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Serum metabolites are associated with all-cause mortality in chronic kidney disease

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Abstract

Chronic kidney disease (CKD) involves significant metabolic abnormalities and has a high mortality rate. Because the levels of serum metabolites in patients with CKD might provide insight into subclinical disease states and risk for future mortality, we determined which serum metabolites reproducibly associate with mortality in CKD using a discovery and replication design. Metabolite levels were quantified via untargeted liquid chromatography and mass spectroscopy from serum samples of 299 patients with CKD in the Modification of Diet in Renal Disease (MDRD) study as a discovery cohort. Six among 622 metabolites were significantly associated with mortality over a median follow-up of 17 years after adjustment for demographic and clinical covariates, including urine protein and measured glomerular filtration rate. We then replicated associations with mortality in 963 patients with CKD from the African American Study of Kidney Disease and Hypertension (AASK) cohort over a median follow-up of ten years. Three of the six metabolites identified in the MDRD cohort replicated in the AASK cohort: fumarate, allantoin, and ribonate, belonging to energy, nucleotide, and carbohydrate pathways, respectively. Point estimates were similar in both studies and in meta-analysis (adjusted hazard ratios 1.63, 1.59, and 1.61, respectively, per doubling of the metabolite). Thus, selected serum metabolites were reproducibly associated with long-term mortality in CKD beyond markers of kidney function in two well characterized cohorts, providing targets for investigation.

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STATEMENT OF COMPETING FINANCIAL INTERESTS

No authors have conflicts of interest. Assay costs were discounted as part of a collaboration agreement between Metabolon and JC, LI, AL to develop metabolomics estimates of GFR and for which a provisional patent has been filed 8/15/2014: PCT/US2015/044567.

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Keywords

Chronic kidney disease; metabolomics; mortality; uremic toxins

INTRODUCTION

Chronic kidney disease (CKD) is a public health problem with a global prevalence of 13.4% and mortality rate of 1.2 million per year worldwide.^{1–3} The kidney is an important regulator of homeostasis by filtration, reabsorption, secretion, synthesis, and degradation of metabolites in numerous pathways.⁴ As such, kidney disease directly leads to a wide range of circulating metabolic abnormalities.⁵

Untargeted serum metabolite profiling, which is a non-directed, high-throughput approach to identify and quantify metabolites, may provide insight into subclinical disease states and risk for future adverse events beyond the prognostic information available from reduced glomerular filtration rate (GFR). The metabolome refers to small molecules between 50 and 1 500 Daltons in size that are the intermediates and products of metabolism, including amino acids, carbohydrates, cofactors, vitamins, lipids, nucleotides, peptides, and xenobiotics. The metabolome has multiple endogenous and exogenous determinants, including the genome, transcriptome, and proteome, and diet and the microbiome. Metabolites that associate with subsequent risk of adverse events may shed light on underlying pathophysiology or represent novel treatment targets.

To date, an untargeted metabolomics approach to identify metabolites associated with mortality has only been used in the general population.^{6,7} Because of the interrelatedness of kidney function and metabolite levels, associations between metabolites and mortality may be even more informative in CKD. Thus, we examined the associations between over 600 serum metabolites identified in untargeted profiling and the risk of all-cause mortality, using a discovery cohort of participants in the Modification of Diet in Renal Disease (MDRD) Study, followed by replication in the African American Study of Kidney Disease and Hypertension (AASK). In addition, we specifically evaluated associations between known uremic toxins and mortality with and without adjustment for clinical covariates.⁸

RESULTS

Baseline characteristics of discovery study and replication study participants

Study participants in the discovery cohort (MDRD; N=299) had a mean age of 55 years and mean BMI of 27 kg/m²; 42% were female, 8% were African American, 10% had diabetes mellitus, 18% had coronary heart disease, and 9% were current smokers (Table 1). The mean measured GFR was 30 mL/min/1.73 m². The median follow-up period was 16.5 years, ranging from 1.1 to 18.6 years, during which 151 participants (51%) died.

