Serum and adipose tissue fatty acid composition as biomarkers of habitual dietary fat intake in elderly men with chronic kidney disease

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ABSTRACT

Background. Fatty acid (FA) composition in serum cholesterol esters (CE) and adipose tissue (AT) reflect the long-term FA intake in the general population. Because both dietary intake and FA biomarkers associate with renal function, our aim was to identify which CE and AT FAs are useful biomarkers of habitual FA intake in individuals with chronic kidney disease (CKD).

Methods. Cross-sectional analysis was performed in 506 men (aged 70 years) with a glomerular filtration rate (GFR) of <60 mL/min per 1.73 m² from the Uppsala Longitudinal Study of Adult Men cohort. Dietary habits were evaluated with a 7-day dietary record. FA compositions in CE and AT were analyzed by gas–liquid chromatography in two random subsamples of 248 and 318 individuals, respectively.

Results. Both CE and AT linoleic acid and docosahexaenoic acid (DHA) were strongly associated with their corresponding intake, after adjustments for non-dietary factors. The proportions of eicosapentaenoic acid (EPA) and palmitic acid in CE and AT moderately correlated with dietary intake, whereas correlations of other FAs were weaker or absent. Proportions of EPA and DHA in CE and AT were positively associated with the total energy-adjusted fish intake. Results were confirmed in adequate reporters as identified by the Goldberg cutoff method. These relationships held constant, regardless of a GFR above or below 45 mL/min per 1.73 m² or the prevalence of microalbuminuria.

Conclusions. Proportions of EPA, DHA, palmitic and linoleic acid in serum CE and AT are good indicators of their dietary intake in men with CKD. They can be considered valid biomarkers for epidemiological studies and assessment of compliance.

INTRODUCTION

Chronic kidney disease (CKD), defined as decreased glomerular filtration rate (GFR) and/or albuminuria, is highly prevalent worldwide and is recognized as a public health burden [1]. The prevalence of CKD ranges from 23.4 to 35.8% in people older than 64 years [2] and rises to 47% in those above 70 years [3], mostly due to decline in the GFR with age. CKD patients are vulnerable to malnutrition, systemic inflammation, metabolic disorders, premature cardiovascular disease (CVD) and progression to end-stage renal disease (ESRD), which collectively lead to high mortality rates [4–6].

The quantity and quality of dietary fatty acids (FAs) affect clinical outcomes [7–9]. Reports using dietary assessment suggest that an unfavourable dietary FA pattern, generally characterized by high saturated FA (SFA) and low polyunsaturated FA (PUFA) intake, is common in patients with CKD.
and may contribute to CKD-related risk profile and mortality [12]. Dietary assessment methods have, however, several limitations that may weaken both the accuracy and precision of the measurement, such as under-reporting of respondents, interviewer bias and lack of well-matched food composition databases [13].

FA biomarkers in blood or tissues could be more accurate and convenient for estimating the long-term dietary FA intake [13]. Previous studies in populations without CKD have suggested that FA proportions in serum cholesterol esters (CEs), phospholipids, as well as adipose tissue (AT) are good indicators of the corresponding habitual intake of FAs of exogenous origin [14–16]. However, results regarding the effect of chronic disease status on diet-biomarker correlations are still mixed [14, 16]. Because both dietary intake and biomarkers of FA intake are associated with the GFR in community studies [17, 18], it is plausible that renal diseases may modify these associations. Although some studies in CKD patients have used serum FAs as biomarkers of dietary intake [8, 11, 19], it is presently unknown whether these biomarkers validly do so in the context of CKD. The objective of this study was to identify which blood and AT FAs are useful biomarkers of habitual FA intake in individuals with CKD.

