ORIGINAL RESEARCH

A Plasma Long-Chain Acylcarnitine Predicts Cardiovascular Mortality in Incident Dialysis Patients

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***Background*—**The marked excess in cardiovascular mortality that results from uremia remains poorly understood.

***Methods and Results*—**In 2 independent, nested case-control studies, we applied liquid chromatography-mass spectrometry-based metabolite profiling to plasma obtained from participants of a large cohort of incident hemodialysis patients. First, 100 individuals who died of a cardiovascular cause within 1 year of initiating hemodialysis (cases) were randomly selected along with 100 individuals who survived for at least 1 year (controls), matched for age, sex, and race. Four highly intercorrelated long-chain acylcarnitines achieved the significance threshold adjusted for multiple testing ($P<0.0003$). Oleoylcarnitine, the long-chain acylcarnitine with the strongest association with cardiovascular mortality in unadjusted analysis, remained associated with 1-year cardiovascular death after multivariable adjustment (odds ratio per SD 2.3 [95% confidence interval, 1.4 to 3.8]; $P=0.001$). The association between oleoylcarnitine and 1-year cardiovascular death was then replicated in an independent sample (n=300, odds ratio per SD 1.4 [95% confidence interval, 1.1 to 1.9]; $P=0.008$). Addition of oleoylcarnitine to clinical variables improved cardiovascular risk prediction using net reclassification (NRI, 0.38 [95% confidence interval, 0.20 to 0.56]; $P<0.0001$). In physiologic profiling studies, we demonstrate that the fold change in plasma acylcarnitine levels from the aorta to renal vein and from pre- to post hemodialysis samples exclude renal or dialytic clearance of long-chain acylcarnitines as confounders in our analysis.

***Conclusions*—**Our data highlight clinically meaningful alterations in acylcarnitine homeostasis at the time of dialysis initiation, which may represent an early marker, effector, or both of uremic cardiovascular risk. **(*J Am Heart Assoc*. 2013;2:e000542 doi: 10.1161/JAHA.113.000542)**

Key Words: cardiovascular disease • dialysis • metabolism • mortality • risk factors

Between 40% and 50% of deaths among patients with end-stage renal disease (ESRD) are due to cardiovascular causes,[1,2] and the risk of cardiovascular mortality in patients with ESRD is >10-fold the risk observed in the general population.[3,4] Traditional cardiovascular risk factors do not fully account for this excess mortality.[5] ESRD is characterized by various metabolic disturbances linked to cardiovascular outcomes, including insulin resistance and protein-energy wasting, but underlying mechanisms remain incompletely understood.[6–8] Therefore, new insights into the intersections among uremia, metabolism, and cardiovascular risk represent an important opportunity to improve patient care.

Current metabolite profiling (or metabolomics) technologies enable high-throughput, high-resolution phenotyping of human plasma and are able to identify novel disease biomarkers and their underlying metabolic pathways in well-characterized epidemiologic cohorts.[9] For example, we have applied liquid chromatography-mass spectrometry (LC-MS)-based metabolite profiling to plasma obtained from participants in the Framingham Heart Study to identify novel predictors of future type 2 diabetes and chronic kidney disease (CKD).[10–12] Although we and others have also applied metabolite profiling to document numerous metabolite alterations that accompany ESRD, to date, no study has linked these findings with future cardiovascular outcomes.[13,14]

Here, we report the application of metabolite profiling to plasma obtained from participants in the Accelerated Mortality on Renal Replacement (ArMORR) study, a prospective cohort

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Accompanying Figures S1 and S2 and Tables S1 through S5 are available at http://jaha.ahajournals.org/content/2/6/e000542/suppl/DC1

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study of ≈10 000 incident US hemodialysis patients. This cohort has been used to study established therapies (activated vitamin D),[15,16] as well as emerging biomarkers, for example, FGF-23 and plasma gelsolin in ESRD.[17,18] With ≈19% mortality at 1 year, access to archived plasma samples, and detailed demographic and laboratory phenotyping, this cohort provides an ideal opportunity to study uremic cardiovascular risk. To explore the mechanism by which select metabolites might accumulate in ESRD, we also performed metabolite profiling in the context of invasive renal catheterization and before and after hemodialysis in a separate set of subjects.

Methods

Study Population

The Accelerated Mortality on Renal Replacement (ArMORR) study is a prospective cohort study of 10 044 incident hemodialysis patients in any of 1056 US centers operated by Fresenius Medical Care North America between June 2004 and August 2005. Full details have been published previously.[15–17,19] Briefly, all subjects underwent 1 year of follow-up except for those who died (15.2%), voluntarily discontinued dialysis (5%), underwent kidney transplantation (3%), recovered renal function (4%), or transferred to a dialysis unit outside the Fresenius system before completing 1 year of hemodialysis (12%). Plasma samples drawn at the beginning of a dialysis session that would otherwise have been discarded after routine clinical testing were saved and stored in liquid nitrogen. Plasma samples were obtained within 14 days of initiation of outpatient hemodialysis.

We examined mortality according to metabolic profile in 2 nested, case-control samples of this cohort. Cases were defined as subjects who suffered cardiovascular death denoted by International Classification of Diseases version 9 codes during the first year of hemodialysis treatment, and controls were defined as subjects who survived through this period; venous thromboembolism was not included as a cause of cardiovascular death (Table S1). In the discovery sample, we randomly selected 100 cases from the entire ArMORR cohort along with 100 controls frequency matched for age, sex, and race. For the replication study, we randomly selected 100 cases along with 200 controls, again frequency matched for age, sex, and race.

