



Serum Metabolites and Kidney Outcomes: The Atherosclerosis Risk in Communities Study

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Rationale & Objective: Novel metabolite biomarkers of kidney failure with replacement therapy (KFRT) may help identify people at high risk for adverse kidney outcomes and implicated pathways may aid in developing targeted therapeutics.

Study Design: Prospective cohort.

Setting & Participants: The cohort included 3,799 Atherosclerosis Risk in Communities study participants with serum samples available for measurement at visit 1 (1987-1989).

Exposure: Baseline serum levels of 318 metabolites.

Outcomes: Incident KFRT, kidney failure (KFRT, estimated glomerular filtration rate <15 mL/min/1.73 m², or death from kidney disease).

Analytical Approach: Because metabolites are often intercorrelated and represent shared pathways, we used a high dimension reduction technique called Netboost to cluster metabolites. Longitudinal associations between clusters of metabolites and KFRT and kidney failure were estimated using a Cox proportional hazards model.

Results: Mean age of study participants was 53 years, 61% were African American, and 13% had diabetes. There were 160 KFRT cases and 357 kidney failure cases over a mean of 23 years. The

314 metabolites were grouped in 43 clusters. Four clusters were significantly associated with risk of KFRT and 6 were associated with kidney failure (including 3 shared clusters). The 3 shared clusters suggested potential pathways perturbed early in kidney disease: cluster 5 (15 metabolites involved in alanine, aspartate, and glutamate metabolism as well as 5-oxoproline and several gamma-glutamyl amino acids), cluster 26 (6 metabolites involved in sugar and inositol phosphate metabolism), and cluster 34 (21 metabolites involved in glycerophospholipid metabolism). Several individual metabolites were also significantly associated with both KFRT and kidney failure, including glucose and mannose, which were associated with higher risk of both outcomes, and 5-oxoproline, gamma-glutamyl amino acids, linoleoylglycerophosphocholine, 1,5-anhydroglucitol, which were associated with lower risk of both outcomes.

Limitations: Inability to determine if the metabolites cause or are a consequence of changes in kidney function.

Conclusions: We identified several clusters of metabolites reproducibly associated with development of KFRT. Future experimental studies are needed to validate our findings as well as continue unraveling metabolic pathways involved in kidney function decline.

Visual Abstract included

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Progressive kidney decline can result in kidney failure with replacement therapy (KFRT) or death from kidney disease. KFRT is associated with many comorbid conditions that can decrease quality of life, such as anemia, acidosis, and mineral and bone disorders, and has an exceedingly high mortality rate.^{1,2} Further, kidney failure produces significant financial costs for the healthcare system, with annual Medicare spending for patients with KFRT totaling \$35.9 billion in 2017, and the US prevalence is expected to increase by 29%-68% through 2030.^{1,3} In order to better prevent and treat kidney failure, we need to better understand the biological underpinnings of kidney disease development and identify early predictors of kidney outcomes.

Metabolomics has been increasingly used to understand the underlying biology of kidney disease.⁴⁻⁶ Studies have identified several individual metabolites related to risk of incident chronic kidney disease (CKD), such as 5-oxoproline and 1,5-anhydroglucitol in the Atherosclerosis Risk in Communities (ARIC) study.⁴ In populations with diabetes, a handful of candidate metabolites have also

been used to chart glomerular filtration rate trajectories and kidney failure risk.⁷⁻¹¹ However, because metabolites are often highly intercorrelated, the standard approach of screening for associated metabolites using a Bonferroni correction has been criticized as overly conservative (ie, resulting in many false negatives).¹² Grouping metabolites into related clusters before investigating their associations with outcomes may help to limit the number of comparisons and illuminate potentially meaningful biological pathways.

In this study, we used a clustering approach to identify novel metabolomic biomarkers and pathways of KFRT and kidney failure in a general population sample. We employed a dimension reduction technique to create eigenclusters of correlated metabolites, allowing us to explore cumulative effects of highly related metabolites and evaluate for potentially important shared pathways. In the secondary analysis, we examined individual component metabolites from significant eigenclusters to determine which metabolites were most significantly associated with the outcomes.

PLAIN LANGUAGE SUMMARY

Metabolites are small breakdown products of metabolism. Early metabolic markers of kidney damage could help with patient risk stratification and prognostication. However, metabolites are often interrelated, and their shared biological pathways are not well understood. In this study, we used an unsupervised approach to group metabolites and then related these groups to the development of kidney failure. We found several metabolite groups that represent plausible biological pathways disrupted early in the course of kidney disease.

METHODS

Study Population

The ARIC study is a prospective community-based cohort of 15,792 individuals who were recruited and enrolled between 1987 and 1989 from 4 US communities (Forsyth County, North Carolina; Jackson, Mississippi; Minneapolis suburbs, Minnesota; and Washington County, Maryland). Details on the ARIC study design and methods were previously published.¹³ Participants attended follow-up visits every 3 years for the first 9 years of the study, followed by visit 5 between 2011 and 2013, visit 6 between 2016 and 2017, and visit 7 between 2018 and 2019. Institutional review boards at each field center approved the study, and written informed consent was obtained from participants at baseline and follow-up visits. The Johns Hopkins Bloomberg School of Public Health Institutional Review Board approved this study (IRB00012998; IRB00009957; IRB00011012).