Study participants in the replication cohort (AASK; N=963) had a mean age of 55 years and a mean BMI of 31 kg/m²; 39% were female, 100% were African American, 0% had diabetes, 52% had coronary heart disease, and 58% were current smokers. The mean

measured GFR was 47 mL/min/1.73 m². The median follow-up period was 9.7 years, ranging from 1 day to 12 years, during which 220 participants (23%) died.

Metabolites associated with mortality in the discovery study

We investigated 622 non-drug, named metabolites (Supplementary Table 1). The mean molecular weight of the investigated compounds was 298 Daltons, ranging from 74 to 811 Daltons. In global analysis adjusted for multiple clinical covariates including ¹²⁵I-iothalamate clearance and log-transformed 24-hour urine protein, there were 6 metabolites that had statistically significant associations with mortality in the MDRD study (discovery threshold $p < 0.001$). These included: glutamine (hazard ratio (HR) per doubling of metabolite=0.3, $p=0.0002$), alpha-ketoglutarate (HR=1.5, $p=0.0004$), ribonate (HR=1.6, $p=0.0006$), fumarate (HR=1.7, $p=0.0007$), allantoin (HR=1.6, $p=0.0007$), and gamma-glutamylglutamine (HR=0.6, $p=0.00095$) (Figure 1). These metabolites are part of the amino acid, energy, carbohydrate, energy, nucleotide and peptide pathways, respectively (Supplementary Figure 1).

Metabolites associated with mortality in the replication study

Three of the six discovered metabolites in MDRD were significant in AASK in the Bonferroni-corrected replication study (replication threshold $p < 0.0083$) adjusted for clinical covariates including ¹²⁵I-iothalamate clearance and log-transformed 24-hour urine protein: ribonate (HR=1.7, $p=0.0005$), fumarate (HR=1.6, $p=0.001$), and allantoin (HR=1.6, $p=0.001$) (Figure 2). All associations were in the same direction with similar magnitude across the two cohorts, with higher levels of metabolites associated with higher mortality. Kaplan-Meier survival curves stratified at the median levels of ribonate, fumarate, and allantoin showed separation over time (all log-rank $p < .05$ except for allantoin in MDRD; Figure 3).

Metabolites associated with mortality in a meta-analysis

In a meta-analysis of the two cohorts, all 3 replicated metabolites (ribonate, fumarate, allantoin) were significantly associated with mortality after Bonferroni correction ($p < 0.00008$) (Figure 4). Each of the replicated metabolites showed a 1.5 to 1.6-fold higher risk of mortality per metabolite doubling. If all 622 metabolites were considered, five additional metabolites were also statistically significant in the meta-analysis: N-acetylcarnosine (HR=0.67, $p=8.3E-8$), 1-palmitoyl-2-linoleoyl-GPE (HR=1.43, $p=8.2E-6$), malate (HR=1.63, $p=3.2E-5$), 1-linoleoyl-GPE (HR=1.47, $p=6.6E-5$), and hydroxycotinine (HR=1.08, $p=7.2E-5$). Each of these metabolites was positively associated with mortality except for N-acetylcarnosine, which had a protective effect.

Sensitivity analysis

In sensitivity analyses adjusting for baseline medication classes in the MDRD study, the effect estimates for the three metabolites significantly associated with mortality remained largely the same (Supplementary Table 2). In sensitivity analysis adjusting for baseline liver function tests in the AASK study, the effect size for the three replicating metabolites were also largely unchanged (Supplementary Table 3).

Correlations between mortality-related metabolites and other metabolites in their respective pathways

Allantoin, ribonate and fumarate were all positively correlated with each other. Allantoin was negatively correlated with GFR and positively correlated with urea (Supplementary Figure 2 & 3). With respect to other metabolites in the (hypo)xanthine/inosine-containing purine pathway, allantoin was generally only weakly correlated (Supplementary Table 4). Only one other purine-pathway metabolite, N1-methylinosine, was associated with mortality, and this was only in AASK, but not MDRD.

Fumarate was negatively correlated with GFR, but not significantly so. With respect to other metabolites in the tricyclic acid cycle pathway, fumarate had the most consistently strong correlation with malate, which was also associated with mortality in both AASK and MDRD (Supplementary Table 5). Alpha-ketoglutarate was associated with mortality in MDRD, but not AASK.