CKD and Non-CKD Controls

In order to assess metabolic profiles in nondialysis subjects, we recruited 60 subjects referred to the Massachusetts General Hospital (MGH) Exercise Laboratory for diagnostic treadmill testing. Thirty individuals had normal renal function, as defined by an estimated glomerular filtration rate (eGFR) ≥60 mL/min per 1.73 m^2 using the MDRD equation, and 30 had CKD, as defined by an eGFR <60 mL/min per 1.73 m^2.[20]

Arterial and Renal Venous Plasma Sampling From Non-ESRD Subjects

We recruited 9 subjects referred to the MGH Cardiac Catheterization Laboratory for invasive blood sampling, as previously described.[12] The protocol included introduction of a Judkins right catheter into the ostium of a renal vein, with plasma sampling from this renal venous catheter and from a catheter positioned in the abdominal aorta at the level of the renal arteries. Because acylcarnitines were since added to our platform, these samples were reanalyzed using our updated profiling method.

Pre- and Post-Hemodialysis Sampling

We recruited 20 subjects undergoing hemodialysis at the MGH. Plasma was obtained from subjects immediately before and after a single hemodialysis session.

All study protocols were approved by the MGH IRB. The IRB waived the need for informed consent in ArMORR because all personal identifiers were removed from the blood samples and from the clinical data before transfer to the investigators. All other study participants above provided written informed consent.

Metabolite Profiling

For the discovery study in ArMORR, we applied 2 distinct LC-MS based methods to distinct plasma aliquots for each experimental sample. Amino acids, amino acid derivatives, urea cycle intermediates, nucleotides, and other positively charged polar metabolites were profiled as previously described using 10 μL of plasma.[10] Of note, this method has been updated to monitor acylcarnitines; using the same chromatographic and mass spectrometry settings previously described, each acylcarnitine transition was monitored as the $[M+H]^+$ parent mass along with the 85.1 *m/z* product ion. Organic acids, sugars, bile acids, and other negatively charged polar metabolites were profiled as previously described using 30 μL of plasma.[12] For the replication study in ArMORR and the physiologic studies of invasive renal catheterization and before and after hemodialysis, only the method that includes acylcarnitines was employed. For the replication study, we also purchased isotope labeled-oleoylcarnitine-^{13}C1 (Sigma-Aldrich) and spiked it into plasma at varying concentrations to generate a calibration curve (Figure S1), permitting comparison of absolute concentrations of plasma oleoylcarnitine levels across individuals with ESRD (ArMORR cases and controls), as well as CKD and non-CKD controls.

Statistical Analyses

Baseline characteristics were compared between cases and controls using chi square, Wilcoxon rank sum, or *t* tests as appropriate. In the discovery ArMORR sample, baseline levels for 165 metabolites were compared between cases and controls using 2-tailed *t* tests. To account for multiple testing, we used a Bonferroni corrected significance threshold of $P<0.0003$ (0.05/165). Non-parametric testing did not change our results.

The 4 metabolites meeting the significance threshold in the discovery analysis were all long-chain acylcarnitines and Pearson correlations were utilized to demonstrate high intercorrelation between each of these metabolites (Table S2). Pearson correlations were also used to determine the correlation between the metabolites and select clinical parameters. We performed logistic regression analyses to estimate the odds ratio of cardiovascular death at different levels of the most significant metabolite in the discovery cohort, oleoylcarnitine. Oleoylcarnitine was analyzed as a continuous variable (scaled to an SD of 1) and the model was adjusted for variables that differed between cases and controls at baseline (systolic blood pressure [mm Hg], albumin [g/dL], transferrin saturation [%], and phosphorous [mg/dL]). These analyses were repeated in the replication analysis, adjusting for variables that differed between cases and controls at baseline in this sample (initial vascular access [catheter versus no catheter], albumin, and systolic and diastolic blood pressure). Additional logistic regression models further adjusted for variables that have previously been associated with death in individuals undergoing hemodialysis: diabetes mellitus, body mass index, history of coronary artery disease, history of congestive heart failure, average urea reduction ratio, baseline hemoglobin (g/dL), ferritin (ng/mL), parathyroid hormone level (pg/mL), cardiac troponin-T (μg/L), and N-terminal pro-B-type natriuretic peptide (NT-pro BNP, ng/L).[17,21–25] Plasma oleoylcarnitine levels were compared between subjects with normal renal function and other groups (CKD, ESRD cases, and ESRD controls) using 2-tailed *t* tests. Because oleoylcarnitine was the only metabolite brought forward in the logistic regression models and *t* tests, a standard significance threshold of $P<0.05$ was used for these analyses in both the discovery and replication cohorts.

We evaluated the improvement in model performance introduced by the inclusion of oleoylcarnitine to a model adjusted for all significant covariates in the combined study sample using 3 previously described approaches.[26] We used *c*-statistics to compare model discrimination, "category-free" net reclassification improvement (NRI) to assess the ability of the model to correctly reclassify risk groups, and integrated discrimination improvement (IDI) to examine the ability of the model to increase average sensitivity without reducing average specificity.

For the study of individuals undergoing invasive catheterization, we compared arterial and renal venous levels of the 17 different acylcarnitines measured by our platform, using 2-tailed, paired *t* tests. Likewise, we compared pre- and post-hemodialysis acylcarnitine levels using 2-tailed, paired *t* tests. To account for multiple testing using the 17 different acylcarnitines, we used a Bonferroni corrected significance threshold of $P<0.0029$ (0.05/17). All analyses were performed using SAS software version 9.1.3 (SAS Institute).