Serum samples were collected at visit 1 and stored at -80°C until metabolomic profiling was performed. For this study, a random selection of samples from 1,977 Black participants from the Jackson, Mississippi field center was sent for profiling by Metabolon, Inc in 2010, followed by a second random selection of samples from an additional 2,055 participants from all 4 sites profiled by Metabolon in 2014. Participants were excluded from the current study if they had prevalent CKD, defined as an estimated glomerular filtration rate (eGFR) <60 mL/min/ 1.73 m² at baseline ($n = 67$), prevalent KFRT or kidney failure ($n = 3$), were missing covariate measurements ($n = 160$), or missing metabolite measures ($n = 3$). A total of 3,799 participants were included in this analysis. The collection obtained exclusively from Jackson, Mississippi constituted our training dataset. The sample derived from all 4 sites served as our validation dataset.

Assessment of Metabolites

Baseline fasting serum samples were assayed by Metabolon using an untargeted, gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry-based metabolomic protocol.^{14,15} Metabolomic assessment and

data cleaning was previously described.^{4,16,17} Metabolites were excluded if more than 80% of samples had missing values in either our training or validation set, log-scale variance was < 0.01 or variance was missing, exogenous metabolites had missing values, and metabolites were not detected in both sets (Figure S1). A total of 318 metabolites were included in this analysis. Endogenous metabolites with missing values were imputed with the lowest value detected in each set. For quality control, 97 samples were analyzed with both the training set and validation set, with a median correlation coefficient of 0.71 across 285 metabolites, as reported previously.¹⁸

Outcomes

Incident KFRT was defined as the initiation of kidney replacement therapy (either dialysis or transplant), which was identified through linkage with the US Renal Data System registry last updated in July 2017.¹⁹

We also included a composite kidney failure outcome. Incident kidney failure was defined by meeting at least 1 of the following criteria: eGFR <15 mL/min/ 1.73 m² at a study visit, KFRT identified through linkage to US Renal Data System registry, or use of one of the previously validated *International Classification of Diseases, Ninth/Tenth Revision* (ICD-9/10) codes during a hospitalization or in a death certificate.²⁰ The main goal of this outcome was to additionally capture individuals who chose not to initiate kidney replacement therapy or those who died prior to inclusion in the US Renal Data System registry.

Assessment of Covariates

Baseline covariate data was collected at visit 1. Participants' age, sex, race, study center, and smoking status were collected from interviewer-administered questionnaires. Age was modeled as a continuous variable. Similar to previous ARIC analyses, we combined participant's race and study center into a "race-center" variable, because of study centers containing nonuniform racial distributions.²¹ Smoking status was treated as a categorical variable with categorization including current, former, and never/unknown. Two participants had unknown smoking history.

Clinical covariates included diabetes, history of coronary heart disease, use of hypertension medications, body mass index, systolic blood pressure, baseline eGFR, and high-density lipoprotein cholesterol. Diabetes was defined as nonfasting blood glucose ≥ 200 mg/dL, fasting blood glucose ≥ 126 mg/dL, self-reported history of diabetes mellitus, diagnosed by a physician, or reported use of diabetic medications. History of coronary heart disease was defined as meeting 1 of these criteria: self-reported diagnosis of myocardial infarction, prior coronary revascularization, or silent myocardial infarction on electrocardiogram. Systolic blood pressure was measured 3 times with a random zero sphygmomanometer after the participant rested for 5 minutes. The first measurement was dropped, and the remaining 2 measurements were averaged.²²

Creatinine was measured at visit 1 in serum samples using the modified kinetic Jaffe method. eGFR was calculated using the creatinine-based 2009 chronic kidney disease Epidemiology equation.²³

Statistical Analysis

We used Cox proportional hazards regression models to estimate the association between exposure (clusters and individual metabolites) and kidney outcomes. Time at-risk began at visit 1 (1987-1989) and concluded at the earliest occurrence of the respective outcome (KFRT or kidney failure), death, loss to follow-up, or administrative censoring. The administrative censoring date for KFRT was December 31, 2017. For kidney failure, participants were censored on December 31, 2019.

We performed longitudinal analyses using clusters of metabolites as the exposure for the primary analysis. To do this, metabolite residuals were estimated separately in each set by regressing the log₂-transformed metabolite on all covariates. We selected model covariates based on known risk factors for adverse kidney outcomes.²⁴ Our model adjusted for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking status, eGFR, and high-density lipoprotein cholesterol. Clusters were then formed using metabolite residuals from the training dataset with the Netboost dimension reduction technique.²⁵ Netboost clusters metabolite residuals using a sparse hierarchical clustering based on Spearman correlations and then aggregates clusters using the first principal component.²⁶ Clustering was performed with a minimum cluster size of 2, a soft power exponent of 2, and a module dissimilarity threshold of 0.2. The clustering was then applied to the validation dataset and principal components refitted for each module. Metabolite cluster values were normalized to their standard deviation in each set. Then, we assessed associations between metabolite clusters and KFRT and kidney failure separately within our training and validation sets and pooled estimates using fixed effects meta-analysis. For clusters significantly associated with KFRT or kidney failure, we tested the proportional hazards' assumption with direct visualization and time interaction.

For those clusters with significant associations with either KFRT or kidney failure in the meta-analysis, we assessed associations of individual component metabolites with the outcomes. For clusters that were significantly associated with both outcomes, we also assessed the associations between the first principal component and baseline characteristics using χ^2 test and analysis of variance for categorical and continuous variables, respectively, to better understand which phenotypes may be represented by these clusters. Bonferroni correction was used to adjust the statistical significance level for the number of exposures tested. Statistical analyses were conducted using R (<http://www.r-project.org>) 4.0.5 and Stata 16.1.

RESULTS

Study Population Characteristics

Baseline characteristics of the 3,799 participants are shown in Table 1. The training dataset was 100% African American, whereas the validation dataset was 28% African American. Participants from the training set were more often female, had higher baseline eGFR, and reported greater usage of antihypertensive medications than participants in the validation set.

