Metabolomic Alterations Associated with Cause of CKD

Morgan E. Grams,*†‡ Adrienne Tin,‡‡ Casey M. Rebholz,‡‡ Tariq Shafi,‡‡ Anna Köttgen,‡‡ Ronald D. Perrone,* Mark J. Samak,§ Lesley A. Inker,‡ Andrew S. Levey,‡ and Josef Coresh‡‡

Abstract

Background and objectives Causes of CKD differ in prognosis and treatment. Metabolomic indicators of CKD cause may provide clues regarding the different physiologic processes underlying CKD development and progression.

Design, setting, participants & measurements Metabolites were quantified from serum samples of participants in the Modification of Diet in Renal Disease (MDRD) Study, a randomized controlled trial of dietary protein restriction and BP control, using untargeted reverse phase ultraperformance liquid chromatography tandem mass spectrometry quantification. Known, nondrug metabolites (n=687) were log-transformed and analyzed to discover associations with CKD cause (polycystic kidney disease, glomerular disease, and other cause). Discovery was performed in Study B, a substudy of MDRD with low GFR (n=166), and replication was performed in Study A, a substudy of MDRD with higher GFR (n=423).

Results Overall in MDRD, average participant age was 51 years and 61% were men. In the discovery study (Study B), 29% of participants had polycystic kidney disease, 28% had glomerular disease, and 43% had CKD of another cause; in the replication study (Study A), the percentages were 28%, 24%, and 48%, respectively. In the discovery analysis, adjusted for demographics, randomization group, body mass index, hypertensive medications, measured GFR, log-transformed proteinuria, and estimated protein intake, seven metabolites (16-hydroxypalmitate, kynurenate, homovanillate sulfate, N2,N2-dimethylguanosine, hippurate, homocitrtuline, and 1,5-anhydroglucitol) were associated with CKD cause after correction for multiple comparisons (P<0.0008). Five of these metabolite associations (16-hydroxypalmitate, kynurenate, homovanillate sulfate, N2,N2-dimethylguanosine, and hippurate) were replicated in Study A (P<0.007), with all replicated metabolites exhibiting higher levels in polycystic kidney disease and lower levels in glomerular disease compared with CKD of other causes.

Conclusions Metabolomic profiling identified several metabolites strongly associated with cause of CKD.


Introduction

Different causes of CKD have different pathologic signatures, require different therapies, and progress at different rates (1,2). Because of these health implications, the 2012 Kidney Disease: Improving Global Outcomes guideline for the evaluation and management of CKD recommended incorporating cause of CKD along with level of GFR and albuminuria in CKD staging (2). Polycystic kidney disease (PKD) in particular is characterized by a relentless decline in GFR, with few effective therapies available (3). Recently, impairment in distinct metabolic processes such as fatty acid oxidation has been hypothesized as a factor in PKD pathogenesis and progression (4).

Metabolite profiling, or an unbiased assessment of small molecules in a biologic specimen, has recently been applied to studies of prognosis in CKD (5–9). A metabolomic approach quantifies low–molecular weight biomarkers influenced by genetic variation, diet, medications, a person’s microbiome, liver function, and, particularly in CKD, GFR. Because kidney function affects many aspects of metabolic health, abnormal metabolomic profiles may mediate the pathogenesis and prognosis of CKD (10–12). Metabolites that differ according to CKD cause above and beyond level of GFR, proteinuria, diet, and medication use may provide novel, disease-specific treatment targets or the potential for early, noninvasive diagnostic techniques.

Designed as a two-by-two factorial randomized clinical trial in two substudies, the Modification of Diet in Renal Disease (MDRD) Study is a unique population of patients with expert-adjudicated cause of CKD, carefully monitored protein intake, and measured GFR using urinary iothalamate clearance (13). Using global metabolomic profiling of stored serum, we investigated the associations of individual metabolites with CKD cause, classified as PKD, glomerular disease, and CKD of other cause, excluding CKD attributed to diabetes mellitus. We used the smaller MDRD substudy (Study B) for discovery and the larger MDRD substudy (Study A) for replication of metabolite associations with cause of CKD.

Correspondence: Dr. Morgan E. Grams, 2024 East Monument, Room 2-618, Baltimore, MD 21205. Email: mgrams2@jhmi.edu
Materials and Methods

Study Population

The MDRD Study was a clinical trial of dietary protein restriction and BP target implemented in a two-by-two factorial design (13). The trial was composed of two substudies on the basis of enrollment GFR: study A consisted of patients with GFR between 25 and 55 ml/min per 1.73 m², and study B consisted of patients with GFR between 13 and 24 ml/min per 1.73 m². Study A randomized patients to usual protein diet or a low-protein diet (1.3 or 0.58 g of protein per kilogram of body weight per day, respectively), and study B randomized patients to a low-protein diet or a very low-protein diet with ketoacid and amino acid supplementation (0.58 and 0.28 g/kg per day, respectively). Both studies randomized patients to usual versus low target BP. A heterogeneous group of patients was recruited into the trial, with noteworthy exclusions being patients with diabetes mellitus treated with insulin and kidney transplant recipients; all participants provided informed consent. For the purpose of this study, we selected stored serum samples from the 12-month postrandomization visit. These specimens were aliquoted for creatinine measurement, drawn in the original trial before GFR measurement, refrigerated, and sent to the central laboratory in batches once a week. There, specimens were aliquoted for creatinine measurement, with the remaining sample frozen and stored at −80°C. Metabolomic profiling was performed using an untargeted metabolomic quantification protocol (Supplemental Appendix 1) (15,16).

In order to focus on potential treatment targets, asset unidentified metabolites and drug metabolites were excluded (n=427 and n=79, respectively). After these exclusions, 687 named compounds within 79 pathways were analyzed (Supplemental Appendix 2). Per protocol, metabolite values are normalized by run-day using spiked quality control standards to allow chromatographic alignment, then divided by the median value of the metabolite (7). Missing values were imputed with the minimum value. All metabolites were log-transformed, after which 84.9% had a skewness between −1 and 1.