Ribonate was negatively correlated with GFR and positively correlated with urea. With respect to other metabolites in the pentose metabolism pathway, ribonate had strong correlations with arabitol/xylitol and arabonate/xylonate (Supplementary Table 6). Arabitol/xylitol was associated with mortality in both MDRD and AASK, and arabonate/xylonate was associated with mortality in MDRD only.

Association between uremic retention solutes and mortality

Among the 21 available uremic toxins identified by the European Uremic Toxin Workgroup⁹, a few had strong associations with mortality in unadjusted analyses, but none met a Bonferroni-adjusted threshold for statistical significance in analysis adjusted for clinical covariates in more than one study (Supplementary Table 7).

DISCUSSION

This study is a rigorous evaluation of the association between metabolites identified through untargeted profiling and mortality in 1 262 people with CKD using a discovery and replication design, as well as careful adjustment for kidney function with protocol-measured GFR by ¹²⁵I-iothalamate clearance and proteinuria. Three biologically plausible metabolites were consistently associated with higher risk of mortality in both the MDRD and AASK studies: fumarate, allantoin, and ribonate. For all three metabolites, Kaplan-Meier survival curves separated at the medians of the metabolites showed good discrimination, with higher levels of metabolites associated with higher mortality. These metabolites may represent targets for treatment and investigation of pathophysiologic mechanisms.

Our study showed three metabolites that were reproducibly associated with mortality in both the MDRD study and AASK. One of the identified metabolites, allantoin, has previously been proposed as a marker of oxidative stress. Levels of allantoin have been shown to rise in muscle after intense physical activity and in serum in rheumatoid arthritis.^{10,11} Allantoin is a byproduct of urate (or uric acid), which is non-enzymatically converted to allantoin in superoxidative states. However, allantoin can also be produced from uric acid in a reaction catalyzed by the enzyme uricase. Uricase is not endogenous to humans,¹² but it can be

present in gut bacteria. Animal studies have suggested that uric acid metabolism is disrupted in CKD, with diminished expression of xanthine oxidase (the enzyme which catalyzes the formation of uric acid) and increased intestinal secretion of uric acid.¹³ In ESRD, there is a greater proportion of uricase-containing bacteria compared to healthy controls and higher levels of serum allantoin but not uric acid, hypoxanthine or xanthine.^{14,15} This is consistent with our findings of only weak correlations between allantoin and other metabolites in the (hypo)xanthine-inosine-containing purine pathway. Higher levels of allantoin could thus be a proxy for higher oxidative stress, or represent an adaptation for poor kidney function, or represent higher uric acid production, which itself has been associated with increased blood pressure and mortality.^{16,17}

The second replicated metabolite, fumarate, is an intermediate in the citric acid cycle, the product of succinate, and precursor of malate. Fumarate is also an inhibitor of the hydroxylation of hypoxia-inducible factor (HIF). Under normal conditions, hydroxylation of HIF allows it to be ubiquitinated by Von Hippel Lindau (VHL) complex and thus degraded. If hydroxylation of HIF fails to occur (such as hypoxic conditions or elevated fumarate), HIF evades ubiquitination by VHL and can activate the transcription of vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), platelet derived growth factor (PDGF), and transforming growth factor- α (TGF- α).¹⁸ In diabetic kidney disease, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform 4 (NOX4)¹⁹ is upregulated in podocytes, which in turns inhibits fumarate hydratase, causing a chronic accumulation of fumarate. In animal studies, the loss of fumarate hydratase impaired the cellular antioxidant response, inhibited hydroxylation of HIF, and caused disordered growth via TGF- β .^{19,20}

The third replicated metabolite, ribonate, arises from dehydrogenation and dephosphorylation of ribose-5-phosphate, an intermediate of the pentose phosphate pathway. Experiments in rats demonstrated that experimentally-induced diabetes led to increased activity of the pentose phosphate pathway's key enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and that this activity correlated positively with the degree of renal hypertrophy.^{21,22} Urine ribonate has also been shown to be increased in non-proteinuric patients with diabetes and low GFR.²³ We observed strong correlations between pentose pathway metabolites, with consistent associations with mortality, supporting a role for this pathway in mortality among patients with CKD.