Metabolite Clusters

Clustering using Netboost in the training dataset formed 43 clusters of metabolites, with 4 of the 318 metabolites not included in a cluster (Table S1). There was a median of 6 metabolites per cluster, with a range of 2 to 33 metabolites per cluster (Figure S2).

Associations of Metabolite Clusters with KFRT and Kidney Failure

A total of 160 participants developed KFRT during a mean follow-up of 23 years. Four metabolite clusters (clusters 26, 5, 34, and 1) were significantly associated with KFRT in meta-analysis, adjusting for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking, eGFR, and high-density lipoprotein cholesterol (Figure 1). Cluster 26 had a hazard ratio of 1.31 per standard

Table 1. Baseline Characteristics of Participants

Characteristics	Training Set	Validation Set
No. of participants	1,773	2,026
Age (y)	52.8 (5.7)	54.2 (5.7)
Female, n (%)	1,130 (63.7%)	1,140 (56.3%)
Black	1,773 (100%)	559 (27.6%)
Forsyth County, North Carolina	0 (0%)	577 (28.5%)
Jackson, Mississippi	1,773 (100%)	430 (21.2%)
Minneapolis Suburbs, Minneapolis	0 (0%)	517 (25.5%)
Washington County, Maryland	0 (0%)	502 (24.8%)
Antihypertensive medication use	673 (38.0%)	537 (26.5%)
Diabetes	282 (15.9%)	230 (11.4%)
History of CHD	68 (3.8%)	119 (5.9%)
Current Smoker	503 (28.4%)	555 (27.4%)
Former Smoker	412 (23.2%)	652 (32.2%)
Never/unknown smoker	858 (48.4%)	819 (40.4%)
eGFR (mL/min/1.73 m ²)	113 (16.6)	102 (15.1)
SBP (mm Hg)	128 (21.0)	122 (19.7)
Cholesterol (mg/dL)	215 (42.5)	215 (42.5)
HDL-C (mg/dL)	55.3 (15.5)	51.4 (19.3)
BMI (kg/m ²)	29.6 (6.1)	28.0 (5.5)

Note: Entries are mean (standard deviation) or n (%).

Abbreviations: BMI, body mass index; CHD, coronary heart disease; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure.

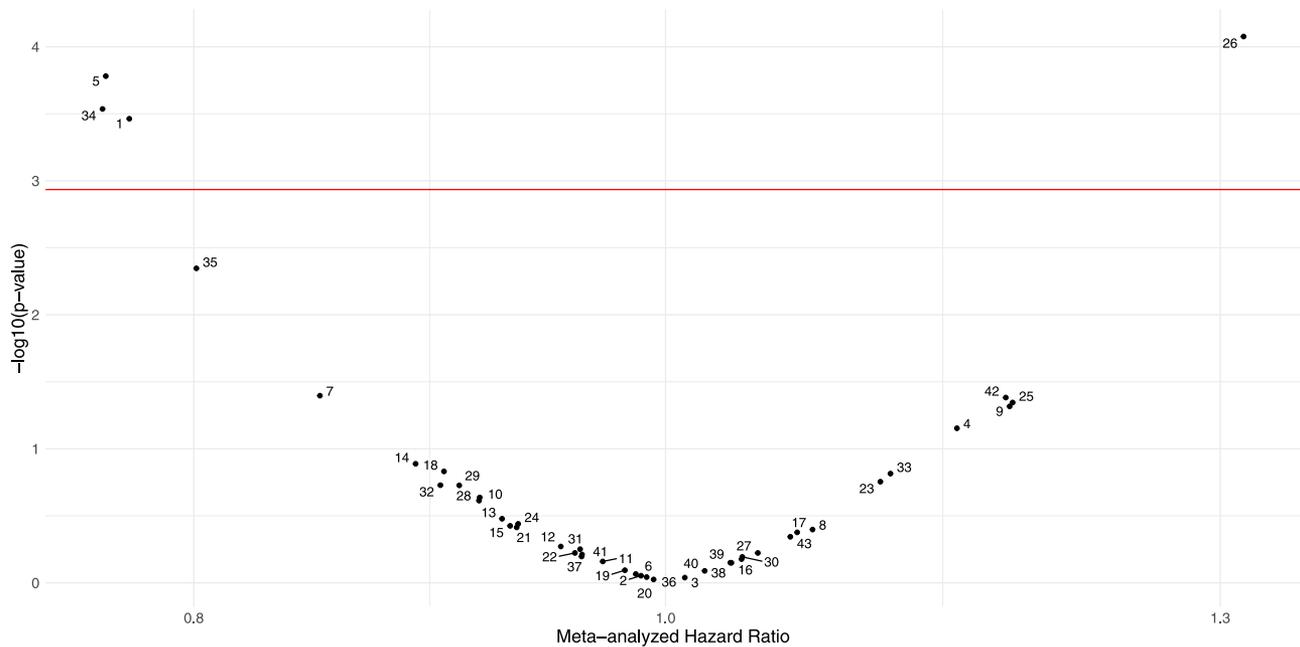


Figure 1. Volcano plot of meta-analyzed associations for clusters with kidney failure with replacement therapy. Adjusted for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking, estimated glomerular filtrated rate based on creatinine, and high-density lipoprotein cholesterol. Red horizontal line represents the Bonferroni-adjusted threshold calculated as $0.05/43$ clusters = 0.001.

deviation (95% confidence interval, 1.15-1.51), whereas clusters 5, 34, and 1 had hazard ratios ranging from 0.77-0.78 per standard deviation (Table 2). Cluster 26 included 6 metabolites that were monosaccharides, disaccharides, or alcohol sugars involved in glycolysis and anaerobic metabolism. These metabolites included dimethylarginine (symmetric dimethylarginine [SDMA] + asymmetric dimethylarginine [ADMA]), glucose, and mannose. Cluster 5 was an assortment of 15 modified and traditional amino acids seen in liver metabolism involving glutathione and gamma-glutamyl transferase. Cluster 34 included 21 metabolites that were primarily lysolipids from the phosphocholine family. Cluster 1 was composed of 4 traditional amino acids and 2 monosaccharides—1,5-anhydroglucitol (1,5-AG) and fructose—with roles in a variety of metabolic pathways such as amino acid metabolism, the urea cycle, glycolysis, and gluconeogenesis.