Statistical Analyses

Patient characteristics at the 12-month clinical visit were compared by CKD cause using chi-squared, Kruskal–Wallis, or t tests, as appropriate. To determine metabolites associated with cause of CKD, linear regression was used, regressing log-transformed metabolites (dependent variable) on nonordered categoric cause of CKD (independent variable), with adjustment for the following potential confounders: race, age, sex, log-transformed GFR measured by urinary clearance of 125I-iothalamate, log-transformed total proteinuria, body mass index, diet randomization group, BP randomization group, estimated protein intake, diastolic BP, and angiotensin-converting enzyme inhibitor and β blocker use, with the last three variables chosen due to significant differences in baseline values by cause of disease. Each analysis was performed separately in the discovery study (study B) and replication study (study A).

The threshold for statistical significance accounted for multiple testing and intrametabolite correlation using the following procedure. The 687 metabolites were included in a principal component analysis, where 63 principal components explained 90% of the metabolite variance. Statistical significance was thus set in the discovery cohort as a P value <0.0008 (0.05 of 63 principal components) (17). In the replication cohort, the threshold for statistical significance was set using a Bonferroni P value of <0.007 (0.05 of 7, where 7 was the number of metabolites tested). For metabolites significantly associated with cause of disease, we evaluated correlations with measured GFR and generated residuals from linear regression of metabolites on the above covariates without adjusting for cause of CKD, and estimated the Spearman correlations of the residuals. This procedure was performed separately within the discovery and the replication studies.

To assess the discriminatory capacity of candidate metabolites for cause of CKD beyond clinical characteristics, a model was built using the following clinical characteristics identified a priori: age, sex, race, systolic and diastolic BP, log-transformed measured GFR, and log-transformed proteinuria. To minimize confounding by randomization arm, study, diet and BP target assignment, and estimated protein intake were also included. The area under the curve (AUC) for this model was calculated for two-way classification (i.e., PKD versus no PKD, and glomerular disease versus no glomerular disease) and then compared with a model that included clinical characteristics and the replicated metabolites in the full MDRD study. Sensitivity and specificity were assessed using the Youden method for determining cut-points (18). Continuous net reclassification index and integrated discrimination index were also calculated to evaluate the two models. Analyses were performed using Stata/MP 14.1 (College Station, TX) and R.
Results
Baseline Characteristics of the Discovery Study (Study B) and Replication Study (Study A)
There were 166 participants in the discovery study: 48 with PKD, 46 with glomerular disease, and 72 with other causes (hypertensive nephrosclerosis, interstitial nephritis, vesicoureteral reflex, single kidney, unknown cause) (Table 1). Average age was 51 years, 84% were white, and 61% were men. Mean GFR was 15 ml/min per 1.73 m², and median proteinuria was 0.4 g/d. Estimated protein intake was 0.6 g/kg per day. In the replication study, there were 423 participants: 119 with PKD, 102 with glomerular disease, and 202 with other causes. Average age was 52 years, 86% were white, and 61% were men. Mean GFR was 35 ml/min per 1.73 m² and median proteinuria was 0.1 g/d. In both studies, participants with PKD or glomerular disease were slightly younger than those with CKD of other cause (49, 48, and 55 years, respectively; P<0.001); white participants were more likely to have PKD, and black and Hispanic participants were more likely to have glomerular disease or CKD of other cause (Supplemental Table 2). Median proteinuria was 0.1 g/d in PKD, 1.3 g/d in glomerular disease, and 0.1 g/d in CKD of other cause. Study randomization arm did not differ by CKD cause; however, there were differences in hypertension treatment, with fewer participants with other CKD cause using angiotensin-converting enzyme inhibitors, and fewer participants with glomerular disease using β blockers.

Discovery of Individual Metabolites Associated with Cause of CKD in Study B
In adjusted analyses, there were seven metabolites significantly associated with cause of disease in the discovery study (Figure 1, Table 2, columns 1–4). These included metabolites involved in amino acid metabolism (kynurenate, homovanillate sulfate, homocitrulline), lipid metabolism (16-hydroxypalmitate), and nucleotide metabolism (N2,N2-dimethylguanosine); a carbohydrate (1,5-anhydroglucitol); and a xenobiotic (hippurate) (Supplemental Table 3). Most demonstrated higher levels in PKD than glomerular disease, with CKD of other causes having intermediate levels, with the exception of two, homocitrulline and 1,5-anhydroglucitol, which were higher in glomerular disease. Of all 687 tested metabolites, slightly more (52%) were higher in PKD compared with other disease, and slightly fewer (49%) were higher in PKD compared with glomerular disease (Supplemental Figure 1). Residuals from the adjusted regressions showed positive correlation between kynurenate, homovanillate sulfate, N2,N2-dimethylguanosine, and hippurate, with little correlation with 16-hydroxypalmitate (Figure 2). 1,5-anhydroglucitol, a monosaccharide that decreases in the setting of hyperglycemia, and homocitrulline, a byproduct of arginine and proline metabolism, were negatively correlated with the others.