Results

Baseline Characteristics of the Discovery Study Sample

The baseline characteristics of the discovery study sample are summarized in Table 1. There were no statistically significant differences in comorbidities, cause of ESRD, type of initial vascular access, or body mass index. Mean systolic blood pressure ($P=0.04$), serum albumin ($P=0.01$), percent transferrin saturation ($P=0.03$), and phosphorous ($P=0.01$) were all lower in the cases compared to controls. Other laboratory values such as parathyroid hormone, ferritin, and hemoglobin were similar between groups.

Select Metabolites Are Associated With Cardiovascular Death in Incident Dialysis Patients

We performed metabolite profiling on baseline plasma obtained within 14 days of study entry. Concordant measurement of serum creatinine by standard clinical tests demonstrated excellent correlation with peak areas reported on the mass spectrometer ($r=0.93$). For the 165 polar metabolites measured by our platform, Figure 1A shows the mean ratio of plasma levels of each analyte in cases versus controls from the discovery sample, plotted against their corresponding *P* values (full results are shown in Table S3). Four acylcarnitines achieved the significance threshold adjusted for multiple testing ($P<0.0003$), with each metabolite level higher in cases than controls: oleoylcarnitine ($P=1.9\times10^{-6}$), linoleylcarnitine ($P=9.7\times10^{-6}$), palmitoylcarnitine ($P=5.0\times10^{-5}$), and stearoylcarnitine ($P=1.8\times10^{-4}$). Box plots for these 4 metabolites in cases and controls are shown in Figure 1B.

Because the 4 significant metabolites in our discovery analysis were all long-chain acylcarnitines, we tested the magnitude of their intercorrelation and found that all were strongly correlated to each other (Table S2). The most significant metabolite, oleoylcarnitine, had a correlation coefficient of 0.91 to 0.92 with the other 3 metabolites ($P<0.001$ for all correlations). We thus evaluated oleoylcarnitine alone in subsequent analyses.

Table 1. Baseline Characteristics of the Discovery Study Sample

	Cases (n=100)	Controls (n=100)
Age, y	69.6±13.7	69.5±13.6
Male	53% (53)	53% (53)
Race		
White	69% (69)	69% (69)
Black	24% (24)	24% (24)
Other	7% (7)	7% (7)
Coexisting conditions		
Coronary artery disease	20% (20)	16% (16)
Cancer	3% (3)	4% (4)
Lipid disorders	9% (9)	14% (14)
Congestive heart failure	18% (18)	17% (17)
Liver disease	2% (2)	1% (1)
Cause of end-stage renal disease		
Diabetes*	49% (49)	48% (48)
Hypertensive renal disease	35% (35)	40% (40)
Glomerulonephropathy	4% (4)	3% (3)
Vascular access		
Fistula	19.3% (18)	29.9% (29)
Graft	11.8% (11)	14.4% (14)
Catheter	68.8% (64)	55.7% (54)
Body mass index, kg/m^2	25.8±8.2	27.4±6.9
Systolic blood pressure†, mm Hg	140.3±29.5	148.4±24.5
Diastolic blood pressure, mm Hg	71.4±23.5	73.4±12.8
Urea reduction ratio	68.7±9.2	68.6±11.2
Laboratory data		
Hemoglobin, g/dL	10.2 (9.4 to 11.0)	10.4 (9.8 to 11.4)
Albumin†, g/dL	3.4 (3.2 to 3.7)	3.7 (3.3 to 3.9)
Ferritin, ng/mL	178 (87 to 344)	149 (74 to 365)
Transferrin saturation†, %	17 (13 to 22)	19 (14 to 27)
Phosphorus†, mg/dL	3.9 (3.1 to 5.2)	4.7 (3.7 to 5.7)
Parathyroid hormone, pg/mL	216 (120 to 316)	224 (125 to 347)

Categorical data are percentages (counts). Counts may not equal total n due to missing data. Clinical measures are means±SD. Laboratory values are median (quartile 1 to quartile 3).
*Includes all patients with diabetes.
†P<0.05 for cases vs controls.

We tested the correlation between plasma oleoylcarnitine levels and covariates that were significantly different between cases and controls. Oleoylcarnitine was only weakly correlated with systolic blood pressure (r=−0.39), albumin (r=0.07), percent transferrin saturation (r=−0.15), and phosphorous (r=−0.12) (Figure S2A). Furthermore, oleoylcarnitine only showed a weak correlation to 2 cardiovascular biomarkers measured in ArMORR, NT-pro BNP (r=0.24) and cardiac troponin T (r=0.17) (Figure S2B). Thus, oleoylcarnitine's association with cardiovascular death is not simply a surrogate for imbalances in baseline covariates or available metrics of cardiovascular dysfunction.

Metabolite Associations With Cardiovascular Death Persist After Multivariable Adjustment

We next fit logistic regression models to assess the association between oleoylcarnitine level and cardiovascular death, adjusting for the covariates that differed between cases and controls (systolic blood pressure, albumin, transferrin saturation, and phosphorous). As shown in Table 2, oleoylcarnitine remained associated with cardiovascular mortality following multivariable adjustment as each standard deviation (SD) increment in metabolite was associated with a 2.3-fold increase in the odds of cardiovascular death at 1 year (P=0.001). To further assess the robustness of our findings, we created an additional logistic regression model that adjusted for established predictors of mortality in ESRD (diabetes mellitus, body mass index, coronary artery disease, congestive heart failure, type of access at dialysis initiation, diastolic blood pressure, average urea reduction ratio, hemoglobin, ferritin, parathyroid hormone level, cardiac troponin T, and NT-pro BNP).[21–25] The association of oleoylcarnitine with cardiovascular death at 1 year remained significant even after adjusting for these measures (Table 2).