There were 357 kidney failure events over a mean follow-up of 23 years. The previously identified clusters 26, 5, and 34 were significantly associated with kidney failure in a directionally consistent pattern to their association with KFRT, whereas cluster 1 just missed the Bonferroni-adjusted significance level for kidney failure ($P = 0.0015$ vs Bonferroni $P = 0.0012$) (Figure 2). Three additional clusters were significantly associated with kidney failure (Table 3). Cluster 14 had a hazard ratio of 0.79 per standard deviation (95% confidence interval, 0.72-0.88). Clusters 25 and 9 were both associated with an increased risk of kidney failure. Cluster 14 was associated with kidney failure and consisted of 7 metabolites with

inosine, hypoxanthine, and guanosine serving critical functions for the purine synthesis pathway and the formation of uric acid (Table S2). Cluster 25 represented 5 molecules involved in the urea cycle, such as citrulline and 4-acetamidobutanoate. Cluster 9 included 2 metabolites: homocitrulline, a derivative of ornithine, and N-acetyl-1-methylhistidine, an urea cycle byproduct.

Associations of Individual Metabolites in Implicated Clusters

There were 42 metabolites in the 4 clusters significantly associated with KFRT. Thirteen of these metabolites had a statistically significant association with KFRT after Bonferroni adjustment ($P = 0.05/42$) (Figure 3). These included: 1,5-AG and fructose (cluster 1); glucose and mannose (cluster 26); 2 gamma-glutamyl amino acids and 5-oxoproline, a glutamic acid derivative (cluster 5); and 6 phosphocholine lysolipids (cluster 34). In a secondary analysis of all 318 individual metabolites regardless of cluster membership, tryptophan and tyrosine were also significantly associated with KFRT after Bonferroni adjustment ($P = 0.05/318$) (Figure S3).

A total of 56 metabolites were included in the 6 clusters significantly associated with kidney failure. Of these, 22 metabolites had statistically significant associations with kidney failure after Bonferroni adjustment ($P = 0.05/56$) (Figure 4). These included: glucose and mannose (cluster 26); 8 gamma-glutamyl amino acids and 5-oxoproline (cluster 5); 9 phosphocholine lysolipids (cluster 34); inosine and hypoxanthine (cluster 14); and homocitrulline

Table 2. Association between Clusters and Kidney Failure with Replacement Therapy

Cluster	Component Metabolites	Training Set (N = 1,773; KFRT = 89)			Validation Set (N = 2,026; KFRT = 71)			Meta-Analyzed (N = 3,799; KFRT = 160)		
		HR	95% CI	P	HR	95% CI	P values	HR	95% CI	P
26	dimethylarginine (SDMA + ADMA), glucose, trehalose, mannose, mannitol, and myo-inositol	1.35	1.13-1.61	< 0.001	1.27	1.02-1.57	0.03	1.31	1.15-1.51	< 0.001
5	asparagine, glutamine, 5-oxoproline, gamma-glutamylalanine, gamma-glutamylglutamate, gamma-glutamylglutamine, gamma-glutamylisoleucine, gamma-glutamylleucine, gamma-glutamylthreonine, gamma-glutamylvaline, threonine, glutamate, gamma-glutamylphenylalanine, gamma-glutamyltyrosine, and DSGEGDFXAEAGGVR	0.72	0.60-0.86	< 0.001	0.84	0.68-1.04	0.12	0.77	0.67-0.88	< 0.001
34	stearoylcarnitine, 1-docosahexaenoyl-GPC (22:6n3), 2-myristoyl-GPC, 1-pentadecanoyl-GPC (15:0), 1-palmitoyl-GPC (16:0), 2-palmitoyl-GPC, 1-palmitoleoyl-GPC (16:1), 1-margaroyl-GPC (17:0), 1-stearoyl-GPC (18:0), 2-stearoyl-GPC, 1-oleoyl-GPC (18:1), 2-oleoyl-GPC, 1-linoleoyl-GPC (18:2n6), 2-linoleoyl-GPC, 1-dihomo-linoleoyl-GPC (20:2n6), 1-eicosatrienoyl-GPC (20:3), 1-arachidonoyl-GPC (20:4n6), 2-arachidonoyl-GPC, 1-docosapentaenoyl-GPC (22:5n3), 1-oleoylglycerol (1-monoolein), and 1-linoleoylglycerol (1-monolinolein)	0.81	0.67-0.98	0.03	0.71	0.57-0.88	0.002	0.77	0.66-0.88	< 0.001
1	glycine, serine, alanine, 1,5-AG, ornithine, and fructose	0.75	0.62-0.90	0.002	0.81	0.66-1.01	0.06	0.78	0.68-0.89	< 0.001

Notes: Only clusters with meta-analyzed P values that reached the Bonferroni-adjusted threshold in the multivariable model are shown. Bonferroni-adjusted threshold calculated as 0.05/43 clusters = 0.001. Model adjusted for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking, estimated glomerular filtrated rate based on creatinine, and high-density lipoprotein cholesterol. Bold indicates metabolite was significantly related to KFRT in secondary analysis. Green color indicates metabolite had a positive meta-analyzed correlation with its respective cluster in unadjusted analysis. Red color indicates a negative meta-analyzed correlation.