Replication of Metabolite Associations with Cause of CKD in Study A
All seven identified metabolites were tested for their association with cause of CKD in study A. There were five metabolites that remained significant in the adjusted model (Table 2, columns 5–8). These included 16-hydroxypalmitate, kynurenate, homovanillate sulfate, N2,N2-dimethylguanosine, and hippurate. Of note, the direction of association comparing PKD to CKD of other cause and PKD to glomerular disease was

Table 1. Characteristics of Modification of Diet in Renal Disease Study participants, by substudy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Discovery (Study B)</th>
<th>Replication (Study A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>166</td>
<td>423</td>
</tr>
<tr>
<td>Mean age (SD), yr</td>
<td>51 (12)</td>
<td>52 (12)</td>
</tr>
<tr>
<td>Race, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>9 (5)</td>
<td>33 (8)</td>
</tr>
<tr>
<td>Hispanic/other</td>
<td>17 (10)</td>
<td>25 (6)</td>
</tr>
<tr>
<td>White</td>
<td>140 (84)</td>
<td>365 (86)</td>
</tr>
<tr>
<td>Men, N (%)</td>
<td>102 (61)</td>
<td>259 (61)</td>
</tr>
<tr>
<td>Diet assignment, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low protein</td>
<td>77 (46)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Low protein</td>
<td>89 (54)</td>
<td>208 (49)</td>
</tr>
<tr>
<td>Usual protein</td>
<td>0 (0)</td>
<td>215 (51)</td>
</tr>
<tr>
<td>Moderate BP assignment, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>48 (29)</td>
<td>119 (28)</td>
</tr>
<tr>
<td>Glomerular disease</td>
<td>46 (28)</td>
<td>102 (24)</td>
</tr>
<tr>
<td>Other cause of CKD</td>
<td>72 (43)</td>
<td>202 (48)</td>
</tr>
<tr>
<td>Mean systolic BP (SD), mmHg</td>
<td>132 (16)</td>
<td>129 (17)</td>
</tr>
<tr>
<td>Mean diastolic BP (SD), mmHg</td>
<td>79 (10)</td>
<td>79 (9)</td>
</tr>
<tr>
<td>Mean BMI (SD), kg/m²</td>
<td>25.4 (3.8)</td>
<td>27.3 (4.2)</td>
</tr>
<tr>
<td>Mean estimated protein intake (SD), g/kg per day</td>
<td>0.60 (0.19)</td>
<td>0.95 (0.28)</td>
</tr>
<tr>
<td>Mean GFR (SD), ml/min per 1.73 m²</td>
<td>15 (5)</td>
<td>35 (11)</td>
</tr>
<tr>
<td>Median urine protein (first, third quartiles), g/d</td>
<td>0.4 (0.2, 1.2)</td>
<td>0.1 (0.0, 0.8)</td>
</tr>
<tr>
<td>Kidney biopsy, N (%)</td>
<td>49 (31)</td>
<td>119 (30)</td>
</tr>
<tr>
<td>History of diabetes, N (%)</td>
<td>6 (4)</td>
<td>12 (3)</td>
</tr>
</tbody>
</table>

BMI, body mass index.
similar for all seven metabolites using this model. In contrast, the differences between glomerular disease and CKD of other cause were, in general, smaller and not always maintained (Supplemental Table 4). The correlations between metabolite residuals in the replication study were also largely unchanged (Figure 2). All replicated metabolites were negatively correlated with measured GFR: 16-hydroxypalmitate \((r=-0.11; \ P<0.01)\), kynurenate \((r=-0.66; \ P<0.001)\), homovanillate sulfate \((r=-0.77; \ P<0.001)\), 16-OH-palmitate \((r=-0.82; \ P<0.001)\), and hippurate \((r=-0.53; \ P<0.001)\).

**Additional Discrimination using Identified Metabolites**

Using clinical variables only, two-way classification models (i.e., PKD versus no PKD and glomerular disease versus no glomerular disease) showed good discrimination in the combined discovery and replication cohorts. For example, the AUC from a clinical model of PKD versus no PKD was 0.81 (95% confidence interval [95% CI], 0.77 to 0.85) and the AUC for glomerular disease versus no glomerular disease was 0.83 (95% CI, 0.80 to 0.87). Adding the five replicated metabolites increased the AUC for both classification models, with an AUC of 0.89 (95% CI, 0.87 to 0.93) and 0.85 (95% CI, 0.82 to 0.89), respectively \((P<0.001\) and \(P=0.04\) compared with the clinical model). Similarly, the probability of PKD, glomerular disease, and CKD of other cause differed by cause of disease (Figure 3). Sensitivity, continuous net reclassification index, and integrated discrimination index were also improved for each classification when the five replicated metabolites were added to clinical variables, and specificity was improved for the classification of PKD compared with no PKD (Supplemental Table 5).

**Discussion**

To our knowledge, this is the first study to evaluate the associations between small molecules identified through untargeted global metabolomic profiling and cause of CKD. Leveraging the substudies of the MDRD trial, a rigorously performed clinical trial with measured GFR and close monitoring of protein intake, we identified and replicated five metabolite associations (kynurenate, homovanillate sulfate, hippurate, N2,N2-dimethylguanosine, and 16-hydroxy-palmitate) that demonstrated consistently higher levels in PKD compared with glomerular disease and CKD of other causes. These distinctions in metabolites persisted after adjustment for demographics, GFR, proteinuria, diet, and medication use, and thus might represent pathophysiologic differences in the development of PKD.

One promising metabolite with low correlation to the other identified metabolites was 16-hydroxy-palmitate. 16-hydroxy-palmitate is the \(\omega\)-oxidation product of palmitic acid, one of the most common saturated fatty acids found in humans (19). \(\omega\)-Oxidation is an alternative to \(\beta\)-oxidation, the preferred route for fatty acid metabolism, and is thought to assume a more important role when \(\beta\)-oxidation is defective (20). Thus, the higher levels of 16-hydroxy-palmitate in PKD could represent an impairment in mitochondrial \(\beta\)-oxidation. This observation is consistent with previous studies implicating defects in lipid handling as a driver of CKD progression as well as in mouse models of PKD (4,21–23). Defects in fatty acid metabolism may affect tubular epithelial cells, which rely on fatty acid oxidation as an energy source (24). Tubulointerstitial fibrosis has been associated with impaired fatty acid oxidation, with intracellular fatty acid accumulation in the tubular epithelial cells (23). In a recent mouse model of PKD, Pkd1 knockout cells exhibited defective palmitate oxidation, and restriction of dietary lipids resulted in a small improvement in the Pkd1 knockout mouse kidney-to-body weight ratio (4).

Several of the PKD-associated metabolites have been previously named as uremic toxins, and there may be implications for treatment (25,26). Hippurate is a glycine conjugate of benzoic acid, related to diet and the gut microbiome, that is...

