RESEARCH BRIEF

Plasma Fatty Acids in Chronic Kidney Disease: Nervonic Acid Predicts Mortality

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Although the value of red blood cell fatty acids (FAs) in estimating risk for acute coronary syndrome in the general population is evident, the value of FAs in chronic kidney disease (CKD) is unknown. Here, we provide a pilot analysis in a spectrum of CKD patients. Plasma samples were obtained from 20 incident dialysis patients (CKD stage 5), matched with samples from 10 CKD stage 3-4 patients, and 10 control subjects. Whole plasma FAs were measured using gas chromatography. Whereas neither linoleic acid nor arachidonate acid were altered in CKD, metabolic intermediates of arachidonate synthesis (γ-linolenate and dihomo γ-linolenate) were reduced in CKD. Demming (orthogonal) correlation of FA abundance with estimated GFR identified several saturated and unsaturated FAs in addition to the intermediates; again, neither linoleate nor arachidonate were related. Follow-up data within the CKD stage 5 patients revealed that nervonic acid, a component of membrane sphingolipids and phosphatidylethanolamines, was a significant predictor of all-cause mortality; the age-adjusted relative risk for a 0.15% change is 2.1 (1.4, 3.7; 95% CI; $P = .0008$). These findings support the exploration of FAs in larger studies for validation of the role FAs in cardiovascular risk and mortality in CKD.

RENAL DISEASE IS associated with a marked increased risk for cardiovascular and infectious complications and premature morbidity.[1] Further, the burden of renal-related morbidity and hospitalizations represents a serious challenge to health care.[2,3] Although a portion of the risk for cardiovascular disease (CVD) includes traditional risk factors (such as age, gender, hypertension, dyslipidemia, diabetes, and smoking), there are also risk factors that are either uniquely associated with or unusually enhanced in chronic kidney disease (CKD; such as albuminuria, lipoprotein remnants, anemia, calcium/phosphorus disturbances, oxidative stress, and inflammation).[4] Although many promising CVD biomarkers have been proposed, only few add predictive value to the traditional ones.[5-7] In the context of uremia, the search for biomarkers is complicated by the fact that for many risk factors, risk is actually reversed compared with the general population; this is true not only in end-stage renal disease (ESRD),[8-10] but also in earlier stages.[11] Improved prediction of disease progression or mortality (CVD-related or not) would potentially lead to earlier interventions and improved outcome.[3]

A limiting factor to the discovery of new biomarkers is the lack of variables uncorrelated with, or orthogonal to, established biomarkers; this is because correlated variables reflect the same underlying disturbances they are unlikely to add

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Financial Disclosure: G.C.S. is supported by a grant from GlaxoSmithKline which studies the impact of n-3 FAs on lipid metabolism. G.C.S. additionally receives speakership honoraria from GlaxoSmithKline.

Funding Support: See Acknowledgment(s) on page 282.

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1051-2276/$36.00

doi:10.1053/j.jrn.2011.05.005

substantially to the predictive value. Fatty acid (FA) profiles are matrices where orthogonal predictors could be found, which offer some unique advantages for risk assessment. More than 25 FAs exist in biological membranes in sufficient abundance to measure reliably. Their relative abundances are independent of traditional markers,[12] but would be defined by nutritional status, genetic makeup, and other conditions which could include the presence or severity of renal disease. Thus, the FA profile is a reservoir of multiple orthogonal biomarkers. We have recently demonstrated the value of the red blood cell FA profile in CVD, where multivariate analysis of tissue FA profiles outperformed the standard risk-prediction algorithms, such as the Framingham scoring system, in assessing risk for acute coronary syndrome subjects.[12] This result confirms not only that FAs represent an orthogonal dataset, but also that they contain at least as much, if not more, information about risk as traditional markers do. More recently, we demonstrated in rats that red blood cell FAs are not changed by a myocardial infarction,[13] which addressed the concern that the discrimination was a product of the myocardial infarction itself and substantiated the prospective value of FA-profiles. Because an ideal risk marker also has interventional value, the therapeutic evidence supports this reasoning; at least 3 prospective studies have demonstrated interventional efficacy of omega-3 FAs (n-3 FAs) in secondary prevention of coronary events[14,15] and heart failure,[16] the latter in a context where statins had no preventive effect.[17] There is no prima facie reason to confine the predictive value of FAs to CVD and it is plausible that FAs could improve renal risk estimators; however, data in CKD are limited—with the notable exception of n-3 FAs. n-3 FAs delay the progression to ESRD[18] and in a placebo-controlled trial they are effective at preventing secondary cardiovascular events, but not composite cardiovascular events and mortality.[19] Patients on hemodialysis have lower plasma n-3 FA content,[20] and a small feasibility study indicates erythrocyte polyunsaturated FA abundance predicts mortality in hemodialysis.[21] To better understand the relationship between the FA profile and CKD, we conducted a pilot study of patients naïve to FA interventions and who reflected the progression of renal disease from mild CKD to ESRD. Among patients with stage 5 CKD, incident samples were collected close to the start of dialysis treatment to determine the predictive value of FAs for mortality. We used basic exploratory analysis to identify FA-targets of interest for future biomarker investigation.