**Materials and Methods**

**Study population**

This is a cross-sectional analysis including individuals with reduced kidney function (GFR <60 mL/min per 1.73 m²) from The Uppsala Longitudinal Study of Adult Men (ULSAM) community-based cohort. The ULSAM cohort was initiated in 1970; all 50-year-old men born between 1920 and 1924 who lived in Uppsala, Sweden, were invited to a health survey (described in detail at http://www2.pubcare.uu.se/ULSAM/). Participants returned for subsequent examinations at age 60, 70, 77 and 82 years. The present analyses are based on the third examination cycle of the ULSAM cohort, when participants were approximately 70 years of age (visits performed during 1991 to 1995; n = 1221). Inclusion criteria for this analysis is a serum cystatin C-estimated GFR <60 mL/min per 1.73 m² (n = 543). Additional exclusion criteria were incomplete data on 7-day dietary records (n = 36) and abnormal values of reported energy intake (<3200 or >18 000 kJ/day; n = 1). The present study therefore comprises 506 participants with CKD according to the current Kidney Disease Outcomes Quality Initiative definition [20]. All participants gave written consent, and the Ethics Committee of Uppsala University approved the study.

All investigations were performed under standardized conditions as described elsewhere [21]. The smoking status was defined as current smoking versus nonsmoking. Regular physical activity was defined as the reporting of regular or athletic leisure-time exercise habits according to four physical activity categories (sedentary, moderate, regular and athletic) [22]. Previous CVD was defined as history of any CVD as recorded in the Swedish Hospital Discharge Registry [International Classification of Diseases (ICD-8) codes 390 to 459]. Diabetes mellitus (DM) was defined as fasting plasma glucose ≥7.0 mmol/L, 2-h postload glucose levels ≥11.1 mmol/L or the use of oral hypoglycaemic agents or insulin [23]. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg or use of antihypertensive medications. Hyperlipidaemia was defined as serum cholesterol >6.5 mmol/L and/or serum triglycerides ≥2.3 mmol/L and/or treatment with lipid-lowering medications.

**Laboratory analysis**

Venous blood samples were drawn after an overnight fast and stored at −70°C until analyses. Serum cystatin C was measured by latex-enhanced reagent (N Latex Cystatin C, Dade Behring, Deerfield, IL, USA) with a Behring BN ProSpec analyser (Dade Behring). The assays were performed at the Department of Clinical Chemistry, University Hospital, Uppsala, which is accredited according to the Swedish Board for Accreditation and Conformity Assessment (Swedac) standard ISO/IEC 17025. The total analytical imprecision of the method was 4.8% at 0.56 mg/L and 3.7% at 2.85 mg/L. GFR was calculated from serum cystatin C concentrations (mg/L) by the following formula: $y = 77.24 \times x^{1.2623}$, which has been shown to be closely correlated with iothalamate clearance [24]. Individuals with CKD were further divided into stage 3A and more advanced stage of CKD on the basis of a GFR cut-off value of 45 mL/min per 1.73 m². Urinary albumin excretion rate (UAER) was calculated on the amount of albumin in the urine collected during the night. The assay employed a commercially available radioimmunoassay kit (Albumin RIA 100, Pharmacia, Uppsala, Sweden). Microalbuminuria was defined as UAER ≥30 mg/24 h.

**Dietary assessment and determination of dietary adequate reporters**

Dietary habits were evaluated with an optically readable form of a 7-day dietary record based on a validated pre-coded menu book [25], which was prepared and previously used by the Swedish National Food Administration (NFA) [26]. The participants were given oral instructions by a dietitian on how to perform the dietary registration, and the amounts consumed were reported in household measurements or specified as portion sizes. The daily intake of energy, various FAs, fish and alcohol were calculated by using a database from the Swedish NFA. This method was used to estimate the intake of major specific FAs, e.g. 16:0 and 18:0 in the SFA class. The FA intake was expressed in two different ways: as absolute intake (g/day) and as a percentage of total fat intake by weight [(g/g total fat) × 100], with the latter being comparable with biomarker measurements.

Stringent criteria to identify adequate reporters of energy intake were applied according to the Goldberg cutoff [27]. In this procedure, an acceptable range of energy intake is determined for each subject in relation to the estimated energy expenditure taking the level of physical activity and basal metabolic rate into consideration, i.e. producing a 95% confidence interval (CI) for energy intake required for weight maintenance. Subjects with reported energy intake within the 95%
CI were regarded as adequate reporters, rendering a subpopulation of 250 individuals for verification of the associations reported in the whole material.