Figure 1. | Six metabolites met the discovery significance threshold [dotted line, \(P<0.001\) based on the likelihood ratio test (LRT)] in association between metabolites and cause of CKD in study B of the Modification of Diet in Renal Disease Study. Red font indicates the metabolite associations that were replicated in study A. Black and gray colors alternate over the x axis to separate metabolites by the associated superpathway.
Table 2. Metabolites significantly associated with cause of CKD in the Modification of Diet in Renal Disease Study, sorted by discovery statistical significance

<table>
<thead>
<tr>
<th>Metabolite (Pathway)</th>
<th>Discovery (Study B)</th>
<th>Replication (Study A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent (%) Difference (Polycystic Kidney Disease versus Other)</td>
<td>Percent (%) Difference (Polycystic Kidney Disease versus Glomerular Disease)</td>
</tr>
<tr>
<td>16-hydroxypalmitate(\alpha) (fatty acid, monohydroxy)</td>
<td>48</td>
<td>-3</td>
</tr>
<tr>
<td>Homocitrulline (urea cycle, arginine and proline metabolism)</td>
<td>-20</td>
<td>40</td>
</tr>
<tr>
<td>1,5-anhydroglucitol (glycolysis, gluconeogenesis, and pyruvate metabolism)</td>
<td>-40</td>
<td>-4</td>
</tr>
<tr>
<td>Kynurein(\alpha) (tryptophan metabolism)</td>
<td>22</td>
<td>-21</td>
</tr>
<tr>
<td>Homovanillate sulfate(\alpha) (phenylalanine and tyrosine metabolism)</td>
<td>63</td>
<td>-6</td>
</tr>
<tr>
<td>N2,N2-dimethylguanosine(\alpha) (purine metabolism, guanine containing)</td>
<td>23</td>
<td>-4</td>
</tr>
<tr>
<td>Hippurate(\alpha) (benzoate metabolism)</td>
<td>58</td>
<td>-15</td>
</tr>
</tbody>
</table>

Percent difference reflects difference in metabolite levels in each cause relative to the other, and was calculated as \(100 \times (\exp[\beta] - 1)\), where \(\beta\) is the coefficient from the regression of log-transformed metabolite as the dependent variable on cause of CKD as a nonordered independent variable, adjusted for the following covariates: race, age, sex, log(GFR), body mass index, study diet and BP assignment, estimated protein intake, angiotensin-converting enzyme inhibitor, \(\beta\) blocker, and log(proteinuria). Statistical significance was determined by \(P < 0.0008 = 0.05\) divided by 63, the number of principal components explaining 90% of the metabolite variance in the discovery cohort (\(n = 166\); study B) and \(P < 0.007 = 0.05\) divided by 7, the number of metabolites that were significant in the discovery analysis in the replication cohort (\(n = 423\); study A).

\(\alpha\) Metabolite associations that were replicated in study A.
secreted by the proximal tubule (27). Mouse models demonstrated that treatment with the CIC-2 chloride channel activator lubiprostone attenuated kidney failure–related changes in the gut microbiota, lessened the accumulation of hippurate, and reduced kidney fibrosis and local inflammation (28). As another example, kynurenate levels are elevated in kidney failure, particularly in relation to tryptophan (29). This imbalance might be induced by upregulation of the indoleamine-2,3-dioxygenase (IDO-1) enzyme in the presence of inflammation, with improvement through the use of niacin supplementation or IDO-1 inhibitors (30).

The metabolites identified as higher in PKD compared with other causes of CKD might signify differences in the location of the pathologic-anatomic findings. Hippurate and kynurenate have been implicated as markers of the health of the proximal tubule, a construct that only partially correlates with GFR (31). Hippurate, kynurenate, and homovanillate are thought to be substrates of the organic anion transporter family, located on the basolateral membrane of the proximal tubule, with ATP–binding cassette transporters mediating urinary secretion on the luminal side (32–34).

Lower hippurate clearance has been associated with increased risk of mortality and a trend toward faster CKD progression, independent of urea and creatinine clearance and albumin excretion rate (31). Torres and colleagues (35) demonstrated for PKD that blood flow decline in the kidney parallels increases in total kidney volume, a marker of disease severity, and precedes the decline in GFR. One possibility is that the early decline in blood flow in the kidney in PKD compared with GFR differentially affects the secretion of small molecules by the proximal tubule. N2,N2-dimethylguanosine, a degradation product of transfer RNA excreted by the kidney, also has reduced urinary excretion in patients with kidney failure and may relate to proximal tubule health (36).

Beyond markers of disease processes and potential treatment, other potential uses for disease-associated metabolites include early or minimally invasive detection of disease. In the case of PKD, a metabolomic signature may be particularly relevant in children and younger adults, before ultrasound can detect cysts in the kidney, and in whom the genetic variant is unknown. Although the
metabolomic associations differentiating glomerular disease from CKD of other cause were weaker than that between PKD and other causes, metabolites associated with GN might inform pretest probabilities of finding disease on biopsy or allow for diagnosis in persons in whom kidney biopsy is not feasible. Alternatively, classification of disease on the basis of metabolites might allow for etiologic classification in existing research studies with stored specimens, enabling more rigorous testing of the risk associated with cause of CKD. Additional discovery and replication work in populations with earlier stages of CKD and finer disease classification as well as the development of targeted assays with more accurate quantification may refine these approaches and bring them closer to clinical practice.

Our study design benefits from an unbiased approach to metabolite detection, expert-adjudicated classification of disease, careful assessment of GFR and protein intake, and a rigorous study design with discovery and replication of findings in separate substudies with different ranges of GFR. Five out of seven discovered metabolites were replicated, all of which showed higher levels in PKD than CKD of other causes. On the other hand, differences between glomerular disease and CKD of other cause were weaker. This may represent imperfect classification between glomerular disease and CKD of other causes or heterogeneity within categories. The MDRD Study was rigorous in categorizing participants according to their cause of CKD, but a kidney biopsy was not uniformly required (37). Future work should investigate the feasibility of metabolomic classification in populations with more homogeneous disease classification, as well as populations with greater minority representation (<10% of MDRD Study participants were black, a significant underrepresentation of the population with CKD stage G3 and G4).