Methods

Patient Selection and Experimental Design

Two groups of patients were selected from ongoing prospective cohort studies at the Karolinska Institute.[22,23] Patients were categorized into 2 groups according to Kidney/Disease Outcome Quality Initiative (K/DOQI) guidelines,[24] patients with stage 3-4 ($n = 10$) and stage 5 CKD ($n = 20$) close to the start of dialysis treatment. The subjects with stage 5 CKD were followed up for 2.7 ± 1.7 years, during which period 12 patients died: 6 from CVD, 1 from cessation of dialysis, and 5 from other or unknown causes. In addition, 10 control subjects with no apparent renal disease were investigated. According to the inclusion criteria, CKD patients and control subjects were not age-matched. The Ethics Committee of Karolinska Institutet Hospital approved the study protocols. Signed informed consent was obtained from all patients before inclusion in the study. The estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) equation per Levey et al.[25]

FA Analysis

Plasma FA composition was analyzed from 100 μL according to the methodology described previously.[26] FA methyl esters were generated from plasma by acid transesterification and analyzed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Columbia, MD) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA), using hydrogen as carrier gas. FAs were identified by comparison with a standard mixture of FAs. Sufficient tricosanoic acid (23:0) was added to achieve a final concentration of 5 mg/mL to allow for quantitation of FA concentrations. Results are given as a percentage of total identified FAs after response factor correction. Samples were analyzed in random order along with standards and 2 quality controls.

Statistical Analysis

Group differences were assessed using analysis of variance. Because the eGFR calculated from

serum creatinine (sCr) and FA abundances each have error, Deming's method of orthogonal regression was deemed most appropriate. In general, least-squares regression is used; however, that method assumes that there is no error in measurements along the *x*-axis. In this case, eGFR has error and so the least-squares assumption is not valid. Deming's orthogonal regression minimizes absolute distance from the line, and so incorporates error in both *x*- and *y*-axes—as long as the measurement error is known and expressed as a variance ratio. The variance ratios were exogenously assigned from historical data (FAs) or historical controls from the Sanford Clinical Laboratories (for sCr) (Sioux Falls, SD, USA). Cox proportional hazards models were assessed using age as a covariate. *P*-values of $<.05$ were considered significant unless otherwise stated. Test assumptions were verified and analyses performed using JMP software version 8.0.2.2 (SAS Institute Inc.). Because of the small sample size, the model diagnostics were limited for proportional hazards models.

Results

Demographics

The group demographics are described in Table 1. One patient with stage 5 CKD (who died because of CVD) was excluded owing to poor recovery of FAs. As expected, substantial group differences were present in markers of renal function and nutritional status. Triglycerides (TG) were elevated in the stage 3-4 CKD group and the inflammation measured by human serum C-Reactive Protein (hsCRP) was different; however, post hoc analysis did not reveal the groups.

Group Differences Among FAs

Decreased abundance of polyunsaturated FAs, particularly intermediates of arachidonate (AA) synthesis, was detected among the patients with stage 5 CKD compared with the control subjects (Table 2). The percent abundances of FAs from stage 3-4 CKD were intermediate to the controls and stage 5 CKD, but were not different from either. In the case of eicosapentaenoic acid (EPA), the abundance was the same as controls and greater than in the stage 5 CKD group. The only other FA in which differences were detected was nervonic acid (24:1n9), but post hoc analysis did not reveal which groups were different. We also analyzed differences by plasma FA concentration; however, the variability was much greater because their concentration is a function of plasma lipoprotein concentration; only differences in EPA were found (data not shown).