Replication of Oleoylcarnitine as a Marker of Uremic Cardiovascular Risk

In order to replicate our findings, we performed a second, independent nested, case-control study in ArMORR with 100 additional subjects who died of a cardiovascular cause during the first year of dialysis (cases), plus 200 individuals who survived for at least 1 year (controls), again frequency matched for age, sex, and race. The baseline characteristics for the replication sample are shown in Table S4 and are notable for differences between cases and controls in initial type of vascular access (P=0.003), serum albumin level (P=0.04), and systolic and diastolic blood pressure (P=0.04 and P=0.001, respectively). Oleoylcarnitine remained significantly associated with cardiovascular death after adjusting for these factors (odds ratio per SD increment in oleoylcarnitine [95% confidence interval]=1.4 [1.1 to 1.9]; P=0.008), as well as additional variables (Table 3).

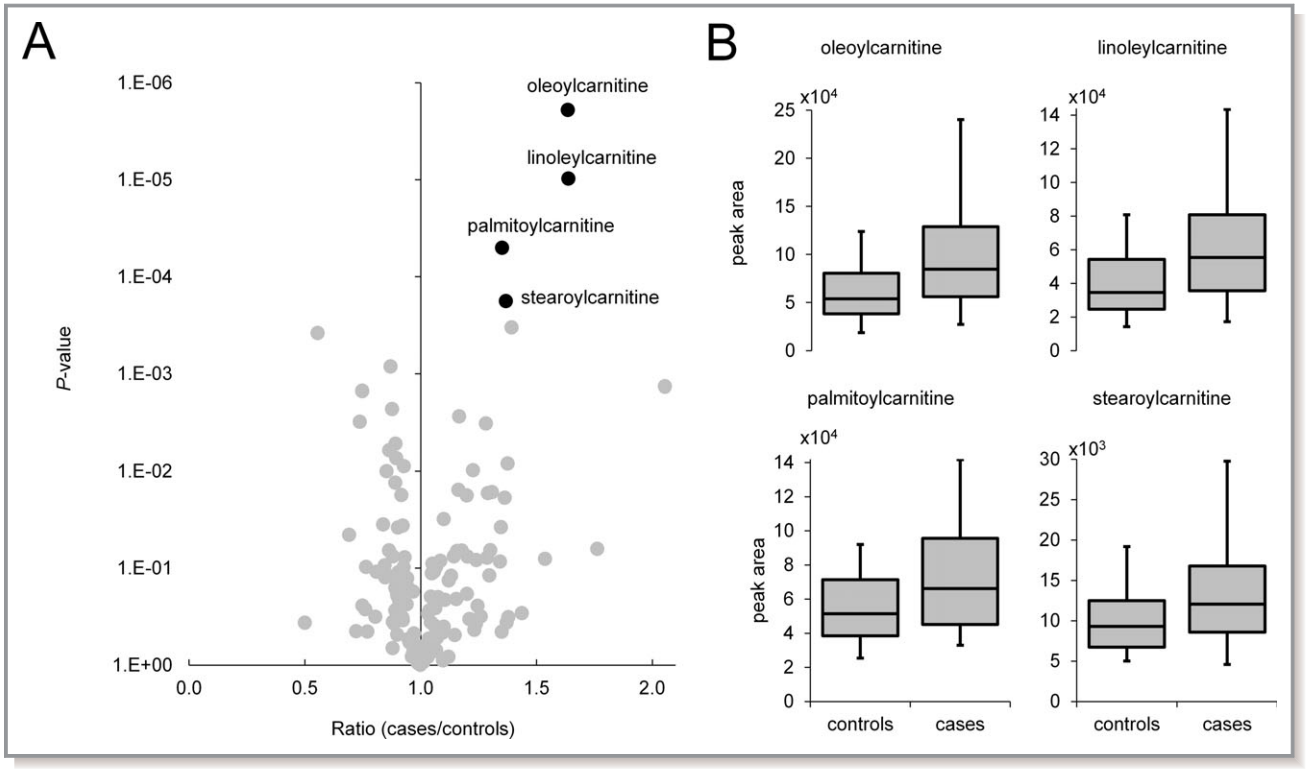

Figure 1. Metabolite profiling identifies markers of cardiovascular death in end-stage renal disease (ESRD). A, The mean ratio of each analyte for cases (n=100) vs controls (n=100) in baseline plasma, with *P* values plotted on the *y* axis. Metabolites that reached the Bonferroni significance threshold of $P<0.0003$ are labeled. B, Median peak areas for oleoylcarnitine, linoleylcarnitine, palmitoylcarnitine, and stearoylcarnitine for cases and controls. Box plots show 75th and 25th percentiles; whiskers show 95th and 5th percentiles.

In order to test whether oleoylcarnitine levels are elevated in subjects with ESRD relative to non-ESRD controls, we profiled plasma obtained from 30 individuals with normal renal function and 30 individuals with CKD but not on dialysis (Table S4). As shown in Figure 2, the mean concentration of plasma oleoylcarnitine was 0.46 mmol/L among individuals with CKD and 0.34 mmol/L among the normal subjects (1.3-fold increase, $P=0.007$). Among the ESRD subjects, the mean concentration of plasma oleoylcarnitine was 2.10 mmol/L among the ESRD controls (4.8-fold increase relative to normal controls, $P<0.001$), and 2.70 mmol/L among the ESRD cases (6.2-fold increase relative to normal controls, $P<0.001$).