Evidence for the biologic role of the other metabolites that were found to be significant in meta-analysis is varied. N-acetylcarnosine, which had a protective association against mortality in our meta-analysis, is used to treat cataracts as its major byproduct, L-carnosine, has an anti-oxidative effect on lens tissue.²⁴ Hydroxycotinine is a primary metabolite of nicotine present in tobacco, a well-known cause of mortality, and has been similarly implicated in a metabolomic study of the general population.^{7,25} The association of hydroxycotinine with mortality even after adjusting for smoking history suggests that hydroxycotinine levels represent either a continuous effect not captured by the binary categorization of smoking, smoking exposure via secondhand smoke, or incorrectly-reported smoking status.⁷ Interestingly, all replicated metabolites were positively correlated with each other, urea, and urate, and negatively correlated with GFR (except malate). Urea is the chief

degradation product of amino acids, whereas urate is created through the degradation of nucleic acids. In CKD, both urea and uric acid have increased flux to the gut with metabolism by gut flora.¹³ Subsequent studies might delineate the role of the microbiome in determining serum levels of these metabolites.

Our study adds to the existing literature by reporting metabolomic associations in populations with CKD, and by evaluating a larger number of metabolites identified through untargeted profiling. Yu et al evaluated the association between 204 metabolites and all-cause mortality in the Atherosclerosis Risk in Communities (ARIC) study, a general population cohort.⁷ Of their eight metabolite associations (gamma-glutamylleucine and uridine which were negatively associated with mortality, and hydroxycotinine, α -hydroxyisovalerate, N-acetylalanine, pregnenolol disulfate, androsteroid monosulfate 2, and glycocholate which were positively associated), only hydroxycotinine was significant in our meta-analysis. Reasons for differences in metabolite associations might include differing baseline characteristics of the study population, notably the much lower GFR in our study. In a separate study, Fischer examined the association of 106 metabolites with all-cause mortality in the Estonian Biobank, another general population cohort.⁶ Most of their metabolomic hits were large molecules or molecular complexes, including α -1-acid glycoprotein, albumin, and very low density lipoprotein (molecular weights of 23 512; 69 367; and 100 000 000 Daltons, respectively), and thus not included in our panel.^{26–28} The fourth metabolite, citrate, had no association with mortality in either AASK or MDRD.

Interestingly, none of the previously named uremic retention solutes had significant associations with mortality in our studies, including indoxyl sulfate, p-cresyl sulfate, and trimethylamine-*N*-oxide (TMAO).^{29,30} Previous studies using targeted assays suggested that indoxyl sulfate, a product of tryptophan metabolism that induces oxidative stress and impairs endothelial healing, was associated with cardiovascular mortality, aortic calcification and pulse wave velocity in CKD patients.^{31–33} A related protein-bound metabolite produced in the gut, p-cresyl sulfate, has been associated with all-cause mortality in CKD patients,³⁴ and TMAO, a product of choline metabolism that suppresses reverse cholesterol transport, has been associated with accelerated atherosclerosis and all-cause mortality.^{35,36} The lack of association in our study may be due to differences in study populations, notably baseline kidney function, or imprecision inherent in untargeted platforms. We also note that adjustment for GFR may affect relationships, since each of the uremic toxins is highly correlated with GFR; not all previous studies included GFR as a covariate.

Our study has several strengths. We used a rigorous study design, quality-controlled metabolomics profiling, discovery and replication cohorts, adjustment for carefully collected clinical covariates including ¹²⁵I-iothalamate clearance and 24-hour urine protein, and correction for multiple comparisons. Previous studies of mortality and the metabolome have evaluated only subjects with preserved GFR, without adjustment for proteinuria and measured GFR. Our study evaluated associations with 622 metabolites, greater than the 204 and 106 metabolites examined thus far, allowing us to identify previously unexamined associations.^{6,7}

However, there were certain limitations as well. As with any observational study, we cannot determine causal effects, and there can be multiple interpretations of a significant association. Significant positive associations between a metabolite and mortality may indicate that the metabolite of interest is directly toxic or that the metabolite of interest is a byproduct of a different pathological process associated with mortality (e.g., hydroxycytinine). Our discovery and replication cohorts were very different in terms of racial distribution and baseline GFR; however, we view these differences as a strength: metabolites that were associated with mortality in both studies provide stronger evidence for a possible biological role. The Metabolon platform detects a large number of compounds in a process designed to minimize process variability; however, this necessitates conservative statistical thresholds and results in fewer volatile compounds to assess. Thus, we may have missed compounds that have a role in mortality. Our discovery cohort was small, limiting power. However, we also showed metabolites that reached statistical significance in meta-analysis including all 1262 participants.

