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Research Paper

Metabolomics and Gene Expression Analysis Reveal Down-regulation of the Citric Acid (TCA) Cycle in Non-diabetic CKD Patients

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ABSTRACT

Chronic kidney disease (CKD) is a public health problem with very high prevalence and mortality. Yet, there is a paucity of effective treatment options, partly due to insufficient knowledge of underlying pathophysiology. We combined metabolomics (GCMS) with kidney gene expression studies to identify metabolic pathways that are altered in adults with non-diabetic stage 3–4 CKD versus healthy adults. Urinary excretion rate of 27 metabolites and plasma concentration of 33 metabolites differed significantly in CKD patients versus controls (estimate range − 68% to +113%). Pathway analysis revealed that the citric acid cycle was the most significantly affected, with urinary excretion of citrate, cis-aconitate, isocitrate, 2-oxoglutarate and succinate reduced by 40–68%. Reduction of the citric acid cycle metabolites in urine was replicated in an independent cohort. Expression of genes regulating aconitate, isocitrate, 2-oxoglutarate and succinate were significantly reduced in kidney biopsies. We observed increased urine citrate excretion (+74%, p = 0.00009) and plasma 2-oxoglutarate concentrations (+12%, p = 0.002) in CKD patients during treatment with a vitamin-D receptor agonist in a randomized trial. In conclusion, urinary excretion of citric acid cycle metabolites and renal expression of genes regulating these metabolites were reduced in non-diabetic CKD. This supports the emerging view of CKD as a state of mitochondrial dysfunction.

1. Introduction

Chronic kidney disease (CKD) is an important public health problem with a high prevalence world-wide (10–14% of the general population) and strongly increased age-standardized death rate (GBD_Collaborators 2015). Yet, there is a paucity of effective treatment options with no major breakthroughs to reduce kidney damage since the introduction of renin-angiotensin system blockers 30 years ago. Lack of relevant animal models is a major limitation to identify novel targets for therapy, and the tradition of CKD patients being excluded from many clinical trials makes drug repurposing from other indications difficult (Ramos et al. 2015; Strippoli et al. 2004). Therefore, studies with patient based pathophysiology research and clinical testing of mechanisms may be highly beneficial.

For decades, the main focus has been on glomerular dysfunction and pathology, but recently it has been suggested that the proximal tubule is important for initiation and progression of CKD (Bonventre 2014; Takaori et al. 2016). This nephron segment has a very high content of mitochondria and is highly dependent on oxidative phosphorylation (Chevalier 2016). Likewise, muscle weakness and atrophy, fatigue, and non-renal organ dysfunction are major CKD symptoms indicating a

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basal defect in cell metabolism. Mitochondrial dysfunction has turned out to be an important mechanism in diabetic kidney disease (DKD) (Hallan and Sharma 2016). A recent metabolomics comparison of 24-h urine samples from subjects with DKD and healthy controls identified 13 metabolites that were reduced in DKD, 12 of which were intermediates in mitochondrial metabolic pathways, suggesting a global suppression of mitochondrial activity (Sharma et al. 2013). Interestingly, 12 of the 13 metabolites were not reduced in diabetes without CKD, suggesting that presence of CKD, and not diabetes alone, was necessary for the observed urine metabolite pattern. Independent studies of kidney biopsy tissue demonstrated reduction of mitochondrial proteins and mitochondrial biogenesis (Sharma et al. 2013).

Metabolomics, the quantitative analysis of small molecules in biological samples, has uncovered numerous abnormalities in the blood of uremic patients. These include accumulation of renally excreted gut metabolites such as p-cresol sulfate (Aronov et al. 2011; Yu et al. 2014), indoxyl sulfate (Aronov et al. 2011; Kobayashi et al. 2014; Yu et al. 2014), and trimethylamine-N-oxide (Mutsaers et al. 2013); altered metabolism of amino acids such as tryptophan (Duranton et al. 2014; Goek et al. 2013; Kobayashi et al. 2014; Rhee et al. 2013; Toyohara et al. 2010), arginine (Duranton et al. 2014; Goek et al. 2013; Nkuipou-Kenfack et al. 2014; Shah et al. 2013; Yu et al. 2014), tyrosine/phenylalanine (Duranton et al. 2014), and glycine (Yu et al. 2014); impaired organic anion transport (Shah et al. 2013; Sharma et al. 2013); and increased anaerobic metabolism(Qi et al. 2012). Although urine is an important bio-sample for metabolomic studies in CKD, there have been relatively few studies evaluating metabolomics in 24-h urine. Furthermore, metabolomics may be a useful tool to identify novel therapeutic targets for CKD and to evaluate the effects of promising interventions. Vitamin D receptor agonists (VDRAs) such as paricalcitol are associated with reduced all-cause and cardiovascular mortality (Duranton et al. 2013; Zheng et al. 2013), reduced urine albumin excretion, and may have other renoprotective and cardioprotective effects (de Borst et al. 2013). However, the mechanisms underlying these effects, as well as other metabolic effects of VDRAs in CKD, remain incompletely understood, limiting full translation to clinical care.

To further investigate the metabolic abnormalities associated with non-diabetic CKD and to explore the effects of VDRAs, we quantified plasma and urine metabolites among participants in a clinical trial of paricalcitol (de Boer et al. 2013). We used an established wide-ranging panel of metabolites that are dysregulated in human inborn errors of metabolism and compared results to healthy controls. Key differences at baseline were replicated in an independent cohort, and expression of genes relevant to the significantly altered metabolites was analyzed in kidney tissue. Finally, we examined the effect of interventional treatment with paricalcitol vs. placebo on blood and urine metabolite profiles in CKD.

2. Methods

2.1. Study Populations

First, we compared 22 non-diabetic CKD stage 3–4 patients and 10 healthy controls to identify abnormalities in plasma and urine present in CKD patients. CKD participants were recruited from nephrology clinics at three medical centers associated with the University of Washington and enrolled in a clinical trial designed to test the effects of paricalcitol on glucose metabolism (NCT01003275) (de Boer et al. 2013). Inclusion criteria for the Paricalcitol study included age ≥ 18 years; estimated GFR 15–59 mL/min/1.73m^2; and fasting serum glucose 100–125 mg/dL. Exclusion criteria included a clinical diagnosis of diabetes or use of glucose-lowering medications; history of maintenance dialysis or kidney transplantation; use within past 8 weeks of prednisone, immunosuppressive medications, or other medications known to strongly affect blood glucose; change in dose of any medication within 8 weeks; and serum calcium >10.1 mg/dL. Healthy control participants were University of Washington employees required to be ≥18 years of age, and the same exclusion criteria were applied. In addition, healthy control participants were required to have an estimated GFR ≥ 60 mL/min/1.73m^2.

Second, major findings were replicated in the Study of Glucose and Insulin in Renal Disease (SUGAR), which included 45 non-diabetic CKD patients and 15 controls matched for age, sex and race (de Boer et al. 2016). SUGAR is a cross-sectional study of insulin and glucose metabolism in moderate to severe non-diabetic CKD. From 2011 to 2014, participants were recruited from nephrology and primary care clinics associated with the University of Washington and the neighboring institutions in Seattle, WA. Inclusion criteria included age ≥ 18 years and estimated GFR < 60 mL/min/1.73m^2. Healthy control participants were individuals with GFR ≥ 60 mL/min/1.73m^2, spot urine albumin-creatinine ratio < 30 mg/g, with the same distribution of age, sex and race as the enrolled participants with CKD. Exclusion criteria included a clinical diagnosis of diabetes, end-stage renal disease (ongoing or imminent maintenance hemodialysis or kidney transplantation) or use of medications known to affect glucose metabolism (e.g. corticosteroids), fasting serum glucose ≥ 126 mg/dL, and hemoglobin < 10 g/dL. All study procedures were approved by the University of Washington Institutional Review Board, and all participants provided written informed consent.