Table 2. Relation of Oleoylcarnitine Level to Risk of Cardiovascular Death in the Discovery Study Sample

	Oleoylcarnitine	
Model	OR (95% CI)	*P* Value
Unadjusted model	2.6 (1.7 to 4.1)	<0.001
Model 2	2.3 (1.4 to 3.8)	0.001
Model 3	2.7 (1.4 to 5.0)	0.002

OR (95% CI) indicates odd ratio per SD increment in metabolite (95% confidence interval). Model 2: Adjusted for variables that differed between cases and controls at baseline (systolic blood pressure, albumin, transferrin saturation, and phosphorus). Model 3: Model 2+diabetes mellitus, coronary artery disease, congestive heart failure, type of vascular access at dialysis initiation (catheter vs no catheter), diastolic blood pressure, body mass index, average urea reduction ratio, hemoglobin, ferritin, parathyroid hormone level, cardiac troponin T, and NT-pro-B-type natriuretic peptide.

Plasma Oleoylcarnitine Levels Reclassify Incident Dialysis Patients At Risk For Death

Next, we quantified how well oleoylcarnitine levels could improve risk prediction across both the discovery and replication samples combined (total n=500). As shown in

Table 3. Relation of Oleoylcarnitine Level to Risk of Cardiovascular Death in the Replication Study Sample

	Oleoylcarnitine	
Model	OR (95% CI)	*P* Value
Unadjusted model	1.6 (1.2 to 2.0)	<0.001
Model 2	1.4 (1.1 to 1.9)	0.008
Model 3	1.5 (1.1 to 2.1)	0.04

OR (95% CI) indicates odds ratio per SD increment in oleoylcarnitine (95% confidence interval). Model 2: Adjusted for variables that differed between cases and controls at baseline (initial vascular access, albumin, systolic and diastolic blood pressure). Model 3: Model 2+diabetes mellitus, coronary artery disease, congestive heart failure, body mass index, average urea reduction ratio, phosphorous, hemoglobin, ferritin, parathyroid hormone level, cardiac troponin T, and NT-pro-B-type natriuretic peptide.

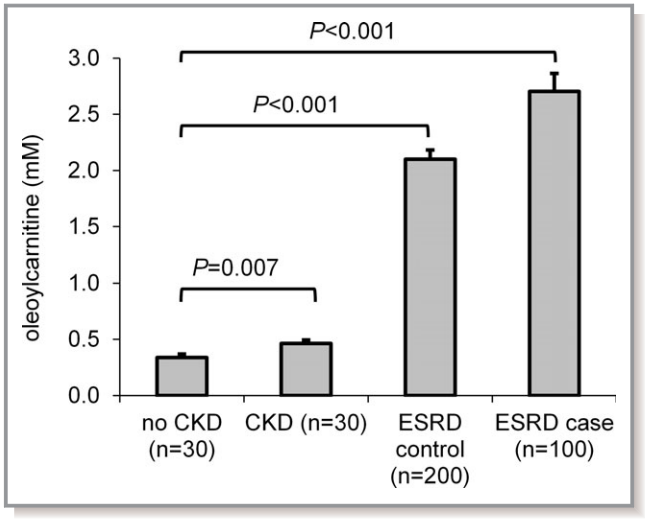

Figure 2. Plasma oleoylcarnitine levels are elevated in CKD and ESRD. Bars show mean concentrations (mmol/L) ±SEM. CKD indicates chronic kidney disease; ESRD, end-stage renal disease.

Table 4, addition of oleoylcarnitine to the other significant mortality predictors in the sample increased the *c*-statistic from 0.67 to 0.70 (P=0.04). Similarly, addition of oleoylcarnitine to the model predicting 1-year cardiovascular death led to a significant improvement in classification accuracy with a category-free net reclassification improvement (NRI) of 0.38 (0.20 to 0.56), P<0.001, and an integrated discrimination index (IDI) of 0.04 (0.02 to 0.06), P<0.001. Results were similar when using a fully adjusted model (Table 4).

Physiologic Studies Demonstrate Differential Renal and Dialytic Acylcarnitine Handling

Acylcarnitines are fatty acid esters of L-carnitine (free carnitine), and vary in length as a function of their fatty acyl moiety. Figure 3 depicts the ratio of all 18 acylcarnitines monitored by our platform in cases versus controls from the discovery sample, along with each corresponding P value. As noted, all 4 metabolites highlighted by the initial discovery analysis were long-chain acylcarnitines (defined as ≥14 carbons), and several other long-chain acylcarnitines had a trend for association with case status. By contrast, L-carnitine, short-chain acylcarnitines (2 to 4 carbons), and medium-chain acylcarnitines (5 to 12 carbons) had no association with case status.

To explore whether differences in residual renal function could impact our findings, we measured acylcarnitine levels in the aorta and renal vein of 9 individuals without ESRD undergoing invasive catheterization.[12] Figure 4 (dark gray bars) demonstrates sharp arterio-venous decreases in medium-chain acylcarnitines, often larger than the drop in creatinine levels, consistent with significant renal clearance of these metabolites. By contrast, free carnitine, short-chain acylcarnitine, and long-chain acylcarnitine levels were not significantly different across the renal circulation. Thus, differences in residual renal function between cases and controls should not underlie difference in plasma long-chain acylcarnitine levels.