In summary, we conducted an untargeted metabolomics screen for risk factors for mortality using a discovery and replication approach. We found fumarate, allantoin, and ribonate to be reproducible risk factors for mortality in two cohorts of participants with CKD. With confirmation in additional studies using absolute quantification of metabolites, the identified metabolites may serve as targets for future investigation for therapeutic and pathophysiologic purposes.

METHODS

MDRD population and design

The MDRD study was a multi-center, randomized clinical trial that investigated the effects of two levels of prescribed dietary protein and two levels of target blood pressure (BP) in slowing GFR decline.³⁷ The trial design and baseline characteristics have been described.^{38,39} In brief, eligible participants were between 18–70 years of age, had a measured glomerular filtration rate (GFR) between 25–55 mL/min/1.73m² in Study A and 13 and 24 mL/min/1.73 m² in Study B, and excluded people with insulin dependent diabetes and urinary total protein excretion <10 g/day.⁴⁰ A total of 840 participants were enrolled between 1989 and 1991. We included the 299 participants with available serum and who had CKD due to hypertension (N=111) or other non-specific causes of nephritis (N=188).⁴¹ Vital status was tracked until 2009 by cohort follow-up and linkage to the National Death Index.

The baseline for our study was the 12-month post-randomization (F12) visit in 1990–1992. Phlebotomy protocol was as follows. Blood was allowed to clot at room temperature for between 30 minutes and 2 hours, centrifuged, and frozen at -70°C for up to a month until shipment on dry ice to the MDRD Central Laboratory, where the samples were aliquoted and frozen at -70°C.⁴² All laboratory processing was performed at the MDRD Central Biochemistry Laboratory at the Cleveland Clinic. This included a 24-hour urine sample, from which total protein was measured by trichloroacetic acid-Ponceau S solution, as well as a fasting morning blood sample, from which serum total and HDL cholesterol were measured on a Hitachi 705, and serum creatinine, uric acid and urea nitrogen were measured on a Beckman Astra 8.^{43,44} Urine creatinine was measured using the modified Jaffe method

with an alkaline picrate assay. GFR was measured by urinary clearance of ^{125}I -iothalamate.
⁴⁵ All self-reported covariates (including cigarette smoking) were assessed at baseline.

AASK population and design

The AASK study was a multi-center, randomized clinical trial that investigated the effects of prescribed antihypertensive agents and two levels of target BP in slowing GFR decline. The trial design and baseline characteristics have been described.^{46,47} In brief, eligible participants were 18–70 years of age, African-American, with hypertension (diastolic BP 95 mmHg) and a GFR of 20–65 mL/min/1.73m². Exclusion criteria included diabetes, known glomerular or polycystic kidney disease, and 24-hour urinary protein to creatinine ratio > 2.5.⁴⁸ A total of 1094 participants were enrolled between 1995 and 1998, among which 963 participants had serum samples and complete event data available for our study. Vital status was tracked until 2007 by cohort follow-up and linkage to the Social Security Death Index.⁴⁸

The baseline for our study was the trial baseline visit in 1995–1998. The phlebotomy protocol was as follows. Blood was allowed to clot at room temperature for 30 minutes, centrifuged, and refrigerated at –20°C for a few days until shipment on dry ice to the AASK Central Laboratory, where the samples were aliquoted and frozen at –70°C.⁴⁹ All laboratory processing was performed at the AASK Central Laboratory at the Cleveland Clinic. This included a 24-hour urine sample, from which total protein was measured by trichloroacetic acid-Ponceau S solution, as well as a fasting morning blood sample, from which serum total and HDL cholesterol were measured on a Hitachi Modular Analytics analyzer, and serum creatinine, uric acid and urea nitrogen were measured on a Beckman CX3 Delta.^{44,48} Urine creatinine was measured using the modified Jaffe method with an alkaline picrate assay. GFR was measured by urinary clearance of ^{125}I -iothalamate.⁵⁰ All self-reported covariates (including cigarette smoking) were assessed at baseline. Participants reported if they smoke occasionally, regularly, or never, the first two of which we coded as “yes” for current smoking.