We also studied gene expression in kidney biopsies from 155 patients from the European Renal cDNA Bank cohort with biopsy-proven, non-diabetic CKD (FSGS or minimal change n = 24, hypertensive nephropathy n = 15, IgA nephropathy n = 27, minimal change disease n = 14, membranous glomerulonephritis n = 21, rapidly progressive glomerulonephritis n = 22, lupus nephritis n = 32). The control kidney biopsies were obtained from healthy kidney transplant donors (n = 31) prior to kidney donation, following the usual clinical protocols.

2.2. Paricalcitol Treatment

CKD participants enrolled in the paricalcitol cross-over intervention trial were allocated to paricalcitol for 8 weeks and placebo for 8 weeks, separated by an 8-week washout period (de Boer et al. 2013). The order of paricalcitol and placebo treatment periods was randomly assigned by the University of Washington Investigational Drug Services and was blinded to both participants and investigators. The active intervention was paricalcitol (19-nor-1,25-(OH)2-vitamin D2) 2 micrograms daily by mouth. Participants were encouraged not to change their use of non-study medications during the course of the study. Healthy control participants were not treated with paricalcitol (de Boer et al. 2013).

2.3. Covariate Data

Demographic data and comorbidities were assessed by questionnaire. Weight and height were measured while wearing light clothing. Blood pressure was measured after resting in a seated position for at least 5 min. Serum creatinine was measured in fasting plasma using a method traceable to isotope dilution mass spectrometer and GFR was estimated with the 2009 CKD-EPI equation (Levey et al. 2009). Timed urine samples (approximately 24-hour) were collected on ice, the urine volume registered, and 10 mL samples frozen at −80 for later analysis; albumin was measured in fresh urine immediately after collection using a turbidimetric assay and used to calculate daily albumin excretion rate.

2.4. Metabolomics

For CKD participants and healthy controls in the paricalcitol trial, plasma and urine samples were analyzed on a gas chromatography mass spectroscopy (GC–MS) platform at ClinMet, Inc. (San Diego, CA). This panel of metabolites has been established over the years to detect inborn errors of metabolism, and the focus on organic acids is also well suited for CKD since the handling of these compounds are central

in kidney function and metabolism. A list of measured metabolites is given in supplemental material (Supplemental Table 3) and includes metabolites from 37 different metabolic pathways. Eleven of these pathways were represented with a sufficiently high number of metabolites so that potential perturbations could be detected with significance. All citric acid cycle metabolites except succinyl CoA and oxalacetate were measured.

Urine aliquots corresponding to 1 μmol of creatinine or 2 mL of plasma were treated with pentafluorobenzyl hydroxylamine to oximate ketoacids prior to lyophilization overnight. Subsequently, the organic acids were extracted by liquid chromatography on silica (42% 2-methyl-2-butanol in chloroform). Solvent was evaporated and the dry residue was silylated with 300 μL of Trisil-N,O-bis (trimethylsilyl) trifluoroacetamide, and finally 1 μL of the reconstituted derivatized sample were injected into a 30 m × 0.32 mm column (Agilent DB-5) in Agilent 5890 gas chromatogram, followed by elution using a 4 °C/min gradient of 70–300°C. Electron impact mass spectrometry using an Agilent 5973 mass selective detector was used to detect the metabolites. Each analyte was identified from the spectrum and the confirmed ratio of qualifying and quantifying ions. The integrated current from the quantifying ion was used to estimate concentration using standard curves with 4–6 calibration points. Peak areas were normalized to internal standards (4-nitrophenol or 2-oxocaproate) added to samples prior to derivatization. Metabolites with results below the lower detection limit in more than two-thirds of all participants were excluded from further analysis in the current study (N = 4 of 66 for urine metabolites, N = 7 of 66 plasma metabolites). A control sample of pooled healthy urines was run every 8 samples to control for day-to-day variation. Citric acid cycle metabolites had coefficient of variation (CV) of 3–10% (Supplemental Table 4). Urine metabolite mass per 1 mmol creatinine was converted to mass excreted per 24 h to reduce problems due to different muscle mass, diet, and circadian rhythm. For details of metabolomics methods in the replication cohort (SUGAR), bioinformatics analyses, and gene expression in human kidney tissue, please see the Supplemental methods.

2.5. Statistical Analysis

Because metabolite distributions were greatly skewed, we applied the natural log transformation $log(x + 1)$ to all metabolites. For each metabolite, we compared the difference in log-metabolites between CKD participants on placebo and normal controls using *t*-tests that assumed unequal variances; for ease of interpretation we present the results as the percent difference between these two groups. To compare the effect of paricalcitol on CKD participants, we likewise performed paired t-tests on the difference in log-metabolites when the participant was on paricalcitol versus on placebo. We defined metabolites with statistically significant differences as those with ≥5% difference between CKD and control combined with a false discovery rate (FDR) ≤ 20%.(Benjamini and Hochberg 1995) Principal components analysis, first scaling each metabolite to unit variance, was applied to investigate systematic patterns of difference between the groups. Main findings were replicated in the SUGAR study with log-transformed urine metabolite concentrations regressed on CKD status, adjusting for age, race (white/non-white), sex, weight and the use of RAAS inhibitors. The p-values were obtained from a two-sided Wald test using sandwich-based robust standard errors. We calculated correlation of eGFR to urine excretion rate and inverse plasma concentrations to illustrate how much of the metabolite variation was caused by filtration. Fractional excretion (FE %) was calculated to illustrate tubular handling of metabolites. All statistical analyses were performed using R for Windows Version 3.1.2.

Metabolic pathway analysis was performed to help identify metabolic pathways potentially associated with non-diabetic CKD. All metabolites found to be significantly different in CKD patients compared to controls were included in these functional analysis performed with MetaboAnalyst 3.0. Enrichment analysis test the probability that significantly differing metabolites should belong to a pathway only by chance, and this web-based tool uses relative between-ness centrality and out-degree centrality measures to calculate compound importance. The pathway impact is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in the pathway. The maximum importance of each pathway is 1 with values ≥ 0.10 indicating significant perturbation of the pathway.

For differential analysis of transcriptomic data, unpaired analyses between healthy controls (living kidney donors) and non-diabetic CKD groups were performed using Significance Analysis of Microarrays (SAM) method implemented in Multi Experiment Viewer (MeV) application (Saeed et al. 2006; Saeed et al. 2003). Genes differentially expressed between two groups with a q-value (False Discovery Rate) below 0.05 were considered significant.