To understand the effects of the hemodialysis procedure on plasma acylcarnitines, we measured acylcarnitine levels in plasma obtained immediately before and after a hemodialysis session from 20 individuals with ESRD (Table S5). As shown in Figure 4 (light gray bars), hemodialysis results in significant clearance of L-carnitine, short-chain acylcarnitines, and medium-chain acylcarnitines. By contrast, the relatively more hydrophobic long-chain acylcarnitines are not appreciably cleared during hemodialysis (a ≈5% to 10% increase in nondialyzed metabolite levels during dialysis is expected given the hemoconcentration attributable to ultrafiltration). These

Table 4. Added Predictive Ability for Mortality With Oleoylcarnitine

	Unadjusted Model	Model 2	Model 3
OR (95% CI)	2.0 (1.6 to 2.5)	1.8 (1.4 to 2.3)	1.8 (1.3 to 2.4)
c-statistics			
Base model	0.66	0.67	0.73
Base model+oleoylcarnitine	N/A	0.70	0.76
P value		0.04	0.07
NRI	N/A	0.38 (0.20 to 0.56)	0.41 (0.19 to 0.63)
P value		<0.001	<0.001
IDI	N/A	0.04 (0.02 to 0.06)	0.04 (0.02 to 0.07)
P value		<0.001	<0.001

IDI indicates integrated discrimination improvement; NRI, Net Reclassification Index; OR (95% CI), odds ratio per SD increment in oleoylcarnitine (95% confidence interval). Models include derivation and replication samples combined (n=500). Model 2: adjusted for variables that differed between cases and controls at baseline in the combined sample (initial vascular access, albumin, percent transferrin saturation, systolic and diastolic blood pressure). Model 3: Model 2+diabetes mellitus, coronary artery disease, congestive heart failure, body mass index, average urea reduction ratio, phosphorous, hemoglobin, ferritin, parathyroid hormone level, cardiac troponin T, and NT-pro-B-type natriuretic peptide.

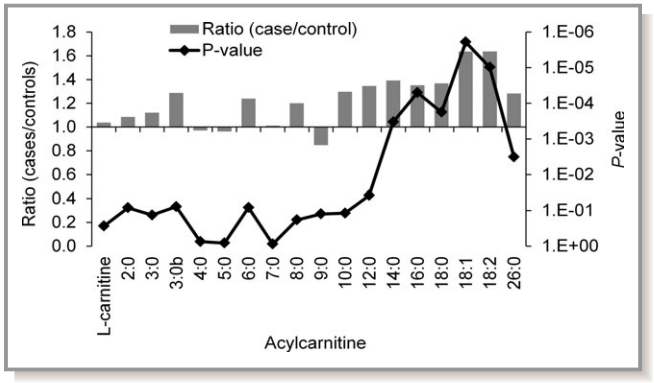

Figure 3. Long-chain acylcarnitines, but not short- or medium-chain acylcarnitines, are associated with cardiovascular mortality. The case to control ratio in accelerated mortality on renal replacement (ArMORR) (left *y* axis), with corresponding *P* values (right *y* axis), for the acylcarnitines monitored by our platform arranged by ascending acyl chain length along the *x* axis is shown. For each acylcarnitine, the first number denotes the total number of carbons in the fatty acyl chain, and the second number (after the colon) denotes the total number of double bonds in the fatty acyl chain; eg, palmitoylcarnitine (16:0), stearoylcarnitine (18:0), oleoylcarnitine (18:1), linoleylcarnitine (18:2). Other common names corresponding to each acylcarnitine are provided in Table S3.

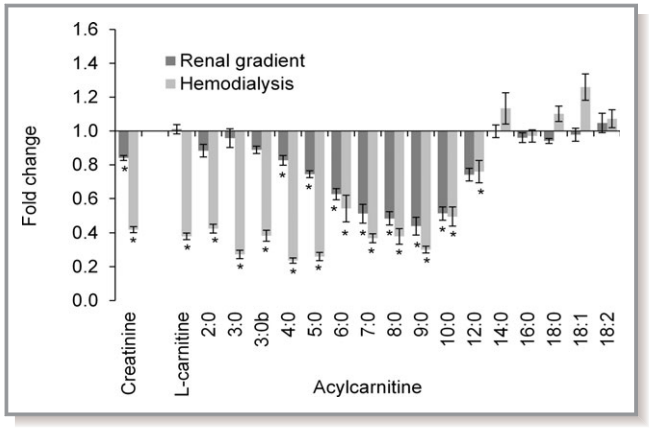

Figure 4. Long-chain acylcarnitines do not undergo renal or dialytic clearance. The fold change in acylcarnitine and creatinine plasma levels from the aorta to renal vein in 9 individuals (dark gray bars) and from pre- to post-hemodialysis in 20 individuals (light gray bars) are shown; data are presented as mean±SEM. *$P<0.0029$. For each acylcarnitine, the first number denotes the total number of carbons in the fatty acyl chain, and the second number (after the colon) denotes the total number of double bonds in the fatty acyl chain; eg, palmitoylcarnitine (16:0), stearoylcarnitine (18:0), oleoylcarnitine (18:1), linoleylcarnitine (18:2). Other common names corresponding to each acylcarnitine are provided in Table S3.

data show that the acute effects of a single dialysis session do not modulate long-chain acylcarnitine levels.