Abbreviations: ADMA, asymmetric dimethylarginine; AG, anhydroglucitol; CI, confidence interval; GPC, glycerophosphocholine; HR, hazard ratio; KFRT, kidney failure with replacement therapy; SDMA, symmetric dimethylarginine.

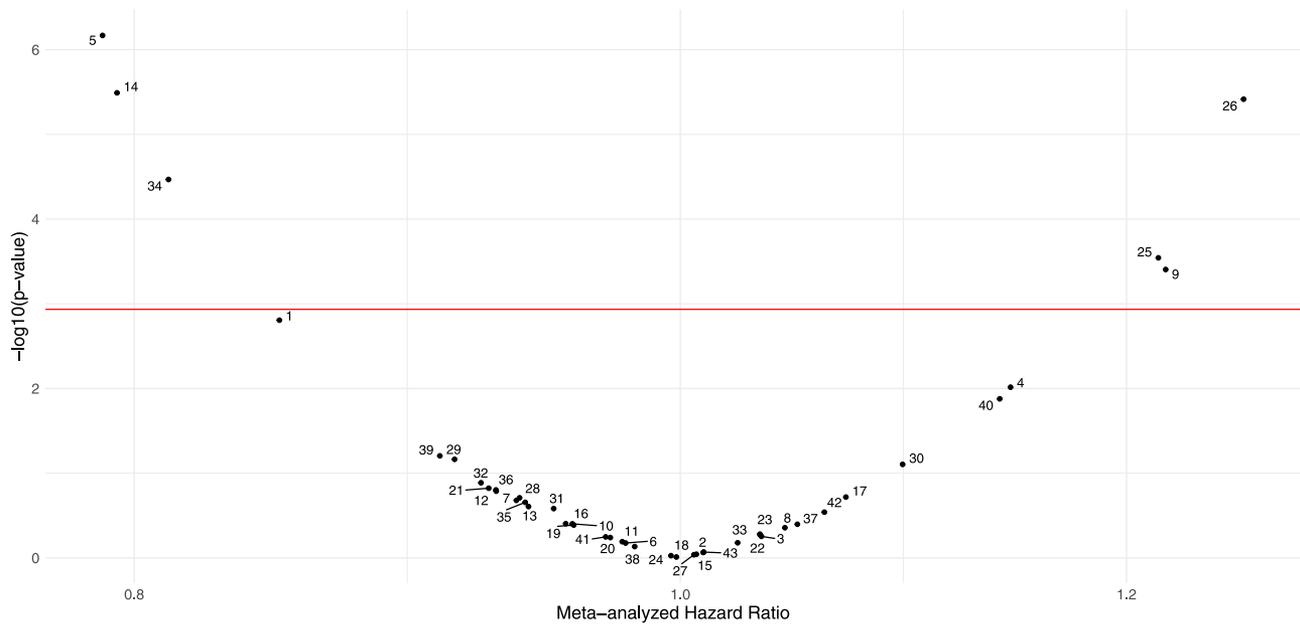


Figure 2. Volcano plot of meta-analyzed associations for clusters with kidney failure. Adjusted for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking, estimated glomerular filtrated rate based on creatinine, and high-density lipoprotein cholesterol. Red horizontal line represents the Bonferroni-adjusted threshold calculated as $0.05/43 \text{ clusters} = 0.001$.

(cluster 9). No individual metabolites from cluster 25 were statistically significantly associated with kidney failure. Nine metabolites from clusters 26, 5, and 34 were significantly associated with both outcomes. In addition, tryptophan, 1-arachidonoylglycerophosphoinositol, and 1,5-AG were significantly associated with kidney failure in the analysis of all 318 metabolites (Figure S4).

Associations of Significant Clusters with Baseline Characteristics

Clusters were consistently associated with specific baseline characteristics in both the training and validation sets (Table S3). Participants with higher and lower exposure to cluster 26 (6 metabolites including glucose and mannose) were more likely to have diabetes, as were those with in cluster 5 (15 metabolites including gamma-glutamyl amino acids and 5-oxoproline). Participants with greater exposure to cluster 5 metabolites had lower cholesterol (Table S4). No baseline characteristic was significantly associated with cluster 34 (Table S5). Race and systolic blood pressure were significantly related to all clusters in the validation set only.

DISCUSSION

In this study, we investigated the association between serum metabolites and KFRT and kidney failure over 3 decades in 3,799 individuals in the general population. Our cluster analysis identified 3 clusters of metabolites significantly associated with both KFRT and kidney failure,

with the clusters representing biomarkers of diabetes, lipid metabolism, and glutamic acid pathways. Several metabolites within these clusters, including glucose and mannose, were positively associated with KFRT and kidney failure, whereas 5-oxoproline, gamma-glutamyl amino acids, phosphocholine lysolipids, and 1,5-AG were inversely associated with both outcomes. A metabolome-wide screen also identified tryptophan as being inversely associated with KFRT and kidney failure. These findings contribute valuable insights into early physiological changes independent of eGFR associated with kidney outcomes.