Unbiased metabolomic profiling is excellent for evaluating a broad range of metabolites, but precision is limited because quantification is relative, not absolute. Samples in MDRD were refrigerated up to a week before shipment, which could affect the profiling of some metabolites. Despite this, the correlations between metabolite creatinine and clinical creatinine were high, as were metabolite correlations among blind duplicates. Subsequent work with targeted assays and samples drawn expressly for the purpose of assessing etiologic differences in the metabolome may provide stronger and previously unidentified associations.

As with all observation studies, causality cannot be determined; this analysis is merely a discovery study and proof-of-concept. Additional work should replicate findings in external cohorts and investigate potential causal roles in animal models. There is no absolute standard for adjusting for multiple comparisons in untargeted metabolomic analyses, where metabolites are often highly correlated (38). Because we structured our study as a discovery and replication cohort, we allowed a slightly more permissive approach in the discovery cohort (17) so as to minimize type II error. In the replication study, we used the conservative Bonferroni correction in order to minimize type I error.

In summary, we evaluated percent differences in metabolites by cause of kidney disease beyond differences in GFR, proteinuria, diet, and medications, identifying a group of metabolites strongly associated with cause of disease. The associated metabolites are biologically plausible and, in the case of 16-hydroxypalmitate, provide some evidence to support the hypothesis of impaired fatty acid metabolism as an underlying driver of PKD. We view this work as hypothesis-generating, which should be followed by future studies in study populations with careful phenotyping and using targeted assays for absolute quantification of metabolites. With additional translational work, metabolites may provide insight into processes underlying differential disease development and progression.

Acknowledgments

M.E.G. receives support from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (K08DK092287 and R01DK108803). A.T. receives support from R01 DK108803. J.C., L.A.I., A.S.L., M.E.G., and M.J.S. receive support from CKD Biomarkers Consortium (NIDDK U01 DK085689). J.C., L.A.I., and A.S.L. receive support from CKD-Epi panel eGFR (R01 DK097702). A.K. was supported by German Research Foundation grants KO 3598/3-1 and KO 3598/4-1. T.S. is supported by R03-DK-104012 and R01-HL-132372. C.M.R. is supported by a mentored research scientist development grant from the NIDDK (K01 DK107782).

Disclosures

Assay costs were discounted as part of a collaboration agreement between Metabolon and J.C., L.A.I., and A.S.L. to develop metabolomic estimates of GFR and for which they have a provisional patent filed on August 15, 2014, entitled “Precise estimation of GFR from multiple biomarkers” (no. PCT/US2015/044567). The technology is not licensed in whole or in part to any company.

References


Received: March 7, 2017 Accepted: July 10, 2017

M.E.G. and A.T. are cofirst authors.

Published online ahead of print. Publication date available at www.jasn.org.


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.2215/CJN.02560317/-/DCSupplemental.
Supplement to:

**Metabolomic Alterations Associated with Cause of Chronic Kidney Disease**

Morgan E. Grams, MD PhD (1,2,3);* Adrienne Tin, PhD (2,3);* Casey M. Rebholz, PhD, MPH, MS (2,3); Tariq Shafi, MBBS MHS (1,2, 3); Anna Köttgen, MD MPH (2,4); Ronald D. Perrone, MD (5); Mark J. Sarnak, MD MS (5); Lesley A. Inker, MD MS (5); Andrew S. Levey, MD (5); Josef Coresh, MD PhD (2,3)

*co-first authors

1) Division of Nephrology, Department of Medicine, Johns Hopkins University, Baltimore MD
2) Department of Epidemiology, Johns Hopkins University Bloomberg School of Public Health, Baltimore MD
3) Welch Center for Prevention, Epidemiology, and Clinical Research, Johns Hopkins University, Baltimore MD
4) Division of Genetic Epidemiology, Faculty of Medicine and Medical Center – University of Freiburg, Freiburg, Germany
5) Division of Nephrology, Tufts Medical Center, Boston, MA

Corresponding author:
Morgan E. Grams, MD PhD
2024 E. Monument, Rm 2-638
Baltimore, MD 21205
Phone: 443-287-1827
Fax: 410-955-0485
Email: mgrams2@jhmi.edu
**Supplemental Table 1.** Expert-adjudicated cause of chronic kidney disease cross-tabulated with clinical center-reported cause of disease in the Modification of Diet in Renal Disease Study participants included in the study population (N=589)

<table>
<thead>
<tr>
<th>Self-reported cause of disease</th>
<th>PKD</th>
<th>Glomerular Disease*</th>
<th>Interstitial Nephritis</th>
<th>VU Reflux/Obstruction</th>
<th>Hypertensive Nephrosclerosis</th>
<th>One kidney</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycystic kidney disease</td>
<td>167</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heredity nephritis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Analgesic nephropathy</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pyelonephritis</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Other interstitial nephritis</td>
<td>0</td>
<td>3</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Obstructive uropathy-acquired</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Obstructive uropathy-congenital</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vesico ureteral reflux</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urinary tract stones</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypertensive nephrosclerosis</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>39</td>
<td>0</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Renal artery stenosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Membranous nephropathy</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Focal sclerosis</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Membranoproliferative glomerulonephritis</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mesangial proliferative glomerulonephritis</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic renal failure with proteinuria</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nephrotic syndrome without biopsy</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>One kidney</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other glomerulonephritis</td>
<td>0</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>167</td>
<td>148</td>
<td>31</td>
<td>26</td>
<td>40</td>
<td>17</td>
<td>160</td>
</tr>
</tbody>
</table>
Supplemental Table 2. Characteristics of Modification of Diet in Renal Disease Study participants, by sub-study and cause of chronic kidney disease

