \)).

Discussion

In the current study, we reproduced the prognostic performance of a urinary metabolite profile taken at a single time point [11] to predict a fast progressive course of disease. When measured in a urine sample at BL, we found that the prognostic value to distinguish fast from
slow progressors of urinary metabolites was similar to currently more established risk markers, such as htTKV. Combining htTKV and the metabolite model predicted fast progressing disease better than these markers individually. Furthermore, we demonstrated that changes in the urinary myoinositol/citrate ratio over a follow-up period of 3 years were associated with a faster progressive course of disease in patients with ADPKD. This probably reflects the association of myoinositol and citrate with GFR. In general, in CKD, urine myoinositol rises and urine citrate falls as GFR declines.

In current clinical practice in ADPKD, conventional classification parameters, including eGFR indexed for age, htTKV adjusted for age, and genetic testing are frequently used for patient risk assessment. They all have their limitations. In literature, varying data on their prog nostic performance for identifying patients with fast pro gressing diseases have been reported [8, 10, 29, 30]. In our study, the value of the urinary myoinositol/citrate ratio (AUC = 0.72) was at least equivalent to that of the established risk model htTKV (AUC = 0.68). Urinary biomarkers improved the prediction model when added to the imaged-based predictor (AUC = 0.75), which is in agreement with other data [26, 29]. The predictive power of our image-based model (AUC = 0.68) was comparable to that of two recent studies in large ADPKD cohorts for distinguishing fast from slow progressors, as defined by the eGFR slope (AUC = 0.61 [29] and AUC = 0.65 [26]).

In line with our data, the prognostic performance was better when conventional risk models were combined with a urinary risk score (AUC = 0.73 [29] and AUC = 0.72 [26]) than of each of the predictors separately.

The two metabolite ratios that we found to be most important in ADPKD progression both included urinary citrate as their denominator. This strengthens its well-documented role in chronic renal dysfunction [31–37]. Citrate is freely filtered by the glomerulus and excretion is regulated by reabsorption, which is mostly influenced by tubular pH. Urinary citrate decreases under conditions of acidosis [38]. Hypocitraturia and acidosis are commonly found in patients with CKD and ADPKD. In ADPKD, acidosis is associated with a greater risk of worsening kidney function [39]. A recent study showed that urinary citrate inversely correlates with more advanced ADPKD progression as reflected by TKV and that its role as a calcium chelator in preventing tubular crystal formation may directly influence the pathophysiology of cyst formation [32]. We showed that a model, including citrate as a single predictor, was not informative enough for risk assessment. A stable or opposite functioning metabolite as nominator was needed to provide sufficient contrast. There is only limited mention of urinary alanine excretion in the literature on chronic renal dysfunction [35, 40]. More data are available on the association between myoinositol and renal function [37, 41, 42]. Myoinositol is mainly synthesized in the kidney, which is an important renal osmolyte involved in protecting cells from hyperosmotic stress. Under healthy conditions, renal myoinositol increases when interstitial osmolality increases, which is mainly driven by elevated tubular reabsorption rather than local production [37]. It is suggested that the reabsorption of myoinositol in damaged renal cells is decreased, resulting in elevated urinary myoinositol excretion. A recent study including 227 patients with varying stages of CKD showed data on the prognostic value of urinary myoinositol in predicting rapid CKD progression from those with stable disease (AUC = 0.78).
The fact that both citrate and myoinositol are commonly reported in literature in the context of chronic renal disease strengthens our finding of these two metabolites as an interesting combination for staging of disease and making a prognosis.

Our study has a number of strengths. First, we included a phenotypically well-defined, large, homogeneous (only PKD1) cohort with standardized longitudinal data and hTKV measurements. Second, the calculation of individual eGFR slopes was based on a mean follow-up duration of almost 4 years and at least three standardized sequential measurements of serum creatinine to allow reliable slope calculation [26]. Third, urine sampling, processing, and storage were standardized, limiting potential bias. Last, an established NMR-based platform was used for biomarker assessment. NMR spectrometry offers quantification and reliable analytical reproducibility. It also allows the measurement of large numbers of samples, qualifying this approach for clinical application.

Our study also has limitations. First, the use of hard study endpoints including ESRD or doubling of serum creatinine was not feasible because the follow-up period was shorter than in some other studies [23, 43]. The use of such outcomes strengthens the reliability and performance for predicting disease progression. Seeing that our cohort does not differ from other studies in other respects, we expect to find similar results for the predictive value of hTKV when the duration of follow-up is extend-

![Fig. 4. Changes in the urinary myoinositol/citrate ratio over time at an individual level within the progressor groups (n = 112). After 3 years of follow-up, the myoinositol/citrate ratio increased in fast progressors (a) (n = 56, p < 0.001), while in slow progressors (b) (n = 56), no such tendency was observed (p = 0.38). Notes: p values for BL versus year 3 (Y3) were calculated using the Mann-Whitney U test (paired version). The dark red dots represent the scaled median values (fast progressors BL = −0.66, Y3 = −0.21; slow progressors BL = −0.86, Y3 = −0.83). Fast and slow progressors were stratified based on an annualized change in eGFR of > or ≤ −3.0 mL/min/1.73 m²/year, respectively.](image)
ed. Considering that the urinary biomarkers that we found improve the predictive value of hTKV, but are still not perfectly discriminative, it would be of great interest to test these biomarkers on a cohort with a longer duration of follow-up. Second, the current study did not include a validation cohort for the longitudinal part of this study because a cohort of sufficient size for validation was not available. For splitting of the cohort into a training and validation cohort, the numbers were not large enough. However, with respect to the cross-sectional analysis, this is our second study investigating urinary metabolites, and while we were using another technique to identify prognostic markers we were able to validate our previous findings. Last, our study design is not suitable for an unequivocal causal or mechanistic interpretation of the identified urinary metabolites. The metabolites that we identified may play a role in renal dysfunction in general and not be specific to ADPKD. Future studies are needed to further unravel their role in pathophysiology and should evaluate the response of biomarker levels to treatment.

In conclusion, urinary metabolic profiling holds promise as an easy-to-perform strategy for risk stratification in ADPKD patients. When urinary biomarkers were combined with an imaging-based predictor, the predictive performance was improved beyond that of single predictors.

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**Statement of Ethics**

The DIPAK observational study was centrally approved by the Medical Ethics Committee of the University Medical Center Groningen (reference: 2013/040) and by the institutional review boards of the respective participating study centers (Leiden University Medical Center, Leiden; Radboud University Medical Center, Nijmegen; Erasmus Medical Center, Rotterdam). The study was performed in adherence to the Declaration of Helsinki. All patients gave written informed consent to participate in the DIPAK study, which included sample storage and use for future studies.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

Study concept and design: S.E.I.D., A.V., D.S., D.J.M.P., O.A.M., and J.W.F.; data acquisition: S.E.I.D. and D.S.; data analysis/interpretation: S.E.I.D., A.V., D.F., and O.A.M.; statistical analysis: S.E.I.D., A.V., D.F., and O.A.M.; study supervision: D.S., O.A.M., and J.W.F. Each author contributed important intellectual content during manuscript drafting or revision and agrees to be personally accountable for the individual’s own contributions and to ensure that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

**Data Availability Statement**

The patient data used for this study are restricted and not publicly available due to privacy and ethical concerns. Please contact the corresponding author, and that data may potentially be shareable with appropriate permissions and oversight.
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