Our findings are generally consistent with previous metabolomic studies of adverse kidney outcomes. Cluster 26, which included glucose and mannose, was significantly associated with increased risk for both KFRT and kidney failure. Hyperglycemia is a known contributor to diabetic kidney disease progression.²⁷ Glucose itself has not been comprehensively studied as a prospective biomarker of KFRT, in part because of its high variability, although it is plausible that excess glucose can generate reactive oxygen species that can lead to nephropathy.²⁸ Likewise, excess mannose can indirectly contribute to reactive oxygen species generation.²⁷ Interestingly, dimethylarginine (SDMA + ADMA) was combined with glyce-mic markers through unsupervised clustering. A previous study of Korean adolescents and adults found that ADMA was associated with obesity and diabetes and that it decreased after an obesity intervention program.²⁹ Furthermore, it has been suggested that ADMA stimulates renal fibrosis under high glucose conditions.³⁰ Elevated

Table 3. Association between Clusters and Kidney Failure

Cluster	Component Metabolites	Training Set (N = 1,773; KF = 173)			Validation Set (N = 2,026; KF = 184)			Meta-Analyzed (N= 3,799; KF = 357)		
		HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
26	dimethylarginine (SDMA + ADMA), glucose, trehalose, mannose, mannitol, and myo-inositol	1.23	1.08-1.41	0.002	1.29	1.11-1.50	< 0.001	1.26	1.14-1.39	< 0.001
5	asparagine, glutamine, 5-oxoproline, gamma-glutamylalanine, gamma-glutamylglutamate, gamma-glutamylglutamine, gamma-glutamylisoleucine, gamma-glutamylleucine, gamma-glutamylthreonine, gamma-glutamylvaline, threonine, glutamate, gamma-glutamylphenylalanine, gamma-glutamyltyrosine, and DSGEGDFXAEGGGVR	0.72	0.63-0.82	< 0.001	0.87	0.76-1.00	0.04	0.79	0.72-0.87	< 0.001
34	stearoylcarnitine, 1-docosahexaenoyl-GPC (22:6n3), 2-myristoyl-GPC, 1-pentadecanoyl-GPC (15:0), 1-palmitoyl-GPC (16:0), 2-palmitoyl-GPC, 1-palmitoleoyl-GPC (16:1), 1-margaroyl-GPC (17:0), 1-stearoyl-GPC (18:0), 2-stearoyl-GPC, 1-oleoyl-GPC (18:1), 2-oleoyl-GPC, 1-linoleoyl-GPC (18:2n6), 2-linoleoyl-GPC, 1-dihomo-linoleoyl-GPC (20:2n6), 1-eicosatrienoyl-GPC (20:3), 1-arachidonoyl-GPC (20:4n6), 2-arachidonoyl-GPC, 1-docosapentaenoyl-GPC (22:5n3), 1-oleoylglycerol (1-monoolein), and 1-linoleoylglycerol (1-monolinolein)	0.83	0.72-0.95	0.009	0.80	0.69-0.91	0.001	0.81	0.73-0.90	< 0.001
14	N-acetylphenylalanine, 3-methoxytyrosine, serotonin (5HT), ADSGEGDFXAEGGGVR, inosine, hypoxanthine, and guanosine	0.78	0.68-0.89	< 0.001	0.81	0.71-0.93	0.003	0.79	0.72-0.88	< 0.001
25	citrulline, acisoga, 4-acetamidobutanoate, N6-carbamoylthreonyladenosine, and N ₂ , N ₂ -dimethylguanosine	1.15	0.98-1.35	0.09	1.27	1.10-1.45	< 0.001	1.22	1.09-1.35	< 0.001
9	N-acetyl-1-methylhistidine and homocitrulline	1.21	1.03-1.42	0.02	1.23	1.06-1.43	0.006	1.22	1.09-1.36	< 0.001

Notes: Only clusters with meta-analyzed P values that reached the Bonferroni-adjusted threshold in the multivariable model are shown. Bonferroni-adjusted threshold calculated as 0.05/43 clusters = 0.001. Model adjusted for age, sex, race-center, systolic blood pressure, antihypertensive medication, diabetes, history of coronary heart disease, smoking, estimated glomerular filtrated rate based on creatinine, and high-density lipoprotein cholesterol. Bold indicates metabolite was significantly related to kidney failure in secondary analysis. Green color indicates metabolite had a positive meta-analyzed correlation with its respective cluster in unadjusted analysis. Red color indicates a negative meta-analyzed correlation.

Abbreviations: ADMA, asymmetric dimethylarginine; CI, confidence interval; HR, hazard ratio; GPC, glycerophosphocholine; KF, kidney failure; SDMA, symmetric dimethylarginine.

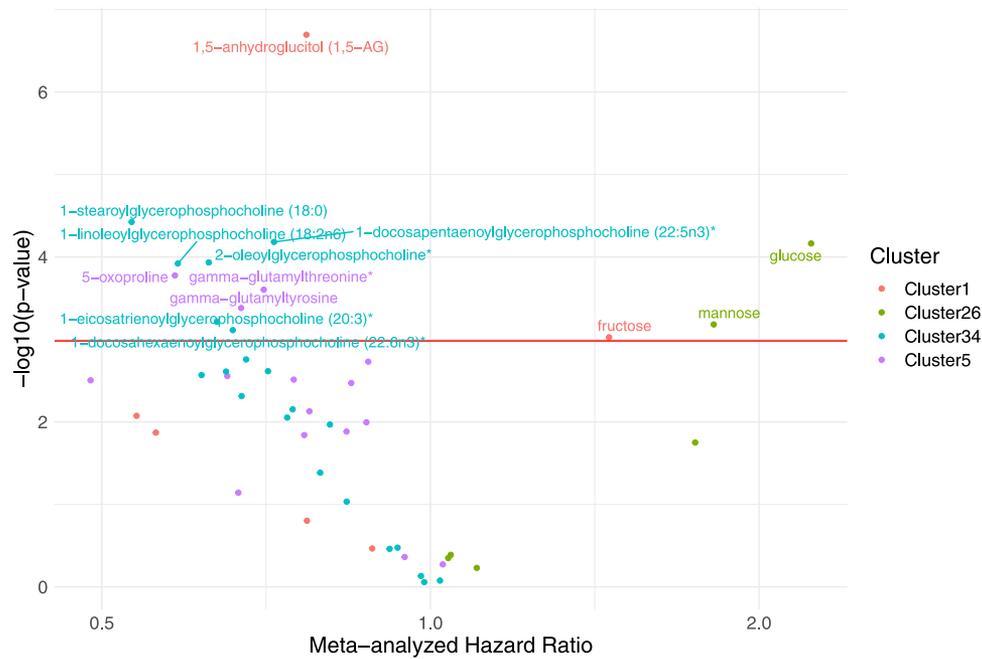


Figure 3. Volcano plot of associations for metabolites in top clusters with kidney failure with replacement therapy. Red horizontal line represents the Bonferroni-adjusted threshold calculated as $0.05/42$ metabolites = 0.001. Asterisk (*) indicates a tier 2 metabolite.