3. Results

3.1. Clinical Characteristics of the Primary Study Population

In the paricalcitol study, the participants with CKD had a mean age of 66 years, and the majority were Caucasian (77%) and male (91%). Almost all participants with CKD were treated for hypertension, 41% had a history of cardiovascular disease, and acid-base balance was in the normal range for most patients. Median estimated glomerular filtration rate (eGFR) and albumin excretion rate were 40 mL/min/1.73m^2 and 59 mg/day, respectively. Healthy control subjects were on average 43 years old, were also predominantly male and Caucasian, had fewer comorbidities than the participants with CKD, and had a median estimated GFR of 92 mL/min/1.73m^2. Fasting glucose levels were higher in participants with CKD than healthy controls, but none had diabetes mellitus based in the ADA diagnostic criteria (Table 1).

3.2. Metabolites in Non-diabetic CKD vs. Healthy Controls in Primary Study Cohort

Plasma concentration and/or urinary excretion of 49 unique metabolites varied significantly between CKD patients and healthy controls after correction for multiple testing (Table 2). The largest relative differences in plasma were for 3-hydroxybutyrate, the most abundant ketone-body, and hippurate, a well characterized gut-microbial end-product, with 113% and 109% higher plasma concentrations in CKD and unchanged urine excretion, respectively. In CKD, long chain fatty acids stearate (C18:0) and palmitate (C16:0) were higher in plasma while urine excretion was lower. Furthermore, there was significantly reduced urinary excretion of central citric acid cycle – also known as the Krebs cycle or the tricarboxylic acid (TCA) cycle – metabolites (citrate −68% and succinate −47%) with unchanged plasma levels. In general, CKD patients had many metabolites with higher blood concentrations and lower urine excretion rates. However, correlations of estimated glomerular filtration rate (eGFR) to urine excretion rates were not significant and explained only a small proportion of the variation for most metabolites (R-squared interquartile range 0.01–0.08). The correlations of plasma levels of metabolites with eGFR were stronger, but still weak (R-squared interquartile range 0.02–0.16, see Supplemental Table 1). Differences in tubular handling of metabolites, measured as fractional excretion (FE%), were small and non-significant for most metabolites. Principal component analysis showed that the measured metabolites segregated participants with CKD from healthy controls (Supplemental Fig. 1).

3.3. Pathway Analysis

To assist in the biological interpretation of our data, we used metabolite enrichment analysis, which is based on several metabolite libraries

Table 1
Characteristics of study participants.

Characteristic	Primary cohort (Paricalcitol trial)		Replication cohort (SUGAR trial)		Gene expression cohort (European Renal Biopsy Bank)	
	CKD (n = 22)	Controls (n = 10)	CKD (n = 45)	Controls (n = 15)	CKD (n = 155)	Controls (n = 31)
Demographics						
Age (years)	66 (12)	43 (11)	62 (14)	56 (12)	46 (17)	48 (12)
Male sex	20 (91%)	7 (70%)	24 (53%)	9 (60%)	85 (55%)	16 (51%)
Race						
Caucasian	17 (77%)	10 (100%)	29 (64%)	12 (80%)	155 (100%)	31 (100%)
African American	3 (14%)	0 (0%)	12 (27%)	2 (13%)	0 (0%)	0 (0%)
Asian	2 (9%)	0 (0%)	4 (9%)	1 (7%)	0 (0%)	0 (0%)
Medical history						
Hypertension	21 (96%)	2 (20%)	40 (89%)	5 (33%)	75 (58%)	None §
Cardiovascular disease	9 (41%)	0 (0%)	18 (40%)	1 (7%)	–	None§
Current smoking	3 (14%)	2 (20%)	9 (20%)	1 (7%)	–	–
Medical treatment						
RAAS inhibitors	18 (82%)	0 (0%)	30 (67%)	5 (33%)	66(63%)	None§
Vitamin D supplements	10 (46%)	5 (50%)	28 (62%)	7 (47%)	–	None§
Physical examination						
Weight (kg)	–	–	88.6 (19.9)	81.7 (20.2)	75.3 (15.9)	Normal§
Body mass index (kg/m^2)	31 (8)	24 (3)	30.3 (6.2)	27.3 (5.9)	26 (4)	Normal§
Systolic BP (mmHg)	132 (16)	117 (10)	134.4 (15.9)	122.4 (14.1)	136 (21)	Normal §
Diastolic BP (mmHg)	77 (11)	75 (9)	80.6 (9.7)	77.2 (9.3)	82(14)	Normal §
Laboratory data						
Median eGFR (mL/min/1.73 m^2)	40 (34–46)	92 (80–101)	36 (23–45)	92 (71–99)	66 (38–94)	105 (85–117)
Median AER (mg/24 h)	59 (21–271)	4 (3–4)	98 (16–279)	6 (3 − 10)	–	Normal §
p-Bicarbonate (mmol/L)	24.0 (2.6)	27.6 (2.3)				
Fasting glucose (mg/dL)	107 (11)	95 (12)	102.5 (8.9)	97.1 (8.9)	–	Normal §

Note: Data are presented as N (%) for categorical variables (some missing data for the replication and the kidney biopsy cohorts), means (SD) or medians (interquartile range) for continuous variables. Estimated GFR (eGFR) was calculated using serum concentrations of creatinine measured at baseline using the 2009 CKD-EPI eq. BP: blood pressure; AER: albumin excretion rate.
§ Kidney biopsy controls were kidney donors. Numerical data were not available except for age, sex and race, but by definition they do not have diabetes, CVD, hypertension, obesity, etc.

consisting of ~1000 entries, to explore whether significantly differing metabolites belong to a common pathway. There was an enrichment of significantly differing compounds in several metabolic pathways (Table 3), including the citric acid cycle (7 significant metabolites while only 0.27 was expected by chance, FDR < 0.001), phenylalanine with subsequent tyrosine and dopamine metabolism (7 metabolites vs. 0.6 expected, FDR < 0.001), short-chain fatty acids butanoate and propanoate metabolism (5 metabolites vs 0.5, FDR = 0.002)), and synthesis and degradation of ketone bodies (2 vs 0.08, FDR = 0.028). Among these pathways, the citric acid cycle and ketone body metabolism had the highest pathway impact scores (0.25 and 0.70, respectively) indicating substantially disturbed pathways due to high numbers and central location of differing metabolites (see note below Table 3). Five of the early citric acid cycle metabolites had significantly reduced urine excretion (citrate, cis-aconitate, isocitrate, 2-oxoglutarate and succinate) but were not significantly different in plasma, while two late citric acid cycle metabolites were significantly increased in plasma only (fumarate and malate).

3.4. Replication of Citric Acid Cycle (TCA) Metabolite Differences

We also evaluated the association of CKD status with urine metabolite excretion in an independent study of non-diabetic CKD stage 3–5, the Study of Glucose and Insulin in Renal Disease (SUGAR) (de Boer et al. 2013). In SUGAR, participants with CKD (median eGFR 37 mL/min/1.73m^2) were matched to control subjects without CKD on age, sex, and race, and the cohort had greater racial and gender diversity than the paricalcitol trial (Table 1). Pathway analyses based on all 140 metabolites measured in SUGAR showed that the citric acid cycle was again the most strongly affected pathway (p = 0.0006 after adjustment for multiple testing and impact score of 0.43). All five TCA cycle intermediates with reduced urine excretion in the paricalcitol cohort (isocitric, citric, succinic, 2-oxoglutarate and cis-aconitate) were also significantly reduced in 24-hour urine samples from the SUGAR cohort after adjusting for age, race, sex, and weight (Table 4).