Measurement of the metabolome

Frozen serum samples from the 12-month visit (F12) for MDRD and the baseline (G1) visit were sent to Metabolon, Inc. (Durham, North Carolina) for metabolite profiling. Upon receipt, samples were stored at –80°C until processing via the following protocol. First, proteins in the serum samples were precipitated using methanol.⁵¹ The extract was then divided into five parts for analysis, two for reverse-phase ultra-high performance liquid chromatography-tandem mass spectrometry (RP/UPLC-MS/MS) with positive ion mode electrospray ionization (ESI), one for RP/UPLC-MS/MS with negative ion mode ESI, one for hydrophilic interaction chromatography UPLC-MS/MS with negative ion mode ESI, and one sample for backup.

Metabolites were identified by comparison of mass to charge ratio (m/z), retention time/index (RI), and chromatographic to a reference spectral library of purified standards.⁵² Peaks were quantified by area under the curve (AUC). Raw area counts for each metabolite in each sample were normalized to correct for variation from instrument inter-day tuning differences

by the median value for each run-day. Several controls were also evaluated: pooled samples comprehensively characterized by Metabolon; ultra-pure water samples to function as process blanks; and several standards known not to interfere with endogenous compounds spiked at fixed concentrations into every analyzed sample. Instruments were tuned and calibrated for mass resolution and mass accuracy daily, and controls were interspersed evenly across injections. Instrument variability was assessed as the median relative standard deviation for the quality control standards that were added into each sample (5% for MDRD, 6% for AASK), and process variability was assessed as the median relative standard deviation for the endogenous metabolites in the pooled matrix samples (11% for MDRD, 10% for AASK). Both estimates of instrument variability and total process variability met Metabolon's internal acceptance criteria.

There were 20 blind duplicate samples run for each study. In MDRD, 68% of the metabolites had a correlation >0.80 , and in AASK, 88% of the metabolites had a correlation >0.80 . We also compared the serum metabolite creatinine (assessed in the untargeted metabolomic profiling approach) and creatinine assessed by the Jaffe method (during the conduct of the trials) in the full populations. In MDRD, the correlation was 0.96. In AASK, the correlation was 0.89.

Analytic Data Preparation

Metabolomic profiling was performed in 2015 for the MDRD study and identified 1 193 metabolites (766 named, 427 unnamed). In AASK, metabolomics profiling was performed in 2017 and identified 1 228 metabolites (833 named, 395 unnamed). Because of advancements in the size of the spectral library between 2016 and 2017, some of the named metabolites identified in AASK were not present in MDRD, and thus we used only the shared metabolites for discovery ($N=622$). We followed a standard data cleaning protocol for both studies: Sample-wise, we excluded samples with $>50\%$ missing in all metabolites (1 sample was removed in MDRD; 1 sample was removed in AASK). Metabolite-wise, we excluded metabolites with $>80\%$ missing across samples in either study ($N=14$). Remaining metabolites with undetectable values were imputed to the minimum measured value of that metabolite. All metabolites were scaled to a median of 1 and $\log^2$ -transformed. Log-transformed values did not exceed 5 standard deviations above the mean and thus no capping was performed. After these steps, 622 metabolites remained for analysis in the MDRD and AASK studies.

Statistical analysis

Baseline covariates were summarized using means and standard deviations or percentages. Cox proportional hazards regression was used to assess the association between each of the metabolites and mortality, with adjustment for age, sex, smoking history, history of coronary heart disease, body mass index, systolic blood pressure, urine protein, measured glomerular filtration rate, and study arms. Study arms in MDRD were study A/B, diet arm, and blood pressure arm. Study arms in AASK were drug arm and blood pressure arm. Adjustment was done for diabetes status and race in the MDRD Study but not AASK, which by design only included African Americans without diabetes. Serum HDL and total cholesterol were not included as covariates since they did not associate with mortality in either cohort.