<table>
<thead>
<tr>
<th></th>
<th>Discovery (Study B)</th>
<th>Replication (Study A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polycystic kidney disease</td>
<td>Glomerular disease</td>
</tr>
<tr>
<td>N</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>Age, mean (SD)</td>
<td>50 (10)</td>
<td>48 (13)</td>
</tr>
<tr>
<td>Race, % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>4.2 (2)</td>
<td>6.5 (3)</td>
</tr>
<tr>
<td>Hispanic/other</td>
<td>6.2 (3)</td>
<td>15.2 (7)</td>
</tr>
<tr>
<td>White</td>
<td>89.6 (43)</td>
<td>78.3 (36)</td>
</tr>
<tr>
<td>Male, % (n)</td>
<td>60.4 (29)</td>
<td>67.4 (31)</td>
</tr>
<tr>
<td>Diet assignment, % (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low protein</td>
<td>43.8 (21)</td>
<td>43.5 (20)</td>
</tr>
<tr>
<td>Low protein</td>
<td>56.2 (27)</td>
<td>56.5 (26)</td>
</tr>
<tr>
<td>Usual protein</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate blood pressure assignment, % (n)</td>
<td>45.8 (22)</td>
<td>41.3 (19)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>132 (17)</td>
<td>132 (13)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>82 (10)</td>
<td>81 (10)</td>
</tr>
<tr>
<td>BMI</td>
<td>25.6 (3.8)</td>
<td>24.7 (3.2)</td>
</tr>
<tr>
<td>Estimated protein intake, g/kg/day, mean (SD)</td>
<td>0.6 (0.23)</td>
<td>0.6 (0.16)</td>
</tr>
<tr>
<td>GFR</td>
<td>12.9 (3.9)</td>
<td>14.7 (4.7)</td>
</tr>
<tr>
<td>Urine protein, g/day</td>
<td>0.2 (0.1, 0.4)</td>
<td>1.1 (0.4, 1.9)</td>
</tr>
<tr>
<td>Kidney biopsy, % (n)*</td>
<td>2.1 (1)</td>
<td>82.2 (37)</td>
</tr>
<tr>
<td>History of diabetes, % (n)</td>
<td>2.1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HbA1c, median (IQR)*</td>
<td>5.7 (5.4-5.9)</td>
<td>5.7 (5.3-5.9)</td>
</tr>
<tr>
<td>Albumin, median (IQR)</td>
<td>4.1 (4-4.3)</td>
<td>4.2 (3.9-4.4)</td>
</tr>
</tbody>
</table>

*Sample size for kidney biopsy N=156 and 398 in Study B and Study A, respectively. Sample size for HbA1c N=161 and 416, respectively.
### Supplemental Table 3. Metabolite identifiers of replicated metabolite hits in PubChem, ChemSpider, Kegg, and HDMB.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Super Pathway</th>
<th>Sub Pathway</th>
<th>Mass</th>
<th>PubChem</th>
<th>ChemSpider</th>
<th>Kegg</th>
<th>HDMB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-hydroxypalmitate</td>
<td>Lipid</td>
<td>Fatty Acid, Monohydroxy</td>
<td>271.2279</td>
<td>10466</td>
<td>10034</td>
<td>C18218</td>
<td>HMDB06294</td>
</tr>
<tr>
<td>homovanillate sulfate</td>
<td>Amino Acid</td>
<td>Phenylalanine and Tyrosine Metabolism</td>
<td>261.0074</td>
<td>29981063</td>
<td>21896746</td>
<td></td>
<td>HMDB11719</td>
</tr>
<tr>
<td>kynurenate</td>
<td>Amino Acid</td>
<td>Tryptophan Metabolism</td>
<td>188.0353</td>
<td>3845</td>
<td>3712</td>
<td>C01717</td>
<td>HMDB00715</td>
</tr>
<tr>
<td>N2,N2-dimethylguanosine</td>
<td>Nucleotide</td>
<td>Purine Metabolism, Guanine containing</td>
<td>312.1303</td>
<td>92919</td>
<td>83878</td>
<td></td>
<td>HMDB04824</td>
</tr>
<tr>
<td>hippurate</td>
<td>Xenobiotics</td>
<td>Benzoate Metabolism</td>
<td>178.051</td>
<td>464</td>
<td>451</td>
<td>C01586</td>
<td>HMDB00714</td>
</tr>
</tbody>
</table>
Supplemental Table 4. P-values of metabolites significantly associated with cause of chronic kidney disease in the adjusted models, by sub-study in the Modification of Diet in Renal Disease Study

<table>
<thead>
<tr>
<th>Metabolite (Pathway)</th>
<th>Discovery (Study B)</th>
<th>Replication (Study A)</th>
<th>Likelihood Ratio Test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value (Polycystic Kidney Disease vs. Other)</td>
<td>P-value (Glomerular Disease vs. Other)</td>
<td>P-value (Polycystic Kidney Disease vs. Glomerular Disease)</td>
</tr>
<tr>
<td>16-hydroxypalmitate (Fatty Acid, Monohydroxy)</td>
<td>3.5E-05</td>
<td>7.7E-01</td>
<td>7.4E-05</td>
</tr>
<tr>
<td>Homocitrulline (Urea cycle, Arginine and Proline Metabolism)</td>
<td>5.0E-02</td>
<td>2.1E-03</td>
<td>1.2E-05</td>
</tr>
<tr>
<td>1,5-anhydroglucitol (Glycolysis, Gluconeogenesis, and Pyruvate Metabolism)</td>
<td>6.2E-05</td>
<td>7.7E-01</td>
<td>7.8E-04</td>
</tr>
<tr>
<td>Kynurenate (Tryptophan Metabolism)</td>
<td>3.5E-02</td>
<td>1.7E-02</td>
<td>8.9E-05</td>
</tr>
<tr>
<td>Homovanillate sulfate (Phenylalanine and Tyrosine Metabolism)</td>
<td>5.5E-04</td>
<td>6.5E-01</td>
<td>4.9E-04</td>
</tr>
<tr>
<td>N2,N2-dimethylguanosine (Purine Metabolism, Guanine containing)</td>
<td>8.9E-04</td>
<td>5.3E-01</td>
<td>4.4E-04</td>
</tr>
<tr>
<td>Hippurate (Benzoate Metabolism)</td>
<td>3.1E-03</td>
<td>3.0E-01</td>
<td>4.2E-04</td>
</tr>
</tbody>
</table>