3.5. Gene Expression for Citric Acid (TCA) Cycle Enzymes

As many of the metabolites from the TCA cycle were consistently reduced in the 24-hour urine collections of subjects with CKD we considered whether gene expression for enzymes relevant to the TCA cycle could also be affected. We therefore evaluated the renal gene expression of citric acid cycle enzymes in 155 non-diabetic CKD patients and 31 healthy kidney donors from the European Renal cDNA Bank (ERCB) with age, blood pressure and eGFR comparable to our primary cohort (Table 1) (Yasuda et al. 2006). There were significantly reduced mRNA levels for ten citric acid cycle enzymes, compared with controls (Fig. 1). Aconitase 1 and 2 (ACO1, ACO2), oxoglutarate dehydrogenase-like (OGDHL), succinate-CoA ligase (SUCLA2, SUCLG1 and SUCLG2), succinate dehydrogenase subunits B and D (SDHB, SDHD), and isocitrate dehydrogenase subunits 3B and 3G (IDH3B, IDH3G) mRNA levels were significantly reduced in the tubulointerstitial compartment. The glomerular compartment displayed very similar findings (Supplemental Fig. 2). This pattern of reduction in gene expression corresponded to the observed reduction of urinary metabolites from the proximal half of the citric acid cycle (e.g. citrate, aconitate, isocitrate, 2-oxoglutarate and succinate, Table 2 and Fig. 1).

3.6. Regulators of Mitochondrial Biogenesis in Non-diabetic CKD

Since citric acid (TCA) cycle is an intra-mitochondrial process, we examined mRNA levels for several key regulators of mitochondrial biogenesis in the same dataset. We found significant reductions of various subunits of the AMP-activated kinase (AMPK), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and peroxisome proliferator-activated receptor alpha (PPAR-α) in non-diabetic CKD kidneys compared with controls (Fig. 2). In contrast, peroxisome proliferator-activated receptor gamma (PPAR- γ) was increased, while nuclear respiratory factor-1 (NRF-1) and transcription factor A, mitochondrial (TFAM) were unchanged. A simplified overview of mitochondrial stimulators, regulators, and their effects is also given (Fig. 2).

Table 2
Differences in urine excretion rate and blood metabolite concentration in the primary cohort, comparing participants with and without CKD.

Metabolite	ID-number	Urine excretion rate			Blood concentration		
		% difference (95% CI)	p-Value	FDR	% difference (95% CI)	p-Value	FDR
Significant in both urine and blood							
Stearate	HMDB00827	**−53 (−77, −4)**	**0.041**	**0.11**	**47 (13, 92)**	**0.0068**	**0.021**
Palmitate	HMDB00220	**−52 (−75, −8)**	**0.03**	**0.092**	**55 (16, 107)**	**0.0059**	**0.02**
Glycolate	HMDB00115	**−45 (−59, −26)**	**0.00026**	**0.0049**	**−19 (−25, −13)**	**9.80E-07**	**5.80E-05**
3-Hydroxyisovalerate	HMDB00754	**−41 (−55, −23)**	**0.00032**	**0.0049**	**25 (10, 42)**	**0.0017**	**0.0093**
Isocitrate	HMDB00193	**−41 (−57, −19)**	**0.0021**	**0.016**	**24 (2, 52)**	**0.033**	**0.061**
Homovanillate	HMDB00118	**−37 (−60, −1)**	**0.047**	**0.12**	**−13 (−21, −3)**	**0.012**	**0.029**
L-2-Hydroxyglutarate	HMDB00694	**−36 (−48, −20)**	**0.0003**	**0.0049**	**6 (2, 11)**	**0.0082**	**0.023**
Hydroxypropionate	HMDB00700	**−29 (−52, 4)**	**0.077**	**0.18**	**18 (3, 36)**	**0.021**	**0.043**
2-Methylcitrate	HMDB00379	**−28 (−41, −12)**	**0.0027**	**0.018**	**13 (7, 19)**	**0.00006**	**0.00094**
3-Hydroxyglutarate	HMDB00428	**−24 (−42, 1)**	**0.06**	**0.15**	**14 (6, 22)**	**0.00077**	**0.0057**
Leucinate	HMDB00665	**−5 (−7, −2)**	**0.0029**	**0.018**	**−8 (−14, −1)**	**0.032**	**0.061**
Significant in blood only							
3-Hydroxybutyrate	HMDB00011	−5 (−27, 24)	0.72	0.78	**113 (22, 272)**	**0.011**	**0.028**
Hippurate	HMDB00714	6 (−34, 71)	0.79	0.83	**109 (55, 181)**	**0.00002**	**0.00057**
Oleate	HMDB00207	−11 (−24, 4)	0.14	0.29	**80 (25, 159)**	**0.0044**	**0.017**
Acetoacetate	HMDB00060	−1 (−20, 24)	0.95	0.95	**76 (3, 203)**	**0.041**	**0.071**
3-Hydroxyadipate	HMDB00345	−16 (−46, 31)	0.42	0.6	**38 (17, 62)**	**0.00035**	**0.0034**
Glycerate	HMDB00139	−25 (−54, 21)	0.22	0.38	**34 (1, 78)**	**0.041**	**0.071**
2-Hydroxybutyrate	HMDB00008	3 (−19, 31)	0.81	0.83	**34 (−1, 82)**	**0.058**	**0.095**
Myristate	HMDB00806	−1 (−4, 1)	0.2	0.36	**29 (−6, 77)**	**0.11**	**0.16**
2-Ethylhydracrylate	HMDB00396	−16 (−41, 20)	0.32	0.5	**28 (11, 47)**	**0.0012**	**0.0072**
L-Malate	HMDB00156	−22 (−52, 27)	0.3	0.48	**22 (7, 40)**	**0.0054**	**0.02**
p-Hydroxyphenylacetate	HMDB00020	−7 (−28, 22)	0.6	0.72	**18 (8, 29)**	**0.00071**	**0.0057**
3-Methylglutaconate	HMDB00522	−4 (−27, 27)	0.78	0.83	**16 (6, 27)**	**0.0025**	**0.011**
Hydroxyphenyllactate	HMDB00755	−8 (−28, 18)	0.48	0.61	**13 (2, 26)**	**0.021**	**0.043**
4-Hydroxyhippurate	HMDB13678	−28 (−72, 84)	0.47	0.61	**10 (3, 18)**	**0.008**	**0.023**
Phenylpyruvate	HMDB00205	−2 (−5, 2)	0.26	0.42	**10 (2, 20)**	**0.017**	**0.038**
Fumarate	HMDB00134	−4 (−17, 12)	0.62	0.72	**7 (2, 12)**	**0.011**	**0.028**
Adipate	HMDB00448	−1 (−31, 42)	0.94	0.95	**6 (2, 9)**	**0.001**	**0.0067**
Hexanoylglycine	HMDB00701	−23 (−48, 14)	0.18	0.35	**6 (1, 11)**	**0.017**	**0.038**
3-Methyladipate	HMDB00555	−10 (−31, 18)	0.43	0.6	**5 (2, 7)**	**0.00022**	**0.0026**
Benzoate	HMDB01870	−5 (−25, 20)	0.65	0.73	**−7 (−11, −2)**	**0.0069**	**0.021**
Methylsuccinate	HMDB01844	4 (−9, 19)	0.52	0.64	**−21 (−35, −5)**	**0.018**	**0.04**
2-Methylacetoacetate	HMDB03771	−5 (−12, 2)	0.14	0.29	**−29 (−43, −13)**	**0.0027**	**0.011**
Significant in urine only							
Citrate	HMDB00094	**−68 (−82, −44)**	**0.00049**	**0.006**	−11 (−35, 22)	0.45	0.56
Succinate	HMDB00254	**−47 (−65, −20)**	**0.0048**	**0.023**	−4 (−13, 7)	0.49	0.60
4-Hydroxybutyrate	HMDB00710	**−46 (−63, −20)**	**0.0049**	**0.023**	5 (−2, 14)	0.16	0.22
2-oxoglutarate	HMDB00208	**−42 (−68, 5)**	**0.071**	**0.17**	9 (−7, 28)	0.27	0.36
cis-Aconitate	HMDB00072	**−40 (−58, −16)**	**0.0051**	**0.023**	−20 (−41, 9)	0.14	0.20
Methylmalonate	HMDB00202	**−40 (−60, −10)**	**0.018**	**0.06**	1 (−4, 6)	0.74	0.84
2-Methyl-3-hydroxybutyrate	HMDB00354	**−39 (−53, −21)**	**0.0011**	**0.011**	3 (−1, 8)	0.097	0.15
Tiglylglycine	HMDB00959	**−37 (−49, −24)**	**0.00005**	**0.0029**	2 (0, 4)	0.03	0.058
Ethylmalonate	HMDB00622	**−37 (−55, −10)**	**0.015**	**0.054**	4 (0, 9)	0.057	0.095
N-acetyl-L-aspartate	HMDB00812	**−31 (−47, −10)**	**0.0085**	**0.033**	6 (−16, 32)	0.62	0.74
Pyroglutamate	HMDB00267	**−30 (−50, −2)**	**0.039**	**0.11**	16 (−5, 43)	0.15	0.20
Glutarate	HMDB00661	**−27 (−41, −10)**	**0.0071**	**0.029**	4 (−3, 10)	0.24	0.33
Glutaconate	HMDB00620	**−24 (−36, −9)**	**0.0049**	**0.023**	1 (−2, 3)	0.7	0.82
Ortho-hydroxyphenylacetate	HMDB00669	**−23 (−37, −6)**	**0.016**	**0.057**	1 (0, 1)	0.1	0.16
Azelate	HMDB00784	**−22 (−31, −10)**	**0.002**	**0.016**	0 (−4, 3)	0.81	0.88
3-Hydroxymethylglutarate	HMDB00355	**−20 (−35, −1)**	**0.037**	**0.11**	2 (1, 2)	0.0021	0.01