FA analysis

FA compositions in serum CE and AT were analysed in two random sub-samples of 248 and 318 CKD men, respectively. Subcutaneous AT was collected with a biopsy from the upper, outer quadrant of the buttocks [28]. The sample was stored at –70°C for some weeks until analysis.

The FA composition was analysed as described previously [29]. Briefly, an extraction with chloroform was conducted. The dry extracts were dissolved in a few drops of chloroform and filled in thin liquid chromatography plates for separation of the lipids. The lipid esters were trans-methylated and the methyl esters were extracted. The FA methyl esters were dissolved in hexane and separated by gas–liquid chromatography (GLC). The Hewlett Packard GLC system used for the analyses was consisted a GC 5890, automatic sampler 7671A, integrator 3392A and 25 m Quadrex Fused Silica capillary column OV-351. The FAs were identified by comparison of the retention times of separation, controlled by Nu Check Prep GLC reference standard GLC-68A. The coefficients of variation (CVs) for all FAs were 1–5.5%, except for 18:0, with a CV of 9.9% [30]. FAs are given as the relative percentage of the sum of the FAs analysed. In this analysis, we compared the eight individual FAs that were estimated from dietary records, that is, 16:0, 18:0, 18:1 n-9, 18:2 n-6, 20:4 n-6, 18:3 n-3, 20:5 n-3, and 22:6 n-3.

Statistical analysis

All statistical analyses were performed using statistical software STATA version 12 (Stata Corporation, College Station, TX, USA). Figures were created with GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA). Values were expressed as mean ± standard deviation (SD) or median (interquartile range) or percentage of total, as appropriate. Spearman’s univariate correlation coefficients (ρ) were calculated to determine correlations between the proportions of specific dietary FAs and their biomarkers in serum CE and AT. Multivariate regression models were fitted to assess the independence of this association by introducing potential confounders such as BMI, smoking status, physical activity, alcohol intake, the prevalence of comorbidities (CVD, DM, hypertension and hyperlipidaemia), GFR and UAER. Data are expressed as standardized regression coefficients (β). Multivariate regression models were used to detect linear trends of marine n-3 PUFA (20:5 n-3 and 22:6 n-3) proportions in serum CE and AT across quartiles or with the linear increase of daily fish intake corrected for the total energy intake. All analyses were repeated in the subset of adequate reporters to eliminate bias of non-adequate reporting. To investigate whether the associations between dietary and biomarker FAs were modified by GFR or UAER, linear trends of FAs in CE and AT across quartiles of the corresponding FAs dietary intake, stratified by GFR (above and below 45 mL/min per 1.73 m²) or UAER (above and below 30 mg/24 h), were tested. The FAs considered important a priori for investigation were 16:0, 18:1 n-9, 18:2 n-6, and 22:6 n-3, because they represent the major dietary sources of SFA, MUFA, n-6 and n-3 PUFA families, respectively. All tests were two-tailed and P < 0.05 was considered significant. Because P values were not adjusted for multiple testing, they have to be considered as descriptive.

RESULTS

General characteristics

Age, BMI, lifestyle parameters, the prevalence of comorbidities, GFR, UAER and nutrient intake of included patients are shown in Table 1. No significant differences were observed between the whole cohort and the random subpopulations in which serum CE and AT FA compositions were measured.

FA compositions in diet, serum CE and AT

Daily dietary FA intakes, as well as the relative proportions of individual FA in the diet, serum CE and AT, are presented in Table 2. SFAs were the most abundant source of fat according to dietary records, followed by MUFA, n-6 and n-3 PUFA. Dietary 16:0, 18:1 n-9, 18:2 n-6 and 18:3 n-3 were the most abundant FAs in their respective subfamilies. In the case of CE, 18:2 n-6 represented about 50% of all FAs, whereas 18:1 n-9 was the most abundant FA in AT.