Plasma FA Correlation With Renal Function

We tested for continuous relationships between eGFR and FA abundance using orthogonal regression analysis on FAs and glomerular filtration rate (GFR) estimated from sCr (Table 3). Among polyunsaturated FAs, nearly the same polyunsaturated FAs identified earlier in the text had a significant relationship between eGFR and FA abundance. In each case, lower eGFR corresponded with lower abundance of polyunsaturated FAs, the

Table 1. Demographics of Controls and Patients With Chronic Kidney Disease

	Controls	Stage 3-4 CKD	Stage 5 CKD	
Parameter	n = 10	n = 10	n = 19	*P*-Value
Age (years)*	61.4 ± 1.5^{A}	79.3 ± 1.5^{A}	60.8 ± 1.1^{B}	<.0001
Body mass index (kg/m^2)	27.0 ± 1.4	27.5 ± 1.4	26.0 ± 1.0	NS
S-albumin (g/L)*	38.4 ± 0.8^{A}	35.4 ± 0.8^{B}	34.2 ± 0.6^{B}	.0008
S-creatinine (μmol/L)†	74 (58, 82)^{A}	208 (177, 331)^{B}	696 (569, 918)^{C}	<.0001
Cholesterol (mmol/L)*	5.3 ± 0.5	5.7 ± 0.5	5.7 ± 0.4	NS
Triglyceride (mmol/L)‡	1.5 (1.1, 2.0)^{A}	2.6 (1.9, 3.7)^{A,B}	2.1 (1.7, 2.7)^{B}	.05
C-reactive protein (serum) (mg/L)‡	0.9 (2.0, 4.3)^{A}	1.0 (2.2, 4.7)^{A}	3.4 (6.0, 10.6)^{A}	.03

CKD, chronic kidney disease; ANOVA, analysis of variance.

*ANOVA, Tukey HSD post hoc test. Values as mean ± SD. Groups sharing a letter (A, B, or C) are not significantly different at $P_{adj} = .05$.

†By Kruskal–Wallis. Values as median (inter-quartile range). Wilcoxon nonparameteric post hoc method.

‡ANOVA on log-transformed data, Tukey honest significant differences post hoc test. Values as mean (95% CI). Groups sharing a letter are not significantly different at $P_{adj} = .05$.

Table 2. Plasma Percent Total FA (95% CI) by Stage 3-4 CKD and Stage 5 CKD Groups

Fatty Acid	Control	Stage 3-4 CKD	Stage 5 CKD
14:0*	0.96 (0.75, 1.2.0)	1.25 (0.98, 1.6.0)	0.92 (0.77, 1.1.0)
16:0*	26.7 (25.0, 28.0)	26.2 (25, 28)	26.4 (25.0, 28.0)
16:1n7*	2.4 (1.9, 3.2)	2.1 (1.6, 2.7)	1.6 (1.4, 2.0)
16:1n7t*	0.20 (0.17, 0.23)	0.24 (0.2, 0.28)	0.21 (0.19, 0.24)
18:0*	9.4 (8.4, 10.0)	8.7 (7.8, 9.7)	8.2 (7.6, 8.8)
18:1n9	23.0 (1.0, 21.0)	24.9 (1.0, 23)	25.4 (0.8, 24.0)
18:1t*,†	0.53 (0.43, 0.64)	0.64 (0.52, 0.77)	0.65 (0.56, 0.74)
18:2 trans*	0.24 (0.20, 0.28)	0.30 (0.25, 0.36)	0.29 (0.25, 0.33)
18:2n6*	20.1 (18.0, 23.0)	19.2 (17.0, 22.0)	21.7 (20.0, 24.0)
18:3n3*	**0.53 (0.06, 0.40)^{A}**	**0.76 (0.06, 0.63)^{A,B}**	**0.69 (0.05, 0.60)^{B}**
18:3n6*	**0.34 (0.26, 0.43)^{A}**	**0.26 (0.20, 0.33)^{A,B}**	**0.22 (0.18, 0.26)^{B}**
20:1n9*	0.31 (0.25, 0.39)	0.32 (0.25, 0.40)	0.31 (0.26, 0.37)
20:2n6*	**0.26 (0.23, 0.30)^{A}**	**0.23 (0.20, 0.27)^{A,B}**	**0.21 (0.19, 0.23)^{B}**
20:3n6	**1.64 (0.01, 1.40)^{A}**	**1.50 (0.01, 1.30)^{A,B}**	**1.24 (0.07, 1.10)^{B}**
20:4n6	5.91 (0.46, 5.00)	5.46 (0.46, 4.50)	5.67 (0.33, 5.00)
20:5n3*	**1.42 (1.10, 1.90)^{A}**	**1.24 (0.95, 1.60)^{A}**	**0.66 (0.54, 0.80)^{B}**
22:5n3	**0.77 (0.045, 0.68)^{A}**	**0.67 (0.045, 0.58)^{A,B}**	**0.63 (0.032, 0.56)^{B}**
22:4n6*	0.19 (0.15, 0.23)	0.16 (0.13, 0.2.0)	0.19 (0.16, 0.22)
22:5n6	0.10 (0.01, 0.08)	0.08 (0.01, 0.07)	0.08 (0.01, 0.07)
22:6n3*	2.99 (2.50, 3.60)	2.85 (2.30, 3.50)	2.30 (2.00, 2.60)
24:0	0.23 (0.02, 0.18)	0.21 (0.02, 0.17)	0.18 (0.02, 0.15)
24:1n9*	**0.46 (0.36, 0.59)**	**0.47 (0.37, 0.60)**	**0.64 (0.53, 0.76)**