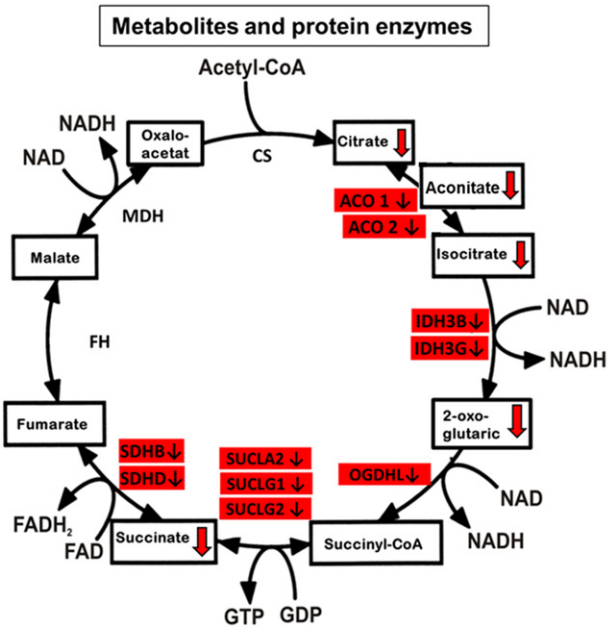
Note: Data are relative difference (% and 95% CI) between non-diabetic CKD and healthy controls. A positive percent difference indicates that the metabolite was higher in CKD than in controls. The significance threshold is ≥5% difference between CKD and controls and Q-values indicating a FDR <20%. The % difference and p-values for metabolites passing this threshold are in bold print. Analytes are ordered by magnitude of difference between cases and controls. p-Values are based on *t*-test on log-transformed values assuming unequal variance.

3.7. Urine and Plasma Metabolites in CKD During Paricalcitol Treatment

Finally, we compared citric acid (TCA) cycle metabolites in plasma and urine at the end of 8 weeks of paricalcitol treatment to those measured at the end of 8 weeks of placebo treatment (Table 5). Among 22 trial participants with CKD, we found that total renal citrate excretion and plasma citrate concentration was higher during paricalcitol treatment (+74%, $p < 0.0001$ and +5%, $p = 0.13$, respectively). Furthermore, higher 2-oxoglutarate excretion and plasma concentrations were also observed during treatment (+34%, $p = 0.08$ and +12%, $p = 0.002$, respectively). There were no changes in the fractional excretion of TCA metabolites (Table 5) or other metabolites (Supplementary Table 2). Distribution of citrate and 2-oxoglutarate in urine and blood is shown for healthy controls, CKD patients without paricalcitol and CKD patients with paricalcitol (Fig. 3). CKD patients also had a small but significantly increase in serum bicarbonate during treatment (24.0 vs 25.5 mmol/L, $p = 0.002$). There was a significant decrease of eGFR after 8 weeks of paricalcitol treatment compared with placebo (33.2 vs 36.5 mL/min/1.73m^2, $p = 0.01$).

4. Discussion

We identified 49 significantly altered metabolites in plasma and urine representing several perturbed metabolic pathways, most



Protein enzymes

Gene Name	Fold Change	Q value
ACO1	**0.9**	**<0.001**
ACO2	**0.9**	**<0.001**
IDH1	1.0	1.000
IDH2	1.0	1.000
IDH3A	1.0	0.071
IDH3B	**0.9**	**<0.001**
IDH3G	**0.9**	**<0.001**
OGDH	1.0	0.056
OGDHL	**0.9**	**<0.001**
SUCLA2	**0.9**	**0.024**
SUCLG1	**0.9**	**<0.001**
SUCLG2	**0.9**	**<0.001**
SDHA		
SDHB	**0.9**	**<0.001**
SDHC		
SDHD	**0.9**	**<0.001**
FH	1.0	1.000
MDH1	1.0	0.121
MDH2	1.0	0.056

Fig. 1. The urinary excretion of citric acid (TCA) cycle metabolites and the renal expression of genes that regulate these metabolites were significantly reduced among participants with versus without non-diabetic CKD. Urine excretion of citric acid cycle metabolites in the proximal part of the pathway were reduced in samples from patients with non-diabetic CKD, as was the mRNA expression of the enzymes catalyzing the proximal steps of the citric acid cycle in the tubulointerstitial compartment in biopsies of patients with non-diabetic CKD.

strikingly the citric acid (TCA) cycle, in non-diabetic CKD patients as compared to healthy controls. Lower urinary excretion of citric acid cycle metabolites were replicated in an independent cohort, and the expression of genes for ten citric acid cycle enzymes were significantly reduced in kidney biopsies of patients with non-diabetic CKD. In participants with CKD, the urinary excretion of citrate and the plasma levels of 2-oxogluarate, which are central citric acid cycle metabolites, were significantly higher during treatment with paricalcitol. These results suggested that non-diabetic CKD is characterized by reduction of citric acid cycle metabolites and enzymes.

