Plasma Metabolomic Profiles in Different Stages of CKD

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Summary
Background and objectives CKD is a common public health problem. Identifying biomarkers adds prognostic/diagnostic value by contributing to an understanding of CKD at the molecular level and possibly defining new drug targets. Metabolomics provides a snapshot of biochemical events at a particular time in the progression of CKD. This cross-sectional metabolomics study ascertained whether plasma metabolite profiles are significantly different in CKD stages 2, 3, and 4.

Design, setting, participants, & measurements An analysis of plasma metabolites, using gas and liquid chromatography coupled to mass spectrometry, was conducted on 30 nondiabetic men ages 40–52 years, with 10 participants each in CKD stages 2, 3, and 4 based on their estimated GFR (calculated by the Modified Diet in Renal Disease formula). Participants were recruited in late 2008, and plasma samples were tested at Metabolon Inc and analyzed in 2012.

Results Comparison of stage 3/stage 2 identified 62 metabolites that differed (P≤0.05), with 39 higher and 23 lower in stage 3 compared with stage 2; comparisons of stage 4/stage 2 identified 111 metabolites, with 66 higher and 45 lower; and comparisons of stage 4/stage 3 identified 11 metabolites, with 7 higher and 4 lower. Major differences in metabolite profiles with increasing stage of CKD were observed, including altered arginine metabolism, elevated coagulation/inflammation, impaired carboxylate anion transport, and decreased adrenal steroid hormone production.

Conclusions Global metabolite profiling of plasma uncovered potential biomarkers of stages of CKD. Moreover, these biomarkers provide insight into possible pathophysiologic processes that may contribute to progression of CKD.


Introduction
CKD encompasses a spectrum of kidney diseases, ranging from kidney damage with normal kidney function to ESRD. The National Kidney Foundation has devised a five-stage classification system for CKD based on the level of GFR (1). Cardiovascular disease (CVD) is a major cause of morbidity and mortality in patients with all forms of CKD and contributes to the complexity of CKD (2–6). This complexity introduces new challenges in predicting and treating patients sufficiently early in the course of CKD to positively alter patient outcome. Until recently, most risk factor analysis of kidney diseases has focused on ESRD. Little is known about the effect of various risk factors at each stage of disease and how these contribute to the rate of progression to ESRD. In addition, there has been little study of the potential differences in risk factors for transition from one stage to the next and whether the risk factors for onset and transition from stage 1 to stage 2 may differ from those for transition to stage 3 and stage 4 CKD or for final development of ESRD. Currently, both the incidence and prevalence of CKD leading to ESRD continue to increase at an alarming rate in the United States. There are at least 19 million people in the United States with some degree of CKD (7), with enormous costs to society (8), prompting the Surgeon General to include CKD as a focus area for improving the nation’s health in Healthy People 2010. An understanding of the characteristics of early stage CKD, as well as the factors that differentially affect the progression of CKD from one stage to the next, is essential for determining appropriate therapy and predicting long-term outcomes.

Metabolomics, which is the most recent systems-biology approach to complement the genomic, transcriptomic, and proteomic efforts to characterize an entire biologic system, is increasingly being used to study kidney function (9–11). Because metabolites represent the end products of the genome and proteome, metabolomics holds the promise of providing an integrated physiologic phenotype of a system. Such metabolic profiling involves a comprehensive measurement of the types and concentrations of metabolites in a system at a specified time, such as each stage of CKD. Metabolomics also provides insight into metabolic pathways and networks downstream of gene expression. Complex metabolite profiles may...
provide the data required to enable the diagnosis, risk stratification, treatment, and evaluation of treatment response of patients. This may be through the identification of single biomarkers as in the more traditional methods or more likely by identification of patterns across many metabolites.

In this cross-sectional study, we determined plasma metabolite profiles of 30 participants; these were non-diabetic men aged 40–52 years, with 10 each in CKD stages 2, 3, and 4 based on their estimated GFR. Our goal was to determine whether there are significant differences in specific metabolites by stage of CKD and whether these may be useful stage-specific biomarkers. A related goal was to consider whether these differences might offer insight into potential pathophysiologic mechanisms that contribute to progression of CKD.