Covariates: race, age, sex, log(GFR), BMI, study diet and BP assignment, estimate protein intake, ACE inhibitor, beta blocker, log(proteinuria)
Statistical significance determined by p< 7.9e-4 (=0.05/63 principal components) in the discovery cohort and p<0.007 (=0.05/7) in the replication cohort.
Bold reflects metabolite associations that were replicated in Study A.
Abbreviations: Other, other cause of CKD.
Supplemental material is neither peer-reviewed nor thoroughly edited by CJASN. The authors alone are responsible for the accuracy and presentation of the material.

**Supplemental Table 5.** Additional measures of discrimination comparing a model with clinical variables only to one that uses both clinical variables and the five replicated metabolites in the Modification of Diet in Renal Disease Study.

<table>
<thead>
<tr>
<th></th>
<th>Polycystic kidney disease vs. no polycystic kidney disease</th>
<th>Glomerular disease vs. no glomerular disease</th>
<th>Other cause of kidney disease vs. non-other cause of kidney disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical variables only</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Youden cutpoint</td>
<td>0.34</td>
<td>0.23</td>
<td>0.45</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.72</td>
<td>0.80</td>
<td>0.72</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.81</td>
<td>0.73</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Clinical variables + metabolites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Youden cutpoint</td>
<td>0.35</td>
<td>0.22</td>
<td>0.41</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.89</td>
<td>0.84</td>
<td>0.82</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.84</td>
<td>0.73</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Clinical variables + metabolites vs. clinical variables only</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous Net Reclassification Index*</td>
<td>0.98 (0.83, 1.13)</td>
<td>0.61 (0.43, 0.78)</td>
<td>0.63 (0.48, 0.79)</td>
</tr>
<tr>
<td>Continuous Integrated Discrimination Index*</td>
<td>0.23 (0.19, 0.26)</td>
<td>0.04 (0.02, 0.06)</td>
<td>0.12 (0.10, 0.15)</td>
</tr>
</tbody>
</table>

*All indices with p<0.001. Net reclassification index reports the proportion of participants who correctly moved up added to the proportion of participants correctly moved down (maximum value: 2). Integrated discrimination index reports the sum of the difference in predicted probabilities, or the sum of the improvement in sensitivity and specificity.
Supplemental Figure 1. Smile plots of association with cause of CKD vs. statistical significance for 687 tested metabolites in the discovery cohort (Study B) in the Modification of Diet in Renal Disease Study. Red denotes the five replicated metabolites, and the % denotes the percent of metabolites that were increased or decreased.
Supplemental Appendix 1. Information on Metabolomic Profiling Procedures

Samples were sent to Metabolon, Inc. on dry ice. On receipt, samples were inventoried and immediately stored at -80°C until processing. Samples were processed by first adding several recovery standards, then using methanol to precipitate proteins. The extract was divided into five fractions: two for separate reverse phase ultraperformance liquid chromatography tandem mass-spectrometry (RP/UPLC-MS/MS) with a positive ion mode electrospray ionization (ESI), one for RP/UPLC-MS/MS with negative ion mode ESI, one for hydrophilic interaction ultra-performance liquid chromatography (HILIC-UPLC-MS/MS) with negative ion mode ESI, and one sample for back-up. These platforms are designed to provide complementary information regarding metabolites. Controls were analyzed concomitantly with the experimental samples, including a pooled matrix sample serving as a technical replicate, extracted water samples for negative controls, and certain QC standards that do not interfere with endogenous compounds. The latter were added to each analyzed sample to provide monitoring for instruments and chromatographic alignment. Instrument variability was assessed as the median relative standard deviation for the QC standards (5%) and process variability was assessed as the median relative standard deviation for the endogenous metabolites in the pooled matrix samples (11%). Both values met Metabolon’s internal acceptance criteria. After analyzing via the four methods, each of which used a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution, data were extracted and processed using internal hardware and software. Peaks were identified through comparison to the Metabolon library, which catalogs purified, authenticated standards (currently >3300 compounds) and recurrent unknown entities. Experimental peaks were compared to the library with respect to retention time/index, mass to charge ratio, and chromatographic data; only compounds that matched on all three criteria are identified. The area under the curve was then used to quantify peaks, with correction for run-day blocks, since samples required more than one day of analysis. For the current analysis, 1193 biochemicals were matched with compounds in the Metabolon library, including 766 known biochemicals, authenticated using purified standards, and 427 recurrent unknown biochemicals. Biochemicals were classified into 79 pathways (see table, next page). Correlation between the metabolite creatinine and in-study, clinically-measured creatinine was 0.96. Per protocol, 20 blind duplicate pairs were analyzed. The median metabolite correlation between duplicates was 0.91, and 72% of all metabolites had a correlation >0.80. Correlations for hippurate, 16-hydroxypalmitate, kynurenate, homovanillate sulfate, and N2,N2-dimethylguanosine were 0.97, 0.58, 0.98, 0.93, and 0.92, respectively.
Supplemental material is neither peer-reviewed nor thoroughly edited by CJASN. The authors alone are responsible for the accuracy and presentation of the material.

Supplemental Appendix 2. Number of metabolites within 79 identified pathways.