FA, fatty acid.
Tested using ANOVA; FAs with overall *P*-value <.05 are in bold.
Post hoc differences determined using Tukey's honest significant differences.
Groups sharing a letter (A, B) are not significantly different. Given no adjustment for type II error, 2 false positives expected.
*Log-transformed to achieve normal distribution or equal variance.
†Combination of 18:1n9t (elaidic acid) and 18:1n11t (vaccenic acid).

strongest relationships being with eicosadienoic acid and eicosatrienoic acid (dihomo γ-linolenic acid [dgLA]), which are a by-product and an intermediate to AA synthesis, respectively. Monounsaturated FAs were related to renal function, but in opposite ways: palmitate decreased with reduced renal function, oleic acid (OA) increased with reduced renal function, and suggests that OA might replace polyunsaturated FAs as the renal function declines. Finally, decreased abundance of a single saturated FA, stearic acid, was associated with declining function.

Risk Prediction in Patients With Stage 5 CKD

We also tested whether any FAs predicted mortality risk in the incident dialysis patients after adjusting for age (per year of increase) and using $P \leq .01$ for statistical significance as a means to control for multiple testing error. Notably, nervonic acid predicted mortality at $P = .003$ by proportional hazards model (Table 4). It ranged from 1.6% to 0.38% of total FAs. For every 0.15% increase in nervonic acid abundance, patients were at a 2.1-fold (95% CI: 1.4, 3.7) greater risk for age-adjusted mortality.

Discussion

The major aim of this study was to determine the levels of FAs in different stages of CKD. We reasoned that the existence of group differences between control subjects, patients with stage 3-4, and stage 5 CKD, and/or correlation between FAs and GFR would provide this level of evidence. We have also shown group differences in multiple FAs, many of which correlate with eGFR. Additionally, we sought to establish whether FAs predict risk for mortality in a small group of incident dialysis patients and found that increasing plasma abundance of nervonic acid predicted mortality. Thus, our pilot study suggests that disturbances in FA metabolism provide valuable information in risk prediction of CKD patients.

Polyunsaturated FA metabolism is thought to be centered on the production of AA (20:4n6),

Table 3. Orthogonal* Regression of FAs (by % Total) With eGFR (n = 39)

Fatty Acid	Slope (95% CI)† (%FA/ln [eGFR mL/min])	Correlation	Variance Ratio*
14:0‡	–	–	–
16:0	–	–	–
16:1n7‡	**0.12 (0.005, 0.23)**	**0.33**	**35.6**
16:1n7t‡	–	–	–
18:0‡	**0.06 (0.02, 0.10)**	**0.43**	**6.02**
18:1n9‡,§	**−0.04 (−0.08, −0.01)**	**−0.38**	**11.3**
18:1t‡	–	–	–
18:2n6	–	–	–
18:3n3	–	–	–
18:3n6	**0.05 (0.02, 0.07)**	**0.50**	**5.03**
20:1n9‡	–	–	–
20:2n6‡	**0.10 (0.04, 0.15)**	**0.52**	**31.3**
20:3n6	**0.16 (0.08, 0.24)**	**0.56**	**14.8**
20:4n6	–	–	–
20:5n3‡	**0.26 (0.14, 0.38)**	**0.58**	**10.1**
22:4n6‡	–	–	–
22:5n3‡	**0.07 (0.005, 0.13)**	**0.34**	**10.5**
22:5n6	–	–	–
22:6n3‡	**0.08 (0.002, 0.17)**	**0.32**	**8.71**
24:0	–	–	–
24:1n9‡	–	–	–

eGFR, estimated glomerular filtration rate.
FAs with significant correlation (*P*-value <.05) are in bold.
*Variance ratios were assigned using historical controls.
†With 21 tests, 2 false positives are expected at $\alpha = 0.05$.
‡Log-transformed to achieve normal distribution or variance.
§Combination of 18:1n9t (elaidic acid) and 18:1n11t (vaccenic acid).

which occurs by elongation and desaturation from linoleic acid (LA; 18:2n6) through the intermediates gamma linoleic acid (18:3n6) or eicosadienoic acid (20:2n6) and dgLA (20:3n6). Some specific enzymes mediating the process are the elongase Elovl5 and the desaturases Δ6 Fads2 and Δ8 Fads2.[27] The AA then serves as a precursor for eicosanoid production through cyclooxygenase, lipoxygenase, or cytochrome p450s. Although we did not observe any difference in LA or AA abundance, the levels of metabolic intermediates were uniformly reduced in stage 5 CKD. Past reports have proposed that increased dgLA were indicative of increased flux through this pathway,[28] thereby suggesting that decreased flux is the cause of lower levels in advancing disease. However, the AA:dgLA ratio is a marker of desaturase activity and it was higher with advanced diseases, suggesting that increases in LA-AA flux characterizes advancing CKD and the lower levels of intermediates could reflect "thinning" of the pool. This is in contrast with subjects with metabolic syndrome and obesity, who have higher desaturase activity using this same measure.[29] In contrast to the n6 FA family, n3 FAs were uniformly lower with reduced renal function. Regardless of the cause, these changes likely represent a point of metabolic stress in CKD, even before stage 5 CKD.

Table 4. Hazard Ratios and 95% CI for All-Cause Mortality of Nervonic Acid, 24:1n9, After Adjustment for Age

Parameter	HR (95% CI)	*P*-Value
Nervonic acid (24:1n9, per 0.15% increase)*	2.1 (1.4, 3.7)	.0008
Age (per 1 year increase)	1.2 (1.0, 1.5)	.06

HR, hazards ratio; CI, confidence intervals.
*0.15% increase corresponds to the 0.15 SD for nervonic acid in a normal population.

It is also noteworthy that 16- and 18-carbon FAs were also related to the degree of renal function, with palmitoleic acid (16:1n7) being inversely related to eGFR, and thus directly related to sCr, whereas the opposite was true of OA (18:1n9). It is unknown whether this is a function of changing metabolism of the FAs, or changing nutritional status. A further explanation of the changes in OA is simply that as the polyunsaturated FAs are depleted, they are replaced with OA.

The strongest predictor of death in stage 5 CKD patients was nervonic acid (24:1n9). Dolegowska et al.[30] report that hemodialysis results in increased levels of nervonic acid. However, our samples were obtained close to the start of dialysis suggesting that patients with high initial nervonic acid are less able to sustain the hemodialysis-induced increases. Nervonic acid is found in amphipathic membrane lipids, most abundantly in sphingolipids. Alterations in sphingomyelin metabolism are well known to mediate inflammation and apoptosis in renal disease[31]; however, in the study by Dolegowska et al.,[30] the increased nervonic acid was found in the phosphatidylethanolamine fraction suggesting that fraction as the best target for future investigations. Because our methodology was selective for glycerophospholipids, including phosphatidylethanolamines, our data likely reflect the results of the study by Dolegowska et al. Other investigators have noted rearrangement of multiple FAs (especially increases in erythrocyte polyunsaturated FAs after a hemodialysis session compared with the start), thereby suggesting that the hemodialysis procedure itself induces rearrangements of FAs.[32]

Some caveats and limitations of our study need consideration. First, given the small number of patients included in this pilot study, our results should only be regarded as preliminary. Clearly, additional studies in larger CKD cohorts are needed to study the detailed FA pattern in relation to the uremic phenotype and premature death risk. Because our approach in this study was to identify, instead of validate, the potential plasma FAs related to CKD progression or stage 5 CKD mortality, we did not adjust our findings to account for false positives because doing so would result in a high rate of false negatives. In this study, we tolerate false positives and report the expected prevalence, about 20% of our results. Finally, as this was a post hoc analysis of existing cohorts of CKD patients, FAs were measured in stored plasma samples—in general, the best biomarkers for FA content are thought to be derived from erythrocyte membranes (not plasma), but because plasma is preserved more commonly than formed elements, it is used here. Nevertheless, there is good correlation between plasma and erythrocyte FA content, but although counterintuitive, this is not the case with plasma phospholipids (unpublished observations).

In conclusion, we have demonstrated the potential for FAs as useful risk estimators in renal disease by showing that specific FAs correlate with eGFR and by showing data suggesting that nervonic acid predicts mortality in stage 5 CKD. Because many FAs are uncorrelated with conventional risk estimators, they are likely to add to existing risk estimates.

Acknowledgments

The authors thank Brenda Collison-Schmidt for expert technical help and acknowledge the support from the Diabetes Theme Center at Karolinska Institutet and an unrestricted grant from Abbott laboratories, Nutritional Division.

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