Kidney Medicine

Serum Metabolomic Markers of Protein-Rich Foods and Incident CKD: Results From the Atherosclerosis Risk in Communities Study

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Rationale & Objective: While urine excretion of nitrogen estimates the total protein intake, biomarkers of specific dietary protein sources have been sparsely studied. Using untargeted metabolomics, this study aimed to identify serum metabolomic markers of 6 protein-rich foods and to examine whether dietary protein–related metabolites are associated with incident chronic kidney disease (CKD).

Study Design: Prospective cohort study.

Setting & Participants: A total of 3,726 participants from the Atherosclerosis Risk in Communities study without CKD at baseline.

Exposures: Dietary intake of 6 protein-rich foods (fish, nuts, legumes, red and processed meat, eggs, and poultry), serum metabolites.

Outcomes: Incident CKD (estimated glomerular filtration rate < 60 mL/min/1.73 m^2 with ≥25% estimated glomerular filtration rate decline relative to visit 1, hospitalization or death related to CKD, or end-stage kidney disease).

Analytical Approach: Multivariable linear regression models estimated cross-sectional associations between protein-rich foods and serum metabolites. C statistics assessed the ability of the metabolites to improve the discrimination of highest versus lower 3 quartiles of intake of protein-rich foods beyond covariates (demographics, clinical factors, health behaviors, and the intake of nonprotein food groups). Cox regression models identified prospective associations between protein-related metabolites and incident CKD.

Results: Thirty significant associations were identified between protein-rich foods and serum metabolites (fish, n = 8; nuts, n = 5; legumes, n = 0; red and processed meat, n = 5; eggs, n = 3; and poultry, n = 9). Metabolites collectively and significantly improved the discrimination of high intake of protein-rich foods compared with covariates alone (difference in C statistics = 0.033, 0.051, 0.003, 0.024, and 0.025 for fish, nuts, red and processed meat, eggs, and poultry-related metabolites, respectively; $P < 1.00 \times 10^{-16}$ for all). Dietary intake of fish was positively associated with 1-docosahexaenoylglycerophosphocholine (22:6n3), which was inversely associated with incident CKD (HR, 0.82; 95% CI, 0.75-0.89; $P = 7.81 \times 10^{-6}$).

Limitations: Residual confounding and sample-storage duration.

Conclusions: We identified candidate biomarkers of fish, nuts, red and processed meat, eggs, and poultry. A fish-related metabolite, 1-docosahexaenoylglycerophosphocholine (22:6n3), was associated with a lower risk of CKD.

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Diet biomarkers are needed to objectively assess dietary intake and to improve on self-reported methods for dietary assessment. Urine nitrogen is a well-established biomarker of the total dietary intake of protein.[1] However, few biomarkers are available for specific protein-rich foods, such as nuts, red and processed meat, eggs, poultry, and legumes.[2-4] On the other hand, several biomarkers have been validated as being reflective of the habitual intake of fish (ie, eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]).[5] Protein-rich foods have distinct nutritional characteristics and variable associations with kidney outcomes.[6-8] For example, in a cohort of middle-aged US adults, a higher intake of red and processed meat was associated with an elevated risk of chronic kidney disease (CKD), whereas a higher intake of fish, nuts, and legumes was associated with a lower CKD risk.[7]

Nutritional metabolomics is an approach for characterizing potential dietary biomarkers. The metabolome is responsive to dietary intake,[9] because many metabolites are derived from or replenished by exogenous food sources. Further, many metabolites are filtered and excreted by the kidneys.[10] After adjusting for the glomerular filtration rate, a metabolomic approach is useful for studying associations of metabolites with sources of dietary proteins and with the risk of kidney disease. Metabolomic studies can improve our understanding of the metabolic effects of specific sources of dietary proteins. Identifying metabolomic markers of dietary proteins can also lead to improved dietary assessment and inform dietary recommendations to modify kidney disease risk.