<table>
<thead>
<tr>
<th>Known, Non-Drug Pathways</th>
<th>Number of Metabolites</th>
<th>Known, Non-Drug Pathways</th>
<th>Number of Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Glycation End-product</td>
<td>1</td>
<td>Leucine, Isoleucine and Valine Metabolism</td>
<td>28</td>
</tr>
<tr>
<td>Alanine and Aspartate Metabolism</td>
<td>6</td>
<td>Long Chain Fatty Acid</td>
<td>14</td>
</tr>
<tr>
<td>Aminosugar Metabolism</td>
<td>4</td>
<td>Lysine Metabolism</td>
<td>12</td>
</tr>
<tr>
<td>Ascorbate and Aldarate Metabolism</td>
<td>3</td>
<td>Lysolipid</td>
<td>25</td>
</tr>
<tr>
<td>Bacterial/Fungal</td>
<td>1</td>
<td>Lysoplasmalogen</td>
<td>4</td>
</tr>
<tr>
<td>Benzoate Metabolism</td>
<td>20</td>
<td>Medium Chain Fatty Acid</td>
<td>5</td>
</tr>
<tr>
<td>Carnitine Metabolism</td>
<td>2</td>
<td>Methionine, Cysteine, SAM and Taurine Metabolism</td>
<td>20</td>
</tr>
<tr>
<td>Chemical</td>
<td>22</td>
<td>Mevalonate Metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Creatine Metabolism</td>
<td>3</td>
<td>Monoacylglycerol</td>
<td>14</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>4</td>
<td>Nicotinate and Nicotinamide Metabolism</td>
<td>7</td>
</tr>
<tr>
<td>Dipeptide</td>
<td>9</td>
<td>Oxidative Phosphorylation</td>
<td>1</td>
</tr>
<tr>
<td>Dipeptide Derivative</td>
<td>1</td>
<td>Pantothenate and CoA Metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Disaccharides and Oligosaccharides</td>
<td>1</td>
<td>Pentose Metabolism</td>
<td>4</td>
</tr>
<tr>
<td>Eicosanoid</td>
<td>3</td>
<td>Phenylalanine and Tyrosine Metabolism</td>
<td>36</td>
</tr>
<tr>
<td>Endocannabinoid</td>
<td>5</td>
<td>Phospholipid Metabolism</td>
<td>38</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Choline)</td>
<td>1</td>
<td>Plasmalogen</td>
<td>11</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (also BCAA Metabolism)</td>
<td>3</td>
<td>Polyamine Metabolism</td>
<td>5</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Carnitine)</td>
<td>18</td>
<td>Polypeptide</td>
<td>1</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Glycine)</td>
<td>2</td>
<td>Polyunsaturated Fatty Acid (n3 and n6)</td>
<td>12</td>
</tr>
<tr>
<td>Fatty Acid Synthesis</td>
<td>3</td>
<td>Primary Bile Acid Metabolism</td>
<td>9</td>
</tr>
<tr>
<td>Fatty Acid, Amino</td>
<td>2</td>
<td>Purine Metabolism, (Hypo)Xanthine/Inosine containing</td>
<td>7</td>
</tr>
<tr>
<td>Fatty Acid, Branched</td>
<td>2</td>
<td>Purine Metabolism, Adenine containing</td>
<td>6</td>
</tr>
<tr>
<td>Fatty Acid, Dicarboxylate</td>
<td>16</td>
<td>Purine Metabolism, Guanine containing</td>
<td>2</td>
</tr>
<tr>
<td>Fatty Acid, Dihydroxy</td>
<td>2</td>
<td>Pyrimidine Metabolism, Cytidine containing</td>
<td>2</td>
</tr>
<tr>
<td>Fatty Acid, Monohydroxy</td>
<td>13</td>
<td>Pyrimidine Metabolism, Orotate containing</td>
<td>3</td>
</tr>
<tr>
<td>Fibrinogen Cleavage Peptide</td>
<td>1</td>
<td>Pyrimidine Metabolism, Thymine containing</td>
<td>2</td>
</tr>
<tr>
<td>Food Component/Plant</td>
<td>35</td>
<td>Pyrimidine Metabolism, Uracil containing</td>
<td>7</td>
</tr>
<tr>
<td>Fructose, Mannose and Galactose Metabolism</td>
<td>3</td>
<td>Secondary Bile Acid Metabolism</td>
<td>17</td>
</tr>
<tr>
<td>Gamma-glutamyl Amino Acid</td>
<td>15</td>
<td>Sphingolipid Metabolism</td>
<td>26</td>
</tr>
<tr>
<td>Glutamate Metabolism</td>
<td>6</td>
<td>Steroid</td>
<td>34</td>
</tr>
<tr>
<td>Glutathione Metabolism</td>
<td>3</td>
<td>Sterol</td>
<td>3</td>
</tr>
<tr>
<td>Glycerolipid Metabolism</td>
<td>2</td>
<td>TCA Cycle</td>
<td>9</td>
</tr>
<tr>
<td>Glycine, Serine and Threonine Metabolism</td>
<td>9</td>
<td>Tobacco Metabolite</td>
<td>4</td>
</tr>
<tr>
<td>Glycogen Metabolism</td>
<td>2</td>
<td>Tocopherol Metabolism</td>
<td>6</td>
</tr>
<tr>
<td>Glycolysis, Gluconeogenesis, and Pyruvate Metabolism</td>
<td>5</td>
<td>Tryptophan Metabolism</td>
<td>22</td>
</tr>
<tr>
<td>Guanidino and Acetamido Metabolism</td>
<td>3</td>
<td>Urea cycle; Arginine and Proline Metabolism</td>
<td>18</td>
</tr>
<tr>
<td>Hemoglobin and Porphyrin Metabolism</td>
<td>6</td>
<td>Vitamin A Metabolism</td>
<td>1</td>
</tr>
<tr>
<td>Histidine Metabolism</td>
<td>13</td>
<td>Vitamin B6 Metabolism</td>
<td>2</td>
</tr>
<tr>
<td>Inositol Metabolism</td>
<td>2</td>
<td>Xanthine Metabolism</td>
<td>15</td>
</tr>
<tr>
<td>Ketone Bodies</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>