Correlations of FA biomarkers with dietary intake

In Table 3, Spearman’s univariate correlations show that 18:2 n-6 and 22:6 n-3 in serum CE were strongly correlated with their corresponding intake. The major dietary SFA 16:0 as well as 20:5 n-3 presented moderate ρ values. On the other hand, 18:0, 18:3 n-3 and 20:4 n-6 were not associated with the dietary intake, whereas 18:1 n-9 in CE was negatively correlated with its proportion in the diet. In AT, the correlations with dietary FAs were similar, except that 18:3 n-3 was moderately associated and 18:1 n-9 was not significantly associated with their counterparts in dietary records.

Multivariable regression models investigating independent associations between dietary FA intake and FA biomarkers in serum CE and AT are presented in Table 3. We did not observe substantial differences between crude regression coefficients and standard coefficients in multivariate regression models, which were adjusted for BMI, smoking status, alcohol intake, physical activity, comorbidities, GFR and UAER. As a sensitivity analysis, these regression models were also fitted in the subpopulation of adequate reporters. As shown in Figure 1, the strength of the associations between dietary FAs and their corresponding CE and AT biomarkers were maintained or even improved.

Associations between fish intake and marine n-3 PUFA biomarkers

Biomarkers for marine n-3 PUFA in serum CE and AT were incrementally higher across increasing reported daily fish intake (Figure 2). Daily fish intake, adjusted for total energy intake, was positively associated with the proportions of 20:5 n-3 and 22:6 n-3 in CE (β = 0.21, P < 0.001; β = 0.26, P < 0.001) and AT (β = 0.18, P = 0.001; β = 0.18, P < 0.001). These results were also confirmed in the subpopulation of adequate reporters (data not shown).
Association between dietary FA intake and FA biomarkers in individuals with different GFR and UAER

Figure 3 shows the association between quartiles of dietary reported FA intake of selected FAs (16:0, 18:1 n-9, 18:2 n-6, and 22:6 n-3) with their corresponding biomarkers in CE and AT, with patients stratified by CKD stages (stage 3A or worse). FA biomarkers varied in a monotonic fashion along with increasing dietary intake, regardless of the stratum. Similar patterns were observed by stratifying according to the prevalence of microalbuminuria (data not shown).

DISCUSSION

This is the first study investigating associations of dietary FA intake with FA biomarkers in serum CE and AT in individuals with CKD stages 3–4. An important strength of this study is the availability of both serum CE and AT in the same population.

AT FA composition has been considered a gold standard for the representation of long-term (>1 year) dietary FA intake, due to the slow turnover time [31] and its lack of response to acute disease [32]. Serum CE is more sensitive to recent diet [33] and represents dietary intake over weeks or months [7]. The data on two types of biologic specimens as well as diet therefore offer internal consistency of specimen comparisons. Another advantage is that a 7-day dietary record rather than a food-frequency questionnaire was used for dietary assessment. The ‘real-time’ nature of the former decreases reporting bias related to memory, particularly in such an elderly cohort. Finally, in order to minimize this bias, we applied the Goldberg cutoff and confirmed the main findings in a subgroup of adequate reporters. The fact that ~50% of the study participants did not adequately report their dietary intake highlights the importance of reporting bias in dietary assessment.

We observed strong associations between intakes of the most essential PUFA, i.e. 18:2 n-6, 20:5 n-3 and 22:6 n-3, and
their respective biomarkers in serum CE and AT. As the body can endogenously synthesize neither 18:2 \( n-6 \) nor 18:3 \( n-3 \), these two FAs are mainly derived from dietary plant oils. Both 20:5 \( n-3 \) and 22:6 \( n-3 \) are \( n-3 \) PUFA of marine origin, e.g. from oily fish or fish oils. Although they can be synthesized from dietary 18:3 \( n-3 \) via elongation and desaturation, the efficiency of the conversion from 18:3 \( n-3 \) to 20:5 \( n-3 \) is poor and controversial (0.2–15%) and the conversion to 22:6 \( n-3 \) is even poorer [34, 35]. As expected from its biology, the relationships between dietary intake and biomarkers for these essential