Several Established Uremic Toxins Have Nominal Associations With Cardiovascular Death

Although our study focuses on long-chain acylcarnitines that achieved the Bonferroni-adjusted significance threshold, we also highlight select metabolites that did not reach significance in the comparison between cases and controls in the discovery sample. More specifically, we note that our platform monitors 24 metabolites (Table 5) previously described as potential uremic toxins (http://www.uremic-toxins.org).[27] By *t* test, 5 of these metabolites had nominal associations with case status, with uridine (P=0.01), sorbitol (P=0.02), and creatine (P=0.02) higher in cases than controls and indoxyl sulfate (P=0.002) and niacinamide (P=0.04) lower in cases than controls. In logistic regression, only creatine (P=0.03) and trimethylamine-N-oxide (TMAO, P=0.04) had nominal associations with cardiovascular mortality at 1 year. Because of recent reports implicating TMAO in atherogenesis,[28,29] we measured TMAO in the replication sample as well, but found no difference in levels between cases and controls (P=0.22).

Discussion

ESRD is a state of small molecule disarray. In the current study, we apply LC-MS-based metabolite profiling to a nested case-control study of incident dialysis patients to highlight elevated levels of long-chain acylcarnitines as markers of uremic cardiovascular risk. More specifically, we find that plasma oleoylcarnitine is associated with 1-year cardiovascular mortality, even after adjusting for baseline clinical and laboratory covariates. Furthermore, we replicate the association between oleoylcarnitine and cardiovascular death in an independent study sample and show that oleoylcarnitine levels can improve cardiovascular risk classification accuracy over clinical variables alone. Finally, using physiologic profiling studies, we exclude renal and dialytic clearance of these metabolites as potential confounders in our analysis.

A major role of L-carnitine (free carnitine) is to transport cytosolic long-chain fatty acids—as acylcarnitines—across the inner mitochondrial membrane, thereby delivering these substrates for β-oxidation and subsequent ATP production.[30] Carnitines are particularly abundant in myocardium and skeletal muscle, which preferentially use fatty acids to generate energy. Primary disorders of carnitine homeostasis, for example, with mutations in the sodium ion-dependent L-carnitine transporter, result in whole-body carnitine depletion and can manifest as progressive cardiomyopathy, muscle weakness, and death if untreated (OMIM #212140).[31] Chronic hemodialysis has long been recognized as a secondary state of L-carnitine deficiency (with an increase in the ratio of acylcarnitines to L-carnitine), attributable to dialytic clearance of L-carnitine over time, the loss of renal L-carnitine biosynthesis, and decreased renal excretion of short-chain

Table 5. Uremic Toxins and Risk of Cardiovascular Death

Metabolite	Ratio (Cases/Controls)	*P* Value*	OR (95% CI)	*P* Value†
Indoxyl sulfate	0.75	0.002	0.7 (0.5 to 1.0)	0.09
Uridine	1.23	0.01	1.5 (1.0 to 2.2)	0.08
Sorbitol	1.31	0.02	1.3 (1.0 to 1.9)	0.07
Creatine	1.20	0.02	1.5 (1.0 to 2.0)	0.03
Niacinamide	0.90	0.04	0.7 (0.5 to 1.0)	0.07
Orotate	1.30	0.07	1.3 (0.9 to 1.8)	0.19
Uracil	1.18	0.07	1.2 (0.8 to 1.6)	0.41
Cytidine	1.16	0.07	1.1 (0.8 to 1.6)	0.39
Kynurenic acid	0.84	0.09	0.9 (0.7 to 1.3)	0.66
Symmetric dimethylarginine	1.06	0.11	1.2 (0.9 to 1.7)	0.19
Dimethylglycine	0.93	0.11	0.7 (0.5 to 1.0)	0.08
Hypoxanthine	0.88	0.12	0.8 (0.6 to 1.1)	0.16
Asymmetric dimethylarginine	1.04	0.20	1.2 (0.8 to 1.6)	0.36
Thymine	1.06	0.26	1.1 (0.8 to 1.5)	0.60
Malondialdehyde	0.76	0.27	0.9 (0.7 to 1.2)	0.50
Anthranilic acid	1.35	0.45	1.1 (0.7 to 1.9)	0.66
Urate	0.97	0.47	0.9 (0.7 to 1.3)	0.62
Hippurate	0.90	0.49	1.0 (0.8 to 1.4)	0.80
Xanthine	1.06	0.51	1.1 (0.8 to 1.6)	0.53
Kynurenine	1.04	0.53	1.1 (0.8 to 1.5)	0.64
Quinolinate	1.05	0.63	1.1 (0.8 to 1.5)	0.65
Oxalate	1.02	0.65	1.3 (0.9 to 1.8)	0.13
Xanthosine	0.97	0.81	1.0 (0.7 to 1.4)	0.98
TMAO (discovery)	1.04	0.36	1.4 (1.0 to 2.0)	0.04
TMAO (replication)	0.94	0.22	0.9 (0.7 to 1.1)	0.36‡

OR (95% CI) indicates odds ratio for cardiovascular mortality per SD increment (95% confidence interval); TMAO, trimethylamine-N-oxide.
**P* value from *t* test.
†*P* value from logistic regression model adjusted for variables differing at baseline in the derivation sample (systolic blood pressure, albumin, transferrin saturation, and phosphorus).
‡*P* value from logistic regression model adjusted for variables differing in the replication sample (initial vascular access, albumin, systolic and diastolic blood pressure).

acylcarnitines.[32–34] L-carnitine is FDA-approved for the prevention and treatment of carnitine deficiency in ESRD, although the large number of studies published to date have not identified a clear benefit to this metabolic intervention.[35]