When GFR decreases, serum metabolite concentrations will increase until a new steady state is achieved with elimination again equaling production. Excretion based on 24-hour urine samples is therefore not affected by reduced GFR per se, but can theoretically be influenced by dietary changes, renal reabsorption, extra-renal excretion and metabolism. Citrate metabolism is well studied over decades, and blood and urine levels are generally held to reflect citric acid (TCA) cycle activity since there is no known extra-renal elimination and minimal influence by oral intake (Bashir et al. 2012; Hamm 1990). However, renal excretion can be substantially modulated by acid-base state. Acidosis will increase tubular reabsorption of citrate with subsequent full metabolization to carbon dioxide in proximal tubular cells, but the small difference in plasma bicarbonate between well-regulated CKD stage 3 patients and healthy controls in our study can only explain a small part of the observed decrease in citrate excretion (Brennan et al. 1988; Caudarella et al. 2003; Hamm 1990; Simpson 1983). Although other factors decreasing urinary citrate excretion are known (starvation, potassium depletion, high-protein intake, hyperparathyroidism) (Caudarella et al. 2003), these are, except for the latter, not likely to be of importance for most CKD stage 3 patients. Therefore, our findings of substantially reduced excretion of the majority of citric acid cycle metabolites and reduced gene expression in CKD patients is best explained by reduced local or systemic citric acid cycle activity. A recent study demonstrated reduced urinary excretion of two citric acid cycle metabolites and 11 other metabolites associated with mitochondrial function in participants with diabetic kidney disease compared to healthy control subjects (Sharma et al. 2013).

Diminished citric acid (TCA) activity may be due to a reduction in overall mitochondrial biogenesis, reduced expression of the genes encoding citric acid enzymes, or reduced citric acid cycle substrate

Table 3
Altered metabolic pathways in non-diabetic CKD versus controls.

Pathway	Enrichment analysis						Topology analysis
	Total	Expected	Hits	Raw p	Holm adjust	FDR	Impact
Citric acid cycle (TCA cycle)	20	0.27	7	1.15E−07	9.19E−06	0.0000	0.25
Phenylalanine metabolism	45	0.60	7	1.17E−06	9.24E−05	0.0000	0.14
Glyoxylate & dicarboxylate metab.	50	0.66	7	2.46E−06	1.92E−04	0.0001	0.10
Propanoate metabolism	35	0.47	5	7.35E−05	5.66E−03	0.0015	0.03
Butanoate metabolism	40	0.53	5	1.42E−04	1.08E−02	0.0023	0.11
Tyrosine metabolism	76	1.01	6	3.83E−04	2.87E−02	0.0051	0.11
Ketone bodies metabolism	6	0.08	2	2.49E−03	1.84E−01	0.0284	0.70
Alanine, aspartate, & glutamate metab.	24	0.32	3	3.58E−03	2.61E−01	0.0358	0.00
Fatty acid biosynthesis	49	0.65	3	2.6E−02	1E+00	0.2310	0.00
Valine, leucine, & isoleucine metab.	40	0.53	2	9.77 E−02	1E+00	0.7820	0.00

Note: Enrichment analysis test if the compounds significantly differing between CKD patients and controls are found more often in a specific metabolic pathway than expected by chance. Pathways are ranked according to their statistical significance, i.e. citric acid cycle is on top since our finding of 7 differing metabolites in this pathway is extremely unlikely to happen by chance. In addition, topology analysis, which takes into account the structure of a given pathway to evaluate the relative importance of the differing compounds based on their relative locations, shows that the 7 differing TCA metabolites represent changes in key positions of the network and will trigger more severe impact on the pathway than changes on marginal or relatively isolated positions. Impact scores range 0 to 1, and scores ≥0.1 suggest significant pathway alteration.

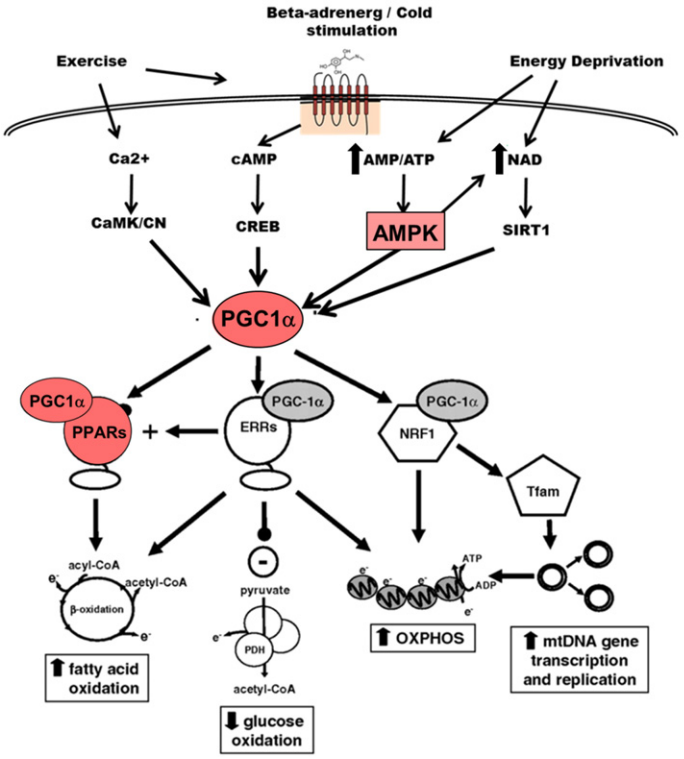
Table 4
Differences in citric acid (TCA) cycle metabolites in 24-hour urine samples in the replication cohort (SUGAR study), comparing participants with non-diabetic CKD to matched controls.

Metabolite	HMDB	% difference (95% CI)	p-value
Citrate	HMDB00094	−81 (−89, −65)	7.3E-08
Aconitate	HMDB00072	−61 (−73, −42)	2.3E-06
Isocitrate	HMDB00193	−61 (−72, −45)	1.0E-07
2-oxoglutarate	HMDB00208	−71 (−81, −54)	1.1E-07
Succinate	HMDB00254	−41 (−59, −15)	4.5E-03
Fumarate	HMDB00134	−37 (−56, −11)	9.3E-03

Note: Log-transformed urine metabolite concentrations were regressed on CKD status, adjusting for age, race (white/non-white), sex, weight, RAAS medication and baseline CVD. The p-values were obtained from a two-sided Wald test using sandwich-based robust standard errors.