Our objectives were to identify serum metabolomic markers of 6 protein-rich foods (fish, nuts, red and processed meat, eggs, poultry, and legumes) using an

PLAIN-LANGUAGE SUMMARY

In this study, we aimed to identify associations between protein-rich foods (fish, nuts, legumes, red and processed meat, eggs, and poultry) and serum metabolites, which are small biological molecules involved in metabolism. Metabolites significantly associated with a protein-rich food individually and collectively improved the discrimination of the respective protein-rich food, suggesting that these metabolites should be prioritized in future diet biomarker research. We also studied associations between significant diet-related metabolites and incident kidney disease. One fish-related metabolite was associated with a lower kidney disease risk. This finding supports the recent nutritional guidelines recommending a Mediterranean diet, which includes fish as the main dietary protein source.

untargeted approach, and to examine whether dietary protein-related metabolites are associated with incident CKD, to understand the early metabolic disturbances related to CKD development. We aimed to study these questions in healthy, free-living, middle-aged US adults to provide broadly generalizable results for dietary assessment and CKD primary prevention.

METHODS

Study Population and Design

The Atherosclerosis Risk in Communities (ARIC) study is a prospective cohort study designed to identify determinants of subclinical atherosclerosis.[11] Between 1987 and 1989 (visit 1), a total of 15,792 middle-aged adults (aged 45-64 years) were enrolled in the ARIC study from 4 US communities (Forsyth County, North Carolina; Jackson, Mississippi; Minneapolis, Minnesota; Washington County, and Maryland) and returned for the following follow-up visits: visit 2 (1990-1992), visit 3 (1993-1995), visit 4 (1996-1998), visit 5 (2011-2013), visit 6 (2016-2017), visit 7 (2018-2019), visit 8 (2020), and visit 9 (2021-2022). Participants provided written informed consent at each visit, and the ARIC study has been approved by Johns Hopkins Bloomberg School of Public Health (IRB00011012) and Johns Hopkins Medicine (IRB00311861; IRB00311999).

At visit 1, fasting blood specimens were collected and stored at −80 °F. Metabolomic profiling was performed by Metabolon, Inc at 2 time periods. In 2010, subgroup 1 was analyzed, consisting of 1,977 African American participants from Jackson, Mississippi. In 2014, subgroup 2 was analyzed, which was a group of 2,055 African American and White participants from all the 4 centers. Participants were excluded for the following reasons: prevalent CKD (defined as an estimated glomerular filtration rate [eGFR] of <60 mL/min/1.73 m^2), missing follow-up times, missing covariates, or missing the dietary intake of legumes (Fig S1). Our study population included 3,726 participants.

Assessment of Protein-Rich Foods

Usual intake over the past year was assessed at visit 1 (1987-1989) using a 66-item food frequency questionnaire (FFQ) adapted from the Willett FFQ.[12,13] We categorized the following 6 protein-rich foods: fish (tuna, dark fish, seafood, and other fish), nuts, red and processed meat (hamburger, red meat main or side dish, hot dogs, processed meat, and bacon), eggs, poultry (chicken or turkey, with or without skin), and legumes (peas, lima beans, baked beans, and lentils). These foods were defined similarly in a prior analysis, with the exception of nuts, which did not include peanut butter in the present study to be more comparable to a prior nutritional metabolomics study.[4,7]

Metabolite Assessment

Metabolites were profiled using an untargeted gas chromatography/mass spectrometry- and liquid chromatography/mass spectrometry-based protocol.[14,15] Known metabolites were identified using a 5-tiered verification system, and all the metabolites in our study were verified with the first 2 tiers. Tier 1 compared metabolites with a reference standard and required metabolites to share at least 2 orthogonal measurements with the standard.[16-18] Tier 2 metabolites were those that did not have a reference standard but were identified based on physiochemical properties or spectral similarities to the metabolite.[17,18] Metabolites from tier 2 are denoted with an asterisk.