serum levels of ADMA and SDMA have been previously documented in kidney failure patients.^{31,32}

Compared to previous approaches, a potential strength of clustering is in identifying common metabolic pathways with disease relevance. Here, we employed an unsupervised correlation network analysis, Netboost, that identifies data-driven clusters, to allow for the detection of yet

unknown clusters and takes full advantage of the components quantified by the measurement platform. We speculate that one such pathway may be present in cluster 5. Cluster 5 was significantly related to both outcomes and contained 5-oxoproline and gamma-glutamyl amino acids. The 5-oxoproline metabolite has been previously linked to incident CKD and advanced CKD.^{4,33} The gamma-glutamyl

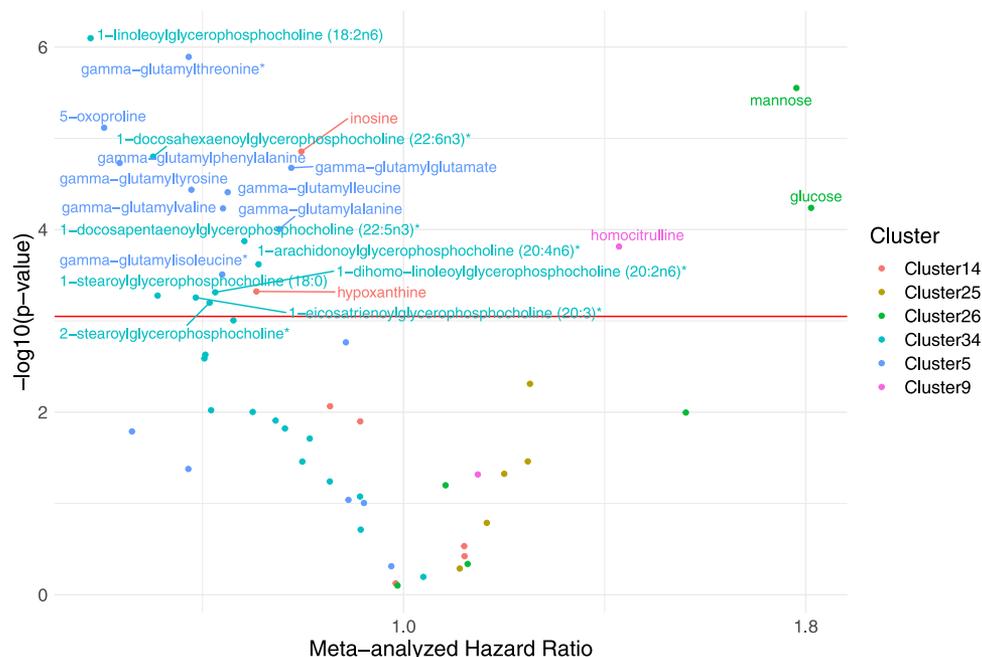


Figure 4. Volcano plot of associations for metabolites in top clusters with kidney failure. Red horizontal line represents the Bonferroni-adjusted threshold calculated as $0.05/56$ metabolites = 0.0009. Asterisk (*) indicates a tier 2 metabolite.

transferase enzyme that acts on gamma-glutamyl amino acids has been identified as a risk factor for adverse kidney outcomes, including kidney failure and mortality.³⁴⁻³⁶ In animal studies, 5-oxoproline formation has been used as a marker of gamma-glutamyl amino acid transport in the kidneys.³⁷ Patients with genetic defects in the gamma-glutamyl amino acid cycle have 5-oxoprolinuria.³⁸ Thus, clustering may be useful for discovering connections between metabolites whose relatedness has yet to be shown in translational studies, or conversely, in identifying new associations that can be further studied in laboratory research.

Lysolipids from the phosphocholine family represented in cluster 34 were also significantly related to both outcomes. Phosphocholine lysolipids are metabolites of phosphatidylcholines, which are components of the cell membrane made from choline and produced during kidney ischemia in animal models.³⁹ One particular lysophospholipid, linoleoylglycerophosphocholine, has been associated with risk of type 2 diabetes and impaired glucose tolerance in individuals without diabetes.⁴⁰⁻⁴² In our study, linoleoylglycerophosphocholine was significantly associated with both outcomes and had the lowest P value for kidney failure. Results from the Framingham Heart Study have previously indicated that changes in choline metabolism could signal tubulointerstitial dysfunction associated with risk of incident CKD.⁴³ In contrast, the 6 significant lysolipids identified in our study were not significantly related to kidney failure in a Joslin Kidney study of patients with type 2 diabetes; however, the latter study was much smaller.⁹