Associations were assessed in each cohort individually. Kaplan-Meier plots for each study were generated with stratification on median levels of the replicated metabolites. Correlations were assessed between metabolites and clinical covariates using Spearman's correlation.

For discovery, the threshold for significance was set *a priori* to a p-value <0.001 given the plan for subsequent replication. Replication used Bonferroni correction for multiple comparisons, dividing 0.05 by the number of comparisons. For example, if 6 metabolites were identified in the discovery cohort, the threshold for significance was 0.05/6 in the replication cohort. In the correlation analysis with 15 metabolites and covariates, the threshold was 0.05/(15 choose 2). In exploratory analyses, we analyzed associations between all 622 metabolites and mortality using fixed effects meta-analysis. In the meta-analysis, the threshold for statistical significance was also set by Bonferroni correction, or 0.05/625 metabolites. All analyses were performed in R (R Foundation, Vienna, Austria) and STATA/IC 14.2 (Stata Corp., College Station, TX).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hazard ratio and p-values for 622 metabolite associations with mortality in the Modification of Diet in Renal Disease Study (N=299)

* The 6 metabolites with associations with mortality at $p < 0.001$ are identified in red. The model adjusted for age, sex, smoking history, history of coronary heart disease, body mass index, systolic blood pressure, log urinary protein, measured glomerular filtration rate, study arms, diabetes and race.

Figure 2.

P-values of the 622 metabolites' adjusted associations with mortality in the Modification of Diet in Renal Disease study (N=299) on the x axis and in the African American Study of Kidney Disease on the y axis (N=963)

* The 3 metabolites with associations with mortality at $p < 0.008$ ($.05/6$) are identified in red. Analyses were adjusted for age, sex, smoking history, history of coronary heart disease, body mass index, systolic blood pressure, log urinary protein, measured glomerular filtration rate, and study arms.

Figure 3.

Kaplan-Meier curves of survival in the Modification of Diet in Renal Disease study (top row) and the African American Study of Kidney Disease and Hypertension (bottom row) stratified by median levels of the replicated metabolites (solid line: median; dotted line, <median)

* Log rank tests in MDRD for fumarate, ribonate, and allantoin (left to right) were associated with p values of 0.03, 0.001, and 0.19. Log rank tests in AASK for fumarate, ribonate, and allantoin (left to right) were associated with p values of 0.03, 2.3E-6, and 0.02.

Figure 4.

Hazard ratios (HR) and 95% confidence intervals (CI) for associations between metabolites and mortality in the Modification of Diet and Renal Disease and African American Study of Kidney Disease and Hypertension individually as well as across both studies using fixed effects meta-analysis.

HRs were estimated in Cox regression models with adjustment for age, sex, smoking history, history of coronary heart disease (cardiovascular disease, coronary artery disease, or peripheral vascular disease), body mass index (BMI), systolic blood pressure (SBP), urine protein, measured glomerular filtration rate (mGFR), and study arms. HRs reflect risk per doubling of metabolite. In the MDRD study, adjustment was also done for diabetes status and race.

Table 1

Baseline characteristics of participants from the Modification of Diet in Renal Disease study (MDRD) and African-American Study of Kidney Disease and Hypertension (AASK) included in the metabolomics study

	MDRD	AASK
N	299	963
Age, mean (SD)	55 (12)	55 (11)
Female, n (%)	126 (42%)	375 (39%)
African-American, n (%)	23 (8%)	963 (100%)
Diabetes, n (%)	31 (10%)	0 (0%)
Coronary heart disease, n (%)	53 (18%)	498 (52%)
Smoking, n (%)	27 (9%)	561 (58%)
Body mass index in kg/m ² , mean (SD)	27 (4)	31 (7)
Systolic blood pressure in mmHg, mean (SD)	130 (18)	150 (24)
Urine Protein in g/day, mean (SD)	0.69 (1.04)	0.53 (0.95)
Glomerular filtration rate in ml/min/1.73 m ² , mean (SD)	30 (13)	47 (14)
High-density lipoprotein in mg/dL, mean (SD)	41 (15)	48 (16)
Total Cholesterol in mg/dL, mean (SD)	203 (42)	212 (46)