Despite substantial interest in the potential benefits of L-carnitine supplementation, to our knowledge no epidemiologic studies have reported the association between carnitine levels and cardiovascular outcomes. Notably, we found no association between plasma L-carnitine levels and 1-year cardiovascular mortality in ArMORR (Figure 3). As noted, L-carnitine deficiency occurs over time on dialysis, and indeed, we found no differences in mean L-carnitine levels in the incident ArMORR subjects compared to non-ESRD controls (data not shown). By contrast, long-chain acylcarnitines such as oleoylcarnitine were markedly elevated in ArMORR subjects relative to non-ESRD controls, and higher levels were associated with an increased risk of cardiovascular death in the ArMORR case-control analyses. Interestingly, in a post hoc analysis of a clinical trial of carnitine supplementation, Murphy et al have reported that higher baseline levels of long-chain acylcarnitines are associated with poorer physical function.[36]

The mechanism underlying the increase in long-chain acylcarnitine levels in cases relative to controls is unclear, yet it is notable that different inborn errors in mitochondrial β-oxidation can result in distinct signatures of plasma acylcarnitine elevation. For example, long-chain acyl-CoA dehydrogenase deficiency (OMIM #609576) results in long-chain acylcarnitine elevations, whereas medium-chain acyl-CoA dehydrogenase deficiency (OMIM #201450) and short-chain acyl-CoA dehydrogenase deficiency (OMIM

#201470) result in medium-chain and short-chain acylcarnitine elevations, respectively. Thus, our data may signal a specific defect in mitochondrial handling and catabolism of long-chain acylcarnitines. Alternatively, because our data show that the hemodialysis procedure acutely decreases plasma levels of short-chain and medium-chain acylcarnitines, we cannot exclude a global defect in acylcarnitine metabolism in cases relative to controls. In this regard, we note that acquired mitochondrial DNA damage, attributed to oxidative stress, has also been associated with mortality in hemodialysis patients.[37]

Although we hypothesize that long-chain acylcarnitine elevations may signal underlying mitochondrial dysfunction, we recognize that select risk markers could also play a direct, functional role in cardiovascular disease pathogenesis. For example, cardiac myocytes exposed to hypoxia exhibit rapid accumulations in long-chain acylcarnitines, and these amphiphilic molecules have been shown to inhibit excitatory Na currents in vitro.[38,39] Furthermore, long-chain acylcarnitines, but not shorter-chain acylcarnitines, are able to increase calcium efflux in a concentration-dependent manner in isolated cardiac sarcoplasmic reticulum vesicles.[40] Most recently, long-chain acylcarnitines, but not medium-chain or short-chain acylcarnitines, were shown to speed the deactivation of hERG channels in HEK293 cells.[41] These effects have been postulated to contribute to the genesis of malignant ventricular arrhythmias that can occur with some inherited disorders of β-oxidation, as well as during myocardial ischemia.[42] If long-chain acylcarnitines do participate directly in the cardiovascular complications of uremia, it will be of interest to determine whether alternative dialytic approaches (eg, high-efficiency convective dialysis), dietary modifications, or medications are able to effectively lower plasma levels.

Decades of research have identified many potential uremic toxins, including dozens of small molecules.[43] However, the majority of these molecules have not been studied in relation to clinical end points. Across 24 previously described uremic toxins monitored by our platform, we did not find any associations with 1-year cardiovascular outcomes in ArMORR that reached our conservative threshold for statistical significance. Prior studies have associated asymmetric dimethylarginine and indoxyl sulfate with mortality in individuals with CKD, and TMAO levels with cardiovascular outcomes in a more general population, but we did not observe such relationships.[28,29,44,45] This may reflect the sample size of our study, differences in study population, or the duration of follow-up.

Several limitations warrant mention. First, we examined baseline metabolite levels in incident dialysis subjects drawn within the first 14 days of starting dialysis. The precise timing of blood draw relative to the number of dialysis sessions received were not available, raising the possibility that dialytic clearance of select molecules could limit the sensitivity of case-control comparisons. Importantly, prior work has shown that long-chain acylcarnitines are not cleared by hemodialysis, a finding corroborated by our data herein.[34,46] Second, although prior studies suggest that the accuracy of ICD-9 codes for cardiac diagnoses are generally high,[47–49] we acknowledge that they are not completely sensitive or specific. Third, because we examined the ability of plasma oleoylcarnitine levels to improve reclassification across both the discovery and replication cohorts combined, these findings will need to be validated in future studies. Such studies could further improve on incremental risk assessment by examining unmatched cohorts, thereby allowing the impact of age, race, and sex to be simultaneously assessed. Fourth, because they were not measured in ArMORR, we did not account for clinical lipid levels in the adjusted models. However, prior studies have shown that traditional lipid parameters are not markers of excess cardiovascular risk in the context of uremia.[50,51] Finally, we note that ArMORR is a study of incident dialysis patients, and that future studies will be required to test whether long-chain acylcarnitine levels are also cardiovascular risk markers among prevalent subjects who survive at least 1 year on dialysis as well as in nondialysis populations.

In summary, biochemical "snapshots" of plasma obtained from a well-characterized epidemiologic cohort identifies oleoylcarnitine as a predictor of 1-year cardiovascular mortality in ESRD. Whereas prior studies have focused on the potential benefit of free carnitine supplementation in prevalent ESRD, our data highlight clinically meaningful alterations in acylcarnitine homeostasis at the time of dialysis initiation. Our findings may also have implications for the increase in cardiovascular risk associated with earlier stages of chronic kidney disease, where we demonstrate modest increases in plasma oleoylcarnitine levels relative to normal controls. Further work is required to more precisely assess the predictive value of long-chain acylcarnitines in additional cohorts (across a spectrum of kidney disease severity) and to determine whether our findings represent a modifiable effector of uremic cardiovascular risk.

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Disclosures

None.

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