availability. Regarding the first point, we observed reduced mRNA levels of AMPK and PGC-1α, key regulators of mitochondrial biogenesis, in kidney tissues from non-diabetic CKD patients. This is the first study to report the reduction of gene expression of several members of the AMPK system in kidney biopsies from patients with CKD. Other recently published studies have also reported reduced kidney PGC-1α in CKD, both diabetic and non-diabetic (Kang et al. 2015; Sharma et al. 2013). Diabetic kidney disease patients had reduced mitochondrial protein in kidney biopsies and reduced mitochondrial mRNA in the urine suggesting global mitochondrial suppression (Sharma et al. 2013). Significantly reduced mitochondrial volume density and other abnormalities were recently found in muscle biopsies and blood leukocytes from CKD patients (Gamboa et al. 2016), and reduced mtDNA copy number was found in diabetic as well as non-diabetic CKD stage 3–4 indicating that these changes start well before end-stage renal disease (Gamboa et al. 2016). Combined, these data suggest that CKD may be associated with a general mitochondrial dysfunction regardless of diabetes status. Secondly, we observed significantly reduced expression of genes for citric acid cycle enzymes in both the glomerular and tubulointerstitial compartments of human kidneys with non-diabetic CKD. The reduction of isocitrate dehydrogenase 3 (IDH3B and IDH3G) in the tubulointerstitial compartment is especially interesting since this is considered to be the major control point in the citric acid cycle (Berg et al. 2002). The second citric acid cycle control point in animal cells, oxoglutaric dehydrogenase (OGDH), was also significantly reduced. Thirdly, it is well known that CKD patients have reduced gluconeogenesis and increased risk of hypoglycemia (Moen et al. 2009). Posphoenol-pyruvate carboxykinase (PEPCK) is the key regulator of gluconeogenesis, but an equally important function could be removal of citric acid cycle intermediates for conversion to pyruvate and fueling of the citric acid cycle (cataplerosis) (Hanson 2009). Reduced PEPCK mRNA abundance and protein expression have been demonstrated in CKD rats (Burki et al. 2015) as well as non-diabetic CKD patients from the ERCB cohort used in our study (fourfold reduction, top 1% of under-expressed genes) (Athey et al. 2012; University-of-Michigan 2016). This could contribute to reduced substrate availability and reduced citric cycle activity.

Fatty acid oxidation and ketone body metabolism are other important mitochondrial processes which are highly integrated with the citric acid (TCA) cycle. Prior studies have suggested impaired long-chain fatty acid beta-oxidation in both chronic and end-stage kidney disease.(Stadler et al. 2015) Carnitine deficiency or inhibition of carnitine palmitoyl transferase-1 (Smogorzewski et al. 1988), which lead to impaired transport of long chain fatty acids into mitochondria for beta-oxidation, may contribute (Matera et al. 2003). Impaired oxidation of long-chain fatty acids is proposed to cause preferential consumption of shorter chain fatty acids because these can translocate into



Gene name	Fold change	Q value
AMPK subunits		
A1 (PRKAA1)	1.0	0.38
A2 (PRKAA2)	0.9	0.07
B2 (PRKAB1)	0.9	<0.001
B2 (PRKAB2)	0.9	0.036
G1 (PRKAG1)	1.0	0.42
G2 (PRKAG2)	0.9	0.06
PGC-1α (PPARGC1A)	0.7	<0.001
PPAR-Y	1.4	<0.001
PPAR-α	0.9	<0.001
NRF-1	1.0	0.27
TFAM	1.0	1.00

Fig. 2. Expression of mRNA regulating mitochondrial biogenesis was significantly different comparing participants with versus without non-diabetic CKD. A. Increased AMPK activity induces activation of PGC-1α, a major regulator of mitochondrial biogenesis. Activated PGC-1α sets in motion several transcriptional programs to stimulate replication of mitochondrial DNA, as well as expression of mitochondrial enzymes such as those involved in fatty acid oxidation, citric acid cycle or the electron transport chain. B. Compared with healthy controls, subjects with non-diabetic CKD showed reduced mRNA levels for two subunits of the AMPK protein (PRKAB1, PRKAB2), as well as PGC-1α, while PPARγ mRNA was increased. PRKAA1 to PRKAG2: AMPK subunits; PARGC1A: PPARγ coactivator 1α (PGC-1α); AMPK: AMP-dependent kinase; PPARγ: Peroxisome proliferator-activated receptor γ; ERR α: Estrogen related receptor α; NRF: Nuclear respiratory factor; TFAM: Transcription factor A, mitochondrial.

Table 5
Effect of paricalcitol treatment on citric acid cycle (TCA) metabolites in non-diabetic CKD patients.

Metabolites	Urine excretion rate		Blood concentration		Fractional Excretion	
	% difference (95% CI)	p-value	% difference (95% CI)	p-value	% difference (95% CI)	p-value
Citrate	74 (37, 120)	< 0.0001	5 (−2, 13)	0.13	1 (−2, 3)	0.49
Aconitate	19 (−12, 62)	0.25	2 (−5, 10)	0.60	1 (−7.7)	0.89
Isocitrate	28 (2, 61)	0.03	3 (−3, 10)	0.30	1 (−4, 7)	0.66
2-oxoglutarate	34 (−4, 85)	0.08	12 (5, 19)	0.002	0 (−1, 2)	0.66
Succinyl CoA	NA	NA	NA	NA	NA	NA
Succinate	29 (−8, 83)	0.14	4 (−1, 10)	0.10	0 (−2, 2)	0.66
Fumarate	17 (2, 35)	0.03	1 (−1, 4)	0.34	0 (−1, 1)	0.97
Malate	25 (−4, 64)	0.09	2 (−5, 10)	0.59	0 (−1, 2)	0.81
Oxaloacetate	NA	NA	NA	NA	NA	NA
Pyruvate	8 (−19, 46)	0.58	7 (−6, 23)	0.28	0 (0, 0)	0.89

Note: Cell contents are percent differences (95% confidence intervals) comparing values obtained during treatment with paricalcitol to values obtained during treatment with placebo for each trial participant (N = 22).

mitochondria independent of carnitine to undergo beta-oxidation, generating adipate (Mortensen 1992). The result is reduced concentration of low-molecular-weight triacylglycerols and accumulation of high-molecular-weight triacylglycerols (Rhee et al. 2010). Consistent with this theory, we and others find higher concentrations of long-chain fatty acids (palmitate, oleate, stearate and myristate) (Fouque et al. 2006; Shah et al. 2013; Wanner et al. 1988) and adipate (Rhee et al. 2010; Toyohara et al. 2010) in plasma (Table 2). In addition, we find significant alteration in metabolism of two of the most abundant short-chain fatty acids (SCFA; propanoate and butanoate), with marked reduction in urinary excretion of several distal propanoate metabolites (hydroxypropionate, methylcitrate, tiglylglycine, methylmalonate). This could be consistent with reduced propanoate availability due to reduced gut-microbial production or increased consumption. The gut-microbiome is increasingly found to influence inflammatory processes and to produce uremic toxins (Felizardo et al. 2016), and SCFA is hypothesized to be kidney protective by reducing inflammation and improving mitochondrial function (Andrade-Oliveira et al. 2015). A signature of defective fatty acid beta-oxidation was also recently reported in genome-wide transcriptomic analyses of fibrotic human kidneys (Kang et al. 2015). Furthermore, we observed strong perturbations in the ketone body metabolism with doubling of plasma beta hydroxyl butyrate, the main ketone body in humans, and it's precursor acetoacetate. Others have found four-fold increased levels