Materials and Methods
Study Participants
Plasma samples, which were obtained from the University of Pennsylvania from patients recruited in late 2008, were stored at −80°C for this study. Samples from 30 participants with CKD, 10 each in stages 2, 3 and 4, were selected for metabolite analysis at Metabolon Inc in 2012. Informed consent was obtained from all participants. Descriptions of the participants are summarized in Table 1.

Sample Accessioning/Preparation
All mass spectrometry data were collected at Metabolon Inc. Each plasma sample was accessioned into the Metabolon LIMS system and was assigned by the LIMS unique identifier, which was associated with the original source identifier only. The nontargeted metabolic profiling platform utilized for this analysis combined three independent platforms: ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS2) optimized for basic species, UHPLC–MS/MS2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed essentially as described previously (12,13). For each sample, 100 µl was used for analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the plasma with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50 µl 0.1% formic acid in water (acidic conditions) or in 50 µl 6.5 mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC–MS/MS2 analyses, or were derivatized to a final volume of 50 µl for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for 1 hour. In addition, three types of controls were analyzed in concert with the experimental samples: aliquots of a well characterized human plasma pool served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Experimental samples and controls were randomized across platform run days.

LC/MS, LC/MS2
For UHPLC–MS/MS2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Milford, MA) and were analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA) that consisted of an electrospray ionization source and linear ion-trap mass analyzer. The MS instrument scanned 99–1000 m/z and alternated between MS and MS2 scans using dynamic exclusion with approximately six scans per second.

GC/MS
Derivatized samples for GC/MS were separated on a 5% phenylmethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific Inc.) operated at unit mass resolving power with electron impact ionization and a 50–750 atomic mass unit scan range.

Compound Identification
Compounds were identified by automated comparison of the ion features in the experimental samples with a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts,

| Table 1. Study participant baseline characteristics |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Kidney Disease Progression |
|                                | CKD Stage 2      | CKD Stage 3      | CKD Stage 4      |
| Number of patients             | 10               | 10               | 10               |
| Sex                            | Male             | Male             | Male             |
| Ethnicity                      | NHW              | NHW              | NHW              |
| Age (yr)                       | 51.4 ± 3.3       | 58.2 ± 2.6       | 61.5 ± 4.7       |
| Height (cm)                    | 168.1 ± 6.9      | 174.5 ± 5.3      | 175.5 ± 6.1      |
| Weight (kg)                    | 92.9 ± 9.6       | 97.3 ± 7.9       | 100.5 ± 10.0     |
| Body mass index                | 32.8 ± 2.6       | 31.9 ± 2.2       | 32.5 ± 1.8       |
| Estimated GFR (ml/min per 1.73 m²) | 63.6 ± 13.2     | 37.9 ± 9.9       | 27.4 ± 4.4       |

Data are presented as mean ± SD. NHW, non-Hispanic white.
Identification of Thematic Changes

Differences in level of a specific metabolite among a large number may be significant by chance. Therefore, it was of interest to search for thematic differences in which multiple metabolites are significantly higher or lower, which would unlikely be due to chance. Random Forest classification of CKD stage 2 compared with stage 3 and of CKD stage 4 compared with stage 2 identified metabolites based upon their abilities to identify groups. The 30 top-ranking metabolites for the Random Forest classification that compared CKD stages 2 and 4 are listed in Figure 1, denoted as the biochemical importance plot. These metabolites were evaluated, along with related metabolites beyond the top 30, to identify thematic differences. These differences may reveal CKD stage-specific biomarkers. They may also reflect significant alterations of pathophysiology that promote progression from CKD stage 2 to higher stages. Several themes were identified.