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Dietary records (( n = 506 ))</th>
<th>CE (( n = 248 ))</th>
<th>AT (( n = 354 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>14.22 ± 5.34</td>
<td>20.98 ± 1.91</td>
<td>11.77 ± 0.90</td>
</tr>
<tr>
<td>18:0</td>
<td>6.55 ± 2.32</td>
<td>9.66 ± 0.63</td>
<td>0.96 ± 0.19</td>
</tr>
<tr>
<td>MUFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 ( n-9 )</td>
<td>19.32 ± 6.71</td>
<td>28.59 ± 2.18</td>
<td>20.52 ± 2.23</td>
</tr>
<tr>
<td>PUFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n-6 ) PUFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 ( n-6 )</td>
<td>7.77 ± 3.20</td>
<td>11.50 ± 2.51</td>
<td>52.32 ± 4.53</td>
</tr>
<tr>
<td>20:4 ( n-6 )</td>
<td>0.13 ± 0.06</td>
<td>0.19 ± 0.07</td>
<td>5.86 ± 1.08</td>
</tr>
<tr>
<td>( n-3 ) PUFA</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18:3 ( n-3 )</td>
<td>1.16 ± 0.45</td>
<td>1.73 ± 0.53</td>
<td>0.83 ± 0.21</td>
</tr>
<tr>
<td>20:5 ( n-3 )</td>
<td>0.09 ± 0.07</td>
<td>0.14 ± 0.12</td>
<td>1.72 ± 0.76</td>
</tr>
<tr>
<td>22:6 ( n-3 )</td>
<td>0.21 ± 0.13</td>
<td>0.33 ± 0.21</td>
<td>0.96 ± 0.23</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation. MUFA, monounsaturated FAs; PUFA, polyunsaturated FAs; SFA, saturated FAs.

Table 3. Univariate and multivariate regressions between dietary FA intake and FA compositions in plasma cholesterol esters and adipose tissue in individuals with chronic kidney disease

<table>
<thead>
<tr>
<th>FAs in the diet</th>
<th>Cholesterol esters</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman’s correlation (( n = 236 ))</td>
<td>Multivariate regression (( n = 354 ))</td>
</tr>
<tr>
<td></td>
<td>Rho (P)</td>
<td>Std. beta (P)</td>
</tr>
<tr>
<td>16:0</td>
<td>0.26 (&lt;0.001)</td>
<td>0.26 (&lt;0.001)</td>
</tr>
<tr>
<td>18:0</td>
<td>0.07 (0.27)</td>
<td>0.11 (0.10)</td>
</tr>
<tr>
<td>18:1 ( n-9 )</td>
<td>-0.22 (&lt;0.001)</td>
<td>-0.27 (&lt;0.001)</td>
</tr>
<tr>
<td>18:2 ( n-6 )</td>
<td>0.34 (&lt;0.001)</td>
<td>0.36 (&lt;0.001)</td>
</tr>
<tr>
<td>20:4 ( n-6 )</td>
<td>0.02 (0.71)</td>
<td>0.01 (0.83)</td>
</tr>
<tr>
<td>18:3 ( n-3 )</td>
<td>-0.04 (0.53)</td>
<td>-0.07 (0.31)</td>
</tr>
<tr>
<td>20:5 ( n-3 )</td>
<td>0.24 (&lt;0.001)</td>
<td>0.19 (0.003)</td>
</tr>
<tr>
<td>22:6 ( n-3 )</td>
<td>0.38 (&lt;0.001)</td>
<td>0.34 (&lt;0.001)</td>
</tr>
</tbody>
</table>