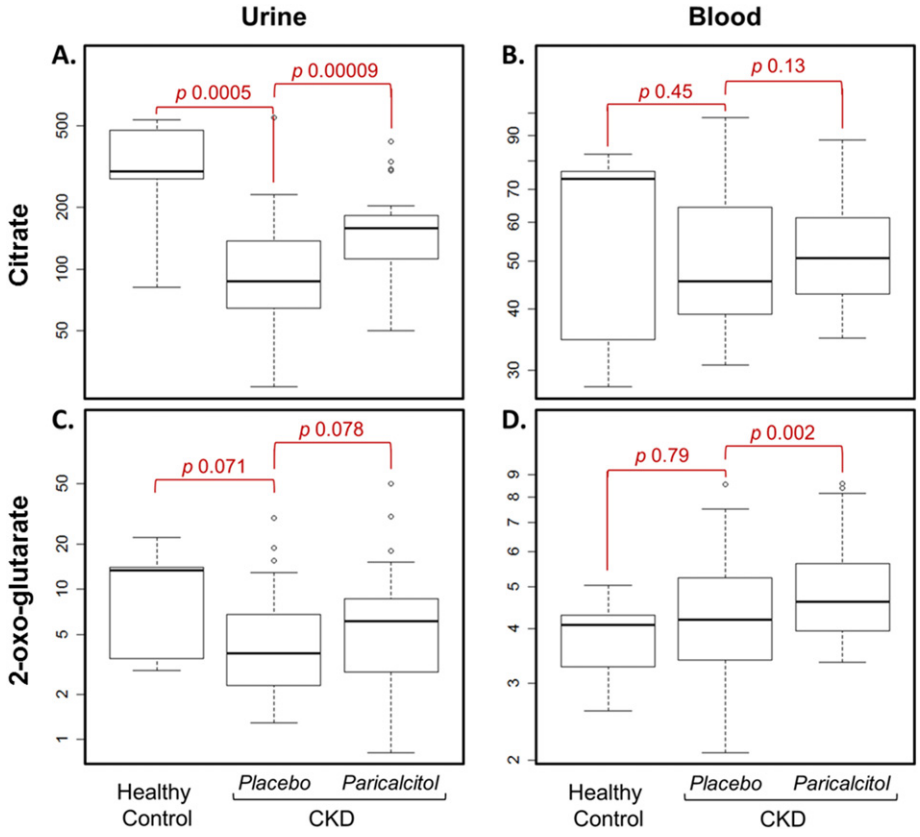


Fig. 3. Urine and blood concentrations of citrate and 2-oxoglutarate in CKD were increased by paricalcitol in non-diabetic CKD. Urine excretion of citrate is reduced in CKD, compared with healthy controls, and is increased significantly after paricalcitol treatment (panel A). Plasma citrate shows a similar trend, however did not reach significance (panel B). Whisker plots depict maximum, upper quartile, median, lower quartile and the minimum values of each metabolite.

of the rate-limiting enzyme in ketogenesis (HMGCS2) in DKD (Zhang et al. 2011). Our data indicate that this could be a general metabolic disturbance in CKD and could reflect a shift in metabolism to burn acetyl-coA. Overall, one could speculate whether these widespread mitochondrial dysfunctions are part of the well-known clinical protein-energy wasting syndrome in CKD and other uremic symptoms (Carrero et al. 2013).

The renoprotective mechanisms of paricalcitol are not well understood. Increased urine excretion of citrate and other metabolites could be explained by improved glomerular function, but we observed a small decrease in GFR estimated from serum creatinine during treatment. Similar results have been found previously and have been attributed to an increased creatinine production with no effect on the measured GFR (Agarwal et al. 2011). The findings are therefore more consistent with paricalcitol having a general effect on TCA cycle activity. 1-25-OH-vitamin D was recently shown to improve mitochondrial activity in human skeletal muscle cells (Ryan et al. 2016; Sinha et al. 2013). Vitamin D has a wide range of effects on several organs and even cancer cells, many of which are not well understood, and some of these could be caused be a redirection of metabolism towards oxidative phosphorylation (Christakos et al. 2016). We could speculate that paricalcitol improve basic cellular functions in podocytes and proximal tubular cell, which are the mainstays against urine albumin losses (Muller-Deile and Schiffer 2014). Alternatively, paricalcitol may increase urine citrate by reducing proximal tubular reabsorption of citrate, for example by altering the acid-base status (Hamm 1990). However, a substantial pH decrease (pH 7.40–pH 7.20, i.e. a 60% increased H + concentration) is needed to give a moderately increased citrate reabsorption (+20–40%) (Brennan et al. 1988; Caudarella et al. 2003; Hamm 1990; Simpson 1983), so the small differences in bicarbonate in our study during paricalcitol treatment of CKD patients (7% change in H + concentration) can only explain a small fraction of the change in citrate excretion. 2-oxoglutarate have an essential role in metabolic control with important effects on mitochondrial potential, tissue respiration, ROS (reactive oxygen species) production, nitrogen metabolism, and glutamate signaling (Bunik and Fernie 2009). Supplementation of 2-oxoglutarate has been found to extend *C. elegans* lifespan by 50%, probably by inhibiting ATP synthase similar to dietary restriction (Chin et al. 2014). Notably, administering 2-oxoglutarate has been shown to increase bone density, possibly by promoting proline hydroxylation, a key component of type I collagen backbone of the bone matrix (Tatara et al. 2005). As such, the paricalcitol-induced increase in plasma 2-oxoglutarate may relate to beneficial effects of VDRAs on bone health. More largescale clinical trials and experimental studies to look into mechanisms are needed.

Strengths of this study are use of an established, quantitative metabolite panel, simultaneous metabolite quantification in urine and plasma, the use of 24-hour urine to quantify urinary excretion, replication of key findings in an independent cohort in a different laboratory, evaluation of gene transcription to complement metabolite measurements, and application of metabolomics to a CKD-relevant treatment (paricalcitol). The main study limitations are the small sample size, the cross-sectional design, lack of complete matching of CKD participants to healthy controls in the primary study, and lack of replication for paricalcitol treatment effects. Our focus on a pre-specified set of metabolites naturally precludes a total evaluation of metabolic abnormalities in CKD, but the metabolomics platforms used survey a variety of biochemical pathways relevant to human errors of metabolism. The large observed differences, the consistency with previous findings, and the replication of key results in an independent population provide reassurance that our results are not due to confounding factors, although further studies should be performed in a larger population with greater ability to account for relevant covariates.

In conclusion, we observed a pattern of significantly altered metabolites consistent with reduced citric acid cycle activity, reduced fatty acid oxidation and increased ketone body metabolism. This could suggest generally decreased mitochondrial function in non-diabetic CKD similar to recent findings in patients with diabetic CKD.

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None of the funders had any role in study design, data collection, data analysis, interpretation, or writing of the manuscript.

Conflict of Interest

K.S. is the founder of Clinical Metabolomics (ClinMet), Inc. All other authors have declared that no conflict of interest exists.

Author Contributions

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Acquisition of data: deBoer, K. Sharma, Kretzler, Ju, Darshi, S. Sharma, Raftery, Barding,

Analysis and interpretation of data: Hallan, Afkarian, Zelnick, Kestenbaum, S·Sharma, Saito, Darshi, Barding, Raftery, Ju, Kretzler, K. Sharma, de Boer

Drafting of manuscript: Hallan, Afkarian

Critical revision: Hallan, Afkarian, Zelnick, Kestenbaum, S·Sharma, Saito, Darshi, Barding, Raftery, Ju, Kretzler, K. Sharma, de Boer.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2017.10.027.

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