Our unsupervised clustering approach identified a third potential metabolic pathway in cluster 1 (6 metabolites including 1,5-AG and fructose), in addition to the speculated pathways in cluster 26 (6 metabolites including dimethylarginine and glucose) and in cluster 5 (15 metabolites including 5-oxoproline and gamma-glutamyl amino acids). 1,5-AG reabsorption in the renal tubules occurs through a common anhydroglucitol/fructose/mannose transport system, which suggests 1,5-AG and fructose are physiologically related.⁴⁴ We found that 1,5-AG was significantly related to both outcomes, and 1,5-AG had the lowest P value for KFRT of all metabolites studied. This is consistent with previous ARIC studies in which 1,5-AG was an early signal of incident CKD and inversely correlated to glucose.^{45,46} High levels of glucose inhibit 1,5-AG's reabsorption in the renal proximal tubule, and thus 1,5-AG is thought to be a marker of hyperglycemic excursions.⁴ Fructose's association with KFRT was notably in the opposite direction of 1,5-AG. This finding is consistent with previous experimental results that demonstrated high levels of fructose accelerate CKD progression.⁴⁷

A few individual metabolites are noteworthy. We found that higher levels of tryptophan were significantly associated with lower risk of both kidney outcomes. Previous studies have found similar associations between

tryptophan-derived metabolites and incident CKD, including the Joslin Kidney study, African American Study of Kidney Disease and Hypertension, and the Framingham Heart Study.^{10,43,48} The German Chronic Kidney Disease study also found that urine 6-bromotryptophan was significantly inversely associated with incident kidney failure.⁴⁹ We also identified a significant association between homocitrulline and risk of kidney failure. One European study has suggested that homocitrulline can serve as a marker for differentiating chronic kidney failure from acute kidney failure.⁵⁰ Citrulline, a structural homolog of homocitrulline, has been previously associated with incident CKD in the Framingham Heart Study, but citrulline was not significantly related to KFRT or kidney failure in our study.⁴³

Several limitations merit consideration. As with all observational studies, we cannot infer causality. It is well known that many metabolites are related to glomerular filtration rate.⁶ This study addressed this by using residuals prior to clustering but determining whether relationships are causal or consequential requires additional experimental evidence. It is also unknown how metabolites may vary over time and if time-varying metabolite changes could modify associations. Metabolite profiling was performed in 2 samples, allowing for the possibility of batch effects. However, we performed analyses separately and then meta-analyzed. The fact that clusters were consistent in direction in both the training and validation sets lends credence to our results. We used Bonferroni correction to assess statistical significance of our results. As aforementioned, this method has been criticized as overly conservative.¹²

Despite these limitations, our study had a number of strengths. We had a large number of events, long follow-up, and more heterogeneous study population relative to previous studies of kidney outcomes.⁹⁻¹¹ We found consistent associations across 2 large, separate samples with the same underlying study population. We used a novel clustering method, providing a unique approach that may be advantageous to use in future metabolomic studies to connect metabolites that may share common pathways.

In conclusion, we identified 7 metabolite clusters that were significantly related to KFRT or kidney failure. These included 10 metabolites that were significantly related to KFRT and kidney failure in individual analyses: glucose, mannose, 1,5-AG, 5-oxoproline, gamma-glutamylthreonine, gamma-glutamyltyrosine, 1-linoleoylglycerophosphocholine (18:2n6), 1-eicosatrienoylglycerophosphocholine (20:3), 1-docosapentaenoylglycerophosphocholine (22:5n3), and 1-docosahexaenoylglycerophosphocholine (22:6n3). We used a novel clustering technique to begin to unravel how correlated metabolites contribute to advanced kidney diseases. Other studies can build on this work by continuing to elucidate common pathways, including the potential pathways we have discussed from cluster 1 (6 metabolites including 1,5-AG and fructose), cluster 26 (6 metabolites including dimethylarginine and glucose), and cluster 5 (15

metabolites including 5-oxoproline and gamma-glutamyl amino acids), and to differentiate causal metabolites from biomarkers of disease.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1: Metabolite exclusion diagram.

Figure S2: Number of metabolites per cluster.

Figure S3: Volcano plot of associations for metabolites with kidney failure with replacement therapy.

Figure S4: Volcano plot of associations for metabolites with kidney failure.

Table S1: Cluster Membership.

Table S2: Metabolic Pathways Associated With Significant Clusters.

Table S3: Association Between Baseline Characteristics and Cluster 26.

Table S4: Association Between Baseline Characteristics and Cluster 5.

Table S5: Association Between Baseline Characteristics and Cluster 34.

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Serum Metabolites and Kidney Outcomes: The Atherosclerosis Risk in Communities (ARIC) Study



Prospective cohort study
Enrollment: 1987 - 1989



N = 3,799 participants in the Atherosclerosis Risk in Communities (ARIC) study



Mean age 53 years
61% Black participants



Mean follow-up
23 years



314 metabolites grouped in
43 clusters

Associations between metabolite clusters and outcomes

Adjusted* hazard ratio per standard deviation, 95% CI, p < 0.001

	Incident KFRT	Kidney failure
Cluster 1 Amino acid metabolism, urea cycle, glycolysis and gluconeogenesis	0.78 (0.68, 0.89)	No significant association
Cluster 5 Glutathione and γ -glutamyl transferase pathways	0.77 (0.67, 0.88)	0.79 (0.72, 0.87)
Cluster 26 Sugar & inositol phosphate metabolism	1.31 (1.15, 1.51)	1.26 (1.14, 1.39)
Cluster 34 Glycerophospholipid metabolism	0.77 (0.66, 0.88)	0.81 (0.73, 0.90)

*adjusted for age, sex, race-center, GFR, diabetes, hypertension, HDL-C, SBP, history of coronary heart disease, smoking status, KFRT, kidney failure with replacement therapy

Conclusion: Several clusters of metabolites are associated with development of kidney failure. Future experimental studies are needed to validate the findings as well as to further unravel metabolic pathways involved in kidney function decline.

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