Multivariate regression models were adjusted for BMI, smoking, alcohol intake, physical activity, cardiovascular disease, diabetes, hypertension, hyperlipidaemia, glomerular filtration rate and urinary albumin excretion rate.
PUFA were indeed the strongest in our study. This agrees with similar reports in non-CKD individuals [14, 15], and these biomarkers can be used as indicators of compliance in supplementation studies [36–39]. We also report that 20:5 *n*-3 and 22:6 *n*-3 biomarkers are largely associated with the fish intake, consistent with a previous report showing a positive association between the frequency of fish servings and *n*-3 PUFA index (erythrocyte 20:5 *n*-3 and 22:6 *n*-3 contents) in 75 haemodialysis patients [11]. However, for 18:3 *n*-3, we did not observe strong associations between dietary FA intake and the biomarker, not even when considering adequate reporters. These results were unexpected and the reason is unclear, but in similar studies the agreement of 18:3 *n*-3 seems also poorer than for the other essential PUFA [14, 16]. The smaller proportion of 18:3 *n*-3 and the relatively higher within-person variability in its measurement may have contributed to these results.

For non-essential FAs, the relationships of dietary with serum CE and AT compositions were weaker or absent, according with those in populations without CKD [14–16, 40]. However, CE and AT 16:0 were fairly good markers of dietary intake in the current population, although less strongly correlated than observed for 18:2 *n*-6 and 22:6 *n*-3. The correlations of SFA with the major MUFA 18:1 *n*-9 were however unexpected and difficult to explain. One might speculate that hepatic SCD-1 activity is suppressed in response to high intake of PUFA [39], food sources which also contain substantial amounts of 18:1 *n*-9 [41]. It is thus possible that high intake of vegetable oils (partly represented as high dietary 18:1 *n*-9 content) may in turn inhibit endogenous synthesis of 18:1 *n*-9 thereby decreasing its levels in the body, and vice versa.

The associations between dietary and biomarker FAs held constant across decreased GFR or elevated UAER groups, suggesting that moderate renal failure does not modify these associations. Likewise, one previous investigation indicates that the status of individuals with other chronic diseases, e.g. CVD, hypertension, and DM, does not modify these relationships either [14]. Nevertheless, we must take into consideration that the included patients were mostly within CKD stages 3A and 3B, and further studies may be necessary including patients with a broader GFR distribution.

Our results need to be interpreted in the light of certain limitations. Errors from dietary assessment, together with physiological within-person variability, are susceptible to weaken the correlations observed [14, 16]. Detailed phenotypic characterization allows us to take into account many non-dietary factors which may confound the dietary and biomarker correlations, but unmeasured or unknown ones cannot be excluded. In this regard, we did not have information regarding the status of individuals with other chronic diseases, e.g. diabetes, hypertension, hyperlipidaemia, glomerular filtration rate and urinary albumin excretion rate.

**FIGURE 1**: Relations between individual FA proportions in dietary records versus serum CE and AT, respectively, expressed as standard coefficients (β) in multivariate regression models, both in all individuals with chronic kidney disease as well as in adequate reporters only. Multivariate regression models were adjusted for BMI, smoking, alcohol intake, physical activity, cardiovascular disease, diabetes, hypertension, hyperlipidaemia, glomerular filtration rate and urinary albumin excretion rate.

**FIGURE 2**: Mean eicosapentaenoic acid (20:5 *n*-3) and docosahexaenoic acid (22:6 *n*-3) proportions in serum cholesterol esters (CE) and AT according to daily fish intake (energy adjusted) quartiles. Bars represent SEM. P for trend <0.01 for all.
the habitual dietary intake of FAs in elderly men with CKD. Dietary fish intake well reflect intake of \( n-3 \) PUFA of marine origin in this population. The weak or lack of association with 18:0, 18:1 \( n-9 \), and 20:4 \( n-6 \) limits their use as biomarkers and thus FA composition does not capture the intake of all FAs. Taken together, these results indicate that specific FA biomarkers could be a valid and objective tool to use in epidemiological studies which aim at linking dietary fat quality and diet-related conditions in CKD. At the same time, they can be considered to measure compliance in dietary intervention studies.

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**CONFLICT OF INTEREST STATEMENT**

BL is employed by Baxter Healthcare Corporation.

**REFERENCES**


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**Original Article**
Determination of uromodulin in human urine: influence of storage and processing

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*¹S.Y. and J.W. contributed equally to this study.

ABSTRACT

Background. Uromodulin (Tamm–Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins.

Methods. Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been

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