Altered Arginine Metabolism

A large difference in relative metabolite concentration was observed for dimethylarginine, as shown in Table 2, as a combination of asymmetric and symmetric dimethylarginine. Dimethylarginine in CKD stage 3 is higher compared with stage 2 (8.1-fold) and in CKD stage 4 compared with stage 2 (4.8-fold). This represents one of the larger metabolite fold increases that were observed in this cross-sectional comparison of CKD stage 2 with higher stages. Other metabolites related to arginine metabolism that were also significantly different in CKD stages 3 and 4 compared with stage 2 include ornithine and citrulline. Ornithine was markedly lower in CKD stages 3 and 4 compared with CKD stage 2.

Elevated Coagulation/Inflammation

The largest fold difference that was observed in comparisons of CKD stage 2 with stage 3, which remained elevated in stage 4, was the increase in coagulation/inflammation factor fibrinopeptide-A and phosphorylated fibrinopeptide-A (Table 2). The higher level of fibrinopeptide-A in CKD stage 3 compared with stage 2 (689-fold) is maintained in CKD stage 4 compared with stage 2 (827-fold). The higher level of phosphorylated fibrinopeptide-A (phosphorylated at serine-3) in CKD stage 3 compared with stage 2 (18-fold) remained elevated in stage 4 compared with stage 2 (45-fold) and was significantly higher in CKD stage 4 compared with stage 3 (2.5-fold). Proline-hydroxyproline dipeptide was significantly higher in CKD stage 3 compared with stage 2 (2.5-fold) and in stage 4 compared with stage 2 (4.5-fold), which may reflect matrix degradation in response to increased coagulation/inflammation.

Impaired Carboxylate Anion Transport

Numerous mono- and di-carboxylate anions are higher in CKD stages 3 and 4 compared with CKD stage 2 (Table 2). A number of these are \( \gamma \)-glutamyl amino acid dipeptides. \( \gamma \)-glutamylglutamine, for example, is higher in CKD stage 3 compared with stage 2 (3.8-fold) and in stage 4 compared with stage 2 (4.8-fold). The \( \gamma \)-glutamyl amino acid dipeptides in Table 2 are involved in the \( \gamma \)-glutamyl cycle,
which is involved in glutathione homeostasis. These increases may reflect increased oxidative stress related to depletion of glutathione. Other carboxylate anions also are increased. 3-Carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF), a known uremic toxin that accumulates in ESRD, is higher in CKD stage 3 compared with stage 2 (18.3-fold) and in stage 4 compared with stage 2 (23.6-fold).

Decreased Adrenal Steroid Hormone Production

A number of adrenal steroid hormones, especially sulfated metabolites, were significantly lower in CKD stage 4 compared with stage 2 (Table 2). These are also anions. However, unlike the situation with carboxylate anions, in which higher levels were observed in higher stages of CKD, the sulfated metabolites were lower in the higher stages of CKD. Cortisol was also significantly lower in CKD stage 4 compared with stage 2 (0.62-fold). This suggests that a decrease in production of adrenal steroid hormones may explain these data.

Discussion

Major differences in metabolite profiles in the various stages of CKD were observed, consistent with altered arginine metabolism, elevated coagulation/inflammation, impaired carboxylate anion transport, and decreased adrenal steroid hormone production. These differences may reveal stage-specific biomarkers of CKD. Of particular interest are the major differences in metabolite profiles related to arginine metabolism and the significance of these changes with respect to impaired production of NO and the effect that this may have on endothelial function. Also of particular interest are the large fold increases in the levels of fibrinopeptide-A in comparisons of CKD stages 3 and 4 with CKD stage 2 and the significance of these increases with respect to development of a procoagulation/proinflammation state.

Arginine Metabolism

There are extensive data on the possible role of asymmetric dimethylarginine in kidney disease owing to its
### Table 2. Ratios of significant changes of specific metabolites by stage of CKD (P≤0.05), mean values, and P values

<table>
<thead>
<tr>
<th>Altered arginine metabolism</th>
<th>Fold of Change</th>
<th>Mean ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylarginine (SDMA + ADMA)</td>
<td>Stages 3/2 8.1</td>
<td>Stages 4/2 4.8</td>
<td>CKD Stage 2 0.497±0.133</td>
</tr>
<tr>
<td>Citrulline</td>
<td>1.6</td>
<td>1.3</td>
<td>0.825±0.194</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.28</td>
<td>0.16</td>
<td>4.33±1.39</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.5</td>
<td>1.5</td>
<td>0.675±0.238</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Elevated coagulation/inflammation</th>
<th>Fold of Change</th>
<th>Mean ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinopeptide A</td>
<td>Stages 3/2 689</td>
<td>Stages 4/2 827</td>
<td>CKD Stage 2 0.002±0.001</td>
</tr>
<tr>
<td>Phosphorylated fibrinopeptide A</td>
<td>18</td>
<td>45</td>
<td>2.5</td>
</tr>
<tr>
<td>Proline-hydroxyproline</td>
<td>2.5</td>
<td>4.5</td>
<td>0.466±0.251</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Impaired carboxylate anion transport</th>
<th>Fold of Change</th>
<th>Mean ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Glutamylleucine</td>
<td>1.3</td>
<td>0.821±0.283</td>
<td>1.07±0.161</td>
</tr>
<tr>
<td>γ-Glutamylisoleucine</td>
<td>1.6</td>
<td>1.7</td>
<td>0.670±0.196</td>
</tr>
<tr>
<td>γ-Glutamylglutamine</td>
<td>3.8</td>
<td>4.8</td>
<td>0.275±0.166</td>
</tr>
<tr>
<td>γ-Glutamylphenylalanine</td>
<td>1.5</td>
<td>1.3</td>
<td>0.903±0.240</td>
</tr>
<tr>
<td>CMPF</td>
<td>18.3</td>
<td>23.6</td>
<td>0.307±0.279</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased adrenal steroid hormone production</th>
<th>Fold of Change</th>
<th>Mean ± SD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydroisoandrosterone sulfate</td>
<td>0.55</td>
<td>1.48±0.878</td>
<td>0.819±0.685</td>
</tr>
<tr>
<td>4-androsten-3-β,17-β-diol disulfate</td>
<td>0.26</td>
<td>3.91±3.76</td>
<td>1.02±0.958</td>
</tr>
</tbody>
</table>

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; CMPF, 3-Carboxy-4-methyl-5-propyl-2- furanpropanoate.

*0.1 > P > 0.05.
ability to inhibit nitric oxide synthase (NOS) and limit production of NO, thereby contributing to vascular complications associated with CKD (18, 19). The presence of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) in human urine was first reported in 1970 (20). Inhibition of NOS by ADMA but not by SDMA was reported in 1992, with the observation that plasma levels of SDMA and ADMA were markedly elevated in CKD and ESRD and the suggestion that inhibition of NOS by ADMA may contribute to CVD, hypertension, and immune dysfunction associated with kidney disease (21). ADMA and SDMA are produced as post-translational modifications of selected arginine residues in specific proteins by methyl transfer from S-adenosylmethionine, which is catalyzed by protein arginine methyltransferases (PRMTs) (22, 23). In turnover of proteins with methylated arginine residues, ADMA and SDMA, as well as mono-methylated arginine residues, are released. Most of the SDMA is released into plasma for clearance by the kidney. ADMA, however, is primarily converted into dimethylamine and citrulline, catalyzed by dimethylarginine dimethylaminohydrolases (DDAH-1 and DDAH-2) with distinct tissue distribution and regulation (24). Hydrolysis catalyzed by DDAH accounts for about 80% of the fate of ADMA. The remaining ADMA is excreted into the urine. It has been suggested that the plasma concentrations of ADMA are determined primarily by DDAH-1, which is highly expressed in kidney (25) and is colococalized with NOS (26). Thus, impaired kidney function may directly dictate the plasma concentrations of ADMA, which can inhibit endothelial NOS (eNOS) at low micromolar concentrations. In addition, methylarginines and dimethylarginines reduce uptake of arginine and other cationic amino acids by inhibition of amino acid transporters CAT-1 and CAT-2, which may also contribute to diminished production of NO (27).

Coagulation/Inflammation

Damage to the endothelium normally exposes collagen and other subendothelial proteins that are recognized by platelet receptors to initiate platelet activation. In addition, tissue factor is exposed on the damaged endothelium and can form a complex with factor VII to initiate the proteolysis cascades that produce thrombin. The activated platelets bind thrombin, which results in release of additional factors to recruit platelets to the site of clot formation. Platelet-bound thrombin also catalyzes the degradation of fibrinogen to form fibrin at the surface of the aggregated platelets, which is then cross-linked to form the clot. Patients with CKD can exhibit defects with any of these aspects of hemostasis, suggesting that CKD is a procoagulation state (28). Impaired platelet activation has been reported in CKD patients with mild-to-moderate CKD (29).

Fibrinopeptide-A is a 16 amino acid peptide derived from the thrombin-catalyzed proteolysis of the N-terminal end of the Aα-chain in fibrinogen. Accumulation of fibrinopeptide-A may reflect diminished capacity to clear this metabolite concomitant with a decrease in estimated GFR or may indicate development of a procoagulation state in the progression of CKD stage 2 to stages 3 and 4. Fibrinopeptide-A is a proinflammatory peptide (30), which suggests that this potential development of a procoagulation state is accompanied by the development of inflammation. Fibrinopeptide-A may be a useful stage-specific biomarker. However, the marked differences in levels of fibrinopeptide-A with stage of CKD may also reflect the development of a procoagulation/proinflammation state and may define the critical point of progression of CKD to states that eventually lead to ESRD. In support of this suggestion, recent studies of several animal models of CKD demonstrated the involvement of coagulation factor Xa and the ability of inhibitors of factor Xa to suppress development of some of the pathologic factors associated with CKD (31).

A critical component of endothelial control of hemostasis is the generation of NO at the appropriate time and levels. In experimental animal studies, eNOS production of NO regulates expression of tissue factor, suggesting that impairment of NO production will result in elevated tissue factor and promotion of coagulation (32). In addition, NO produced by the endothelium acts locally to inhibit platelet aggregation and therefore is essential for dampening the procoagulation response (33). The differences in levels of dimethylarginines (Table 2) observed in comparisons of CKD stage 2 with stage 3 suggests impaired NO production, which therefore may exacerbate the development of a procoagulation/proinflammation state in early stage CKD and enhance its progression (34).

Carboxylate Anions

CMPF is one of a number of uremic toxins that accumulate in ESRD, due to tight binding to albumin, and present problems in removal during hemodialysis (35). CMPF is toxic both to endothelial cells and to proximal tubular cells (36, 37). CMPF was higher in CKD stage 3 compared with stage 2 and higher in stage 4 compared with stage 3.

In summary, in this cross-sectional metabolomics study, we determined metabolite patterns in different stages of CKD. The results demonstrated that, for the specific population that was studied, significant differences in metabolite patterns occur. Specifically, markedly higher levels of dimethylarginine and fibrinopeptide-A in comparisons of CKD stage 3 with CKD stage 2 suggest that these may be stage-specific biomarkers. These differences also point to endothelial dysfunction and suggest that a convergence of impaired NO production and enhanced production of coagulation/inflammation factor fibrinopeptide-A may collectively act to enhance progression of CKD beyond stage 2. These are testable hypotheses.

Study Limitations

This was an initial proof-of-concept study in which to address the question whether metabolomics might provide meaningful data for comparison of differences in CKD by stage. As such, it was a cross-sectional study with a limited study population. The results, although interesting and potentially meaningful for identification of stage-specific biomarkers and for insight into CKD progression, are preliminary to a longitudinal study of metabolite changes in CKD progression. The approved future longitudinal study will utilize the Chronic Renal Insufficiency Cohort population in order to compare CKD progression in diabetic and nondiabetic CKD populations that are further divided by age, sex, and ethnicity.
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Disclosures
E.K. and K.L.P. are employees of Metabolon Inc and, as such, have affiliations with or financial involvement with Metabolon Inc.

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