For metabolomic data cleaning, metabolite values were rescaled to a median of 1 and transformed to a $\log_2$ scale.[19-21] Metabolites with low variance (ie, <0.01) were excluded. Values were capped at 5 standard deviations from the mean metabolite value. Metabolites with missing values for >80% of participants were excluded (subgroup 1: n = 13; subgroup 2: n = 34). For the remaining metabolites, values that were missing or below the lower limit of detection were imputed to the minimum observed value within each subgroup. Our primary analysis was restricted to metabolites that met inclusion criteria in both subgroups (n = 360 metabolites). In secondary analyses, we studied metabolites in one subgroup (subgroup 1: n = 1 metabolite; subgroup 2: n = 365 metabolites).

Incident CKD Outcome

At visits 1 and 2, creatinine was measured in the serum using the modified kinetic Jaffe method, and at visit 4, creatinine was measured in the plasma using the Jaffe method. Serum creatinine was measured at visits 3, 5, 6, and 7 using the Roche enzymatic method. Creatinine values from visits 1-5 were remeasured in 2011-2013, and original values from visits 1, 2, and 4 were recalibrated.[22]

Creatinine values were calibrated using a National Institute of Standards and Technology standard.[23,24] Calibrated creatinine values were then used to estimate the glomerular filtration rate with the 2021 CKD-EPI race-free equation.[25] Incident CKD was defined as follows: new onset of CKD stages 3-5 (eGFR < 60 mL/min/1.73 m^2) with ≥25% eGFR decline relative to visit 1, diagnostic codes for hospitalization or death related to CKD stages 3-5, or end-stage kidney disease identified by linkage to the US Renal Data System registry through December 31, 2017.[26,27]

Assessment of Covariates

During visit 1, trained interviewers administered questionnaires to collect information on sociodemographic characteristics (ie, age, sex, race, and education) and health behaviors (ie, smoking status, alcohol consumption, and physical activity). Trained staff measured participants' weight using a calibrated scale and their height using a stadiometer to calculate the body mass index. Physical activity was quantified as a score from 1-5, incorporating the intensity, duration, and frequency of sport-related physical activity during leisure time.

The FFQ was used to estimate total energy intake. Nonprotein food groups (vegetables, fruits, dairy, whole grains, and refined grains) were categorized using food items reported on the FFQ.

Diabetes included a nonfasting blood glucose level of ≥200 mg/dL, a fasting blood glucose level of ≥126 mg/dL, a self-report of diabetes mellitus diagnosed by a physician, or a self-reported use of diabetic medications within the past 2 weeks. Hypertension was defined using visit measurements (systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg) or the self-reported use of hypertensive medications within the past 2 weeks. The definition of coronary heart disease comprised a self-reported diagnosis of myocardial infarction, prior coronary revascularization, or silent myocardial infarction on electrocardiogram.

Statistical Analysis

We examined participant characteristics for the overall study population and by subgroups. Multivariable linear regression models were used to estimate cross-sectional associations between protein-rich foods (fish, nuts, red and processed meat, eggs, poultry, and legumes) and serum metabolites in each subgroup and then meta-analyzed using fixed-effects inverse-variance weighted models. We adjusted for age, sex, body mass index, total energy intake, eGFR, smoking status, physical activity, education, and alcohol consumption. We additionally adjusted for nonprotein food groups (dietary intake of vegetables, fruits, dairy, whole grains, and refined grains) to identify metabolites specifically related to protein-rich foods. Race and center were covariates for analyses of subgroup 2, given that this subgroup consisted of both African American and White participants from all the 4 centers. Bonferroni correction was used to minimize the possibility of false-positive findings (eg, main analysis: $P = 0.05/[360$ metabolites studied in both subgroups × 6 protein-rich groups$] = 2.31 \times 10^{-5}$; secondary analysis in subgroup 2: $P = 2.28 \times 10^{-5}$).

Spearman correlation coefficients were calculated to explore interrelationships of significant metabolites. We used C statistics to examine the ability of individual metabolites and panels of metabolites to discriminate the highest versus lower 3 quartiles of dietary intake of protein-rich foods beyond covariates. This empirical definition of an elevated protein intake was used owing to the lack of a clinically relevant threshold. Covariates included in C-statistic models were the same as the multivariable linear regression models. To calculate the difference in C statistics from the model with covariates versus the model with covariates and metabolites, we ran 200 bootstrap iterations, computing a difference in C statistics within each iteration, and reported the average of the 200 iterations. The associated P value was computed using the 'boot.pval' R package through the inversion of confidence intervals (R Foundation for Statistical Computing, Vienna, Austria).

Cox regression models were used to estimate associations between dietary protein-related metabolites and incident CKD adjusted for all covariates included in the linear regression models as well as the following CKD risk factors: diabetes, hypertension, and coronary heart disease. Hazard ratios were estimated per doubling of metabolites (1-unit higher in $\log_2$-transformed metabolite levels). To mitigate batch effects, we performed analyses separately in each subgroup and then meta-analyzed results. Significance thresholds were adjusted using Bonferroni correction (eg, fish-related metabolites: 0.05/8 = 0.006).

Deviations in the proportional hazards' assumption were tested by the visual inspection of Schoenfeld residual plots. As a sensitivity analysis, we repeated the analyses on dietary protein-related metabolites and incident CKD, excluding the first 3 years of follow-up. We used Stata version 17 (StataCorp, College Station, Texas) and R version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) to conduct these analyses.

RESULTS

Participant Characteristics

For the 3,726 participants, the mean age was 54 years, 60% were female, and 62% were African American (Table 1). The mean body mass index was 29 kg/m^2, mean eGFR was 102 mL/min/1.73 m^2, and the prevalence of hypertension and diabetes was 44% and 13%, respectively. On average, the participants consumed 1.2 servings/d of red and processed meat, 0.4 servings/d of fish, 0.4 servings/d of poultry, 0.3 servings/d of eggs, 0.3 servings/d of legumes, and 0.1 servings/d of nuts.

All participants from subgroup 1 were African American and from Jackson, Mississippi, whereas 27% of participants

Table 1. Baseline Characteristics of Participants

Characteristics	Overall (n = 3,726)	Subgroup 1 (n = 1,769)	Subgroup 2 (n = 1,957)
Age (y)	54.0 (5.8)	53.3 (5.7)	54.7 (5.7)
Female, n (%)	2242 (60.2)	1136 (64.2)	1106 (56.5)
African American, n (%)	2291 (61.5)	1769 (100)	522 (26.7)
Center, n (%)			
Forsyth County, North Carolina	560 (15.0)	0 (0)	560 (28.6)
Jackson, Mississippi	2168 (58.2)	1769 (100)	399 (20.4)
Minneapolis Suburbs, Minneapolis	504 (13.5)	0 (0)	504 (25.8)
Washington County, Maryland	494 (13.3)	0 (0)	494 (25.2)
Body mass index (kg/m^2)	28.7 (5.8)	29.6 (6.1)	27.9 (5.5)
Estimated glomerular filtration rate (mL/min/1.73 m^2)	101.5 (12.6)	100.4 (14.8)	101.6 (12.2)
Blood glucose level (mg/dL)	110.5 (41.6)	111.8 (43.0)	109.3 (40.2)
Smoking status, n (%)			
Current smoker	1035 (27.8)	499 (28.2)	536 (27.4)
Former smoker	1037 (27.8)	406 (23.0)	631 (32.2)
Never smoker	1654 (44.4)	864 (48.8)	790 (40.4)
Education level, n (%)			
Less than high school	1185 (31.8)	711 (40.2)	474 (24.2)
High school or vocational school	1282 (34.4)	499 (28.2)	783 (40.0)
Some college or more	1259 (33.8)	559 (31.6)	700 (35.8)
Alcohol consumption (g/wk)	36.7 (94.6)	32.6 (101.3)	40.4 (88.0)
Physical activity[a]	2.3 (0.8)	2.1 (0.7)	2.4 (0.8)
Coronary heart disease, n (%)	186 (5.0)	67 (3.8)	119 (6.1)
Hypertension, n (%)	1651 (44.3)	934 (52.8)	717 (36.6)
Diabetes, n (%)	500 (13.4)	279 (15.8)	221 (11.3)
Dietary variables			
Total energy intake (kcal/d)	1621.4 (615.0)	1582.7 (621.3)	1656.3 (607.4)
Fish (servings/d)	0.4 (0.3)	0.4 (0.4)	0.3 (0.3)
Nuts (servings/d)	0.1 (0.2)	0.1 (0.3)	0.1 (0.2)
Red and processed meat (servings/d)	1.2 (0.8)	1.2 (0.8)	1.1 (0.8)
Eggs (servings/d)	0.3 (0.4)	0.3 (0.4)	0.3 (0.4)
Poultry (servings/d)	0.4 (0.3)	0.4 (0.3)	0.4 (0.3)
Legumes (servings/d)	0.3 (0.3)	0.3 (0.3)	0.3 (0.3)

Values are mean (standard deviation) or n (%).
[a]Physical activity was quantified as a score (1-5), accounting for the intensity, duration, and frequency of sport-related physical activity during leisure time.

from subgroup 2 were African American, with 20%-29% from each of the 4 centers. In subgroup 1, 40% of participants had less than high-school education versus 24% in subgroup 2. On average, alcohol consumption was higher in subgroup 2 at 40 g/wk versus 33 g/wk in subgroup 1. The 2 subgroups had similar dietary intakes of protein-rich foods.

Associations of Protein-Rich Foods With Serum Metabolites

There were 30 significant associations between protein-rich foods and serum metabolites (fish, n = 8; nuts, n = 5; legumes, n = 0; red and processed meat, n = 5; eggs, n = 3; poultry, n = 9). Fish intake was significantly associated with 7 lipids, including 3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF, β = 0.99), DHA (β = 0.27), and EPA (β = 0.22) (Table 2 and Table S1). Nut consumption was most strongly associated with tryptophan betaine (β = 1.42), followed by 4-vinylphenol sulfate (β = 0.87). Red and processed meat intake was positively associated with one amino acid (2-hydroxybutyrate) and 4 lipids, including margarate (17:0). Egg consumption was positively associated with one lipid (docosapentaenoate [DPA]). Poultry intake was associated with 6 amino acids (eg, 3-methylhistidine and creatine).

In the secondary analysis, 11 significant associations were observed between protein-rich foods and serum metabolites, including positive associations between red and processed meat and 1-stearoylplasmenylethanolamine and between poultry and sulfate (Table S2).

Fish-related EPA and DHA were strongly related to each other (r = 0.65) (Fig S2). Nut-related metabolites were weakly correlated (Fig S3). Several long odd-chain fatty acids related to red and processed meat were

Table 2. Thirty Significant Associations Between Protein-Rich Foods and Serum Metabolites Per Serving Higher in Protein-Rich Foods

Protein-Rich Food Group	Metabolite	Superpathway	Subpathway	Meta-Analyzed β	Meta-Analyzed SE	Meta-Analyzed *P* Value
Fish	CMPF	Lipid	Fatty acid, dicarboxylate	0.989	0.069	3.41×10^{-47}
Fish	DHA (22:6n3)	Lipid	Polyunsaturated fatty acid (n3 and n6)	0.268	0.023	3.95×10^{-31}
Fish	1-Docosahexaenoylglycerophosphocholine (22:6n3)[a]	Lipid	Lysolipid	0.227	0.031	1.28×10^{-13}
Fish	1-Docosahexaenoylglycerophosphoethanolamine[a]	Lipid	Lysolipid	0.196	0.030	7.64×10^{-11}
Fish	EPA (20:5n3)	Lipid	Polyunsaturated fatty acid (n3 and n6)	0.215	0.035	1.01×10^{-9}
Fish	n-6 DPA (22:5n6)	Lipid	Polyunsaturated fatty acid (n3 and n6)	-0.192	0.038	3.53×10^{-7}
Fish	2-Aminobutyrate	Amino acid	Methionine, cysteine, SAM, and taurine metabolism	0.099	0.023	1.28×10^{-5}
Fish	1-Eicosatrienoylglycerophosphocholine (20:3)[a]	Lipid	Lysolipid	-0.130	0.030	2.04×10^{-5}
Nuts	Tryptophan betaine	Amino acid	Tryptophan metabolism	1.42	0.095	2.01×10^{-50}
Nuts	4-Vinylphenol sulfate	Xenobiotics	Benzoate metabolism	0.873	0.101	4.23×10^{-18}
Nuts	Stearoyl sphingomyelin	Lipid	Sphingolipid metabolism	-0.174	0.026	5.14×10^{-11}
Nuts	Heptanoate (7:0)	Lipid	Medium-chain fatty acid	0.092	0.019	6.58×10^{-7}
Nuts	Catechol sulfate	Xenobiotics	Benzoate metabolism	0.285	0.059	1.20×10^{-6}
Red and processed meat	AHB	Amino acid	Methionine, cysteine, SAM, and taurine metabolism	0.100	0.016	6.39×10^{-10}
Red and processed meat	Margarate (17:0)	Lipid	Long-chain fatty acid	0.057	0.012	1.51×10^{-6}
Red and processed meat	10-Heptadecenoate (17:1n7)	Lipid	Long-chain fatty acid	0.061	0.013	3.78×10^{-6}
Red and processed meat	Decanoylcarnitine	Lipid	Fatty acid metabolism (acyl carnitine)	0.085	0.019	6.99×10^{-6}
Red and processed meat	10-Nonadecenoate (19:1n9)	Lipid	Long-chain fatty acid	0.067	0.015	1.22×10^{-5}
Eggs	n-6 DPA (22:5n6)	Lipid	Polyunsaturated fatty acid (n3 and n6)	0.291	0.029	8.80×10^{-24}
Eggs	Tryptophan betaine	Amino acid	Tryptophan metabolism	-0.427	0.059	4.63×10^{-13}
Eggs	Gamma-CEHC	Cofactors and vitamins	Tocopherol metabolism	-0.227	0.048	1.95×10^{-6}
Poultry	3-Methylhistidine	Amino acid	Histidine metabolism	0.995	0.091	1.21×10^{-27}
Poultry	N1-methyl-2-pyridone-5-carboxamide	Cofactors and vitamins	Nicotinate and nicotinamide metabolism	0.179	0.033	8.80×10^{-8}
Poultry	Urea	Amino acid	Urea cycle; arginine and proline metabolism	0.130	0.025	1.76×10^{-7}
Poultry	Pyroglutamine[a]	Amino acid	Glutamate metabolism	-0.217	0.043	5.15×10^{-7}

(Continued)

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Table 2 (Cont'd). Thirty Significant Associations Between Protein-Rich Foods and Serum Metabolites Per Serving Higher in Protein-Rich Foods

Protein-Rich Food Group	Metabolite	Superpathway	Subpathway	Meta-Analyzed β	Meta-Analyzed SE	Meta-Analyzed *P* Value
Poultry	Tiglyl carnitine	Amino acid	Leucine, isoleucine, and valine metabolism	0.184	0.040	4.26×10^{-6}
Poultry	Pyridoxate	Cofactors and vitamins	Vitamin B6 metabolism	0.172	0.038	4.57×10^{-6}
Poultry	Caffeine	Xenobiotics	Xanthine metabolism	-0.468	0.103	5.02×10^{-6}
Poultry	Beta-hydroxyisovaleroylcarnitine	Amino acid	Leucine, isoleucine, and valine metabolism	0.135	0.030	5.26×10^{-6}
Poultry	Creatine	Amino acid	Creatine metabolism	0.129	0.030	2.08×10^{-5}

Linear regression models are adjusted for age, sex, race (in subgroup 2), study center (in subgroup 2), body mass index, total energy intake, estimated glomerular filtration rate based on creatinine, smoking status, physical activity, education, alcohol consumption, total vegetable intake, total fruit intake, dairy intake, whole grain intake, and refined grain intake. Bonferroni-adjusted *P* value = 0.05/(360 metabolites × 6 protein-rich groups) = 2.31×10^{-5}.
Abbreviations: AHB, 2-hydroxybutyrate; CEHC, carboxyethyl hydroxychroman; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoate; DHA, docosahexaenoate; DPA, docosapentaenoate; EPA, eicosapentaenoate; SAM, S-adenosylmethionine; SE, standard error.
[a]Tier 2 metabolites that did not have a reference standard available but were identified based on physiochemical properties or spectral similarities.

strongly correlated (margarate [17:0], 10-heptadecenoate [17:1n7], and 10-nonadecenoate [19:1n9]) (Fig S4). Egg-related metabolites were weakly correlated (Fig S5). For poultry-related metabolites, pyroglutamine and creatine were strongly inversely correlated (r = -0.64) (Fig S6).

Discrimination of Protein-Rich Foods With Metabolites

The panels of metabolites all significantly improved the discrimination of high intake of protein-rich foods (Table S3). For fish, 7 out of 8 metabolites improved discrimination, with the greatest improvement observed for CMPF (difference in C statistics = 0.019) and DHA (difference in C statistics = 0.016). For nuts, all but one metabolite was significant, with tryptophan betaine improving discrimination the most (difference in C statistics = 0.046), followed by 4-vinylphenol sulfate (difference in C statistics = 0.017). For red and processed meat, 2-hydroxybutyrate and decanoylcarnitine marginally but statistically significantly improved discrimination. For eggs, DPA (n-6; 22:5n6) (difference in C statistics = 0.018) and tryptophan betaine (difference in C statistics = 0.005) improved discrimination. For poultry, 5 metabolites improved discrimination, with 3-methylhistidine offering the greatest improvement (difference in C statistics = 0.016). Smaller combinations of metabolites similarly improved discrimination of the respective protein-rich group relative to the full panel of metabolites.

Associations of Protein-Related Metabolites With Incident CKD

A total of 1,412 (37.8%) participants developed incident CKD over a median follow-up of 24 years (interquartile range, 14-29 years). One fish-related metabolite, 1-docosahexaenoylglycerophosphocholine (22:6n3), was positively associated with fish intake (β = 0.23) (Table 2) and inversely associated with incident CKD (Fig 1). A doubling of 1-docosahexaenoylglycerophosphocholine (22:6n3) was associated with 18% lower risk of incident CKD (hazard ratio, 0.82; 95% confidence interval: 0.75, 0.89; $P = 7.81 \times 10^{-6}$) (Table 3).

Another metabolite, 1-eicosatrienoylglycerophospho choline (20:3), was inversely associated with fish intake (Table 2) and inversely associated with incident CKD (Table 3). In the secondary analysis of metabolites analyzed only in subgroup 2, sulfate was positively associated with poultry (Table S2) and positively associated with incident CKD (Table 3), and 1-stearoylplasmenylethanolamine was positively associated with red and processed meat (Table S2) and inversely associated with CKD (Table 3).

Visual inspection of Schoenfeld residual plots demonstrated deviations that appeared to occur within the first few years of follow-up. Sensitivity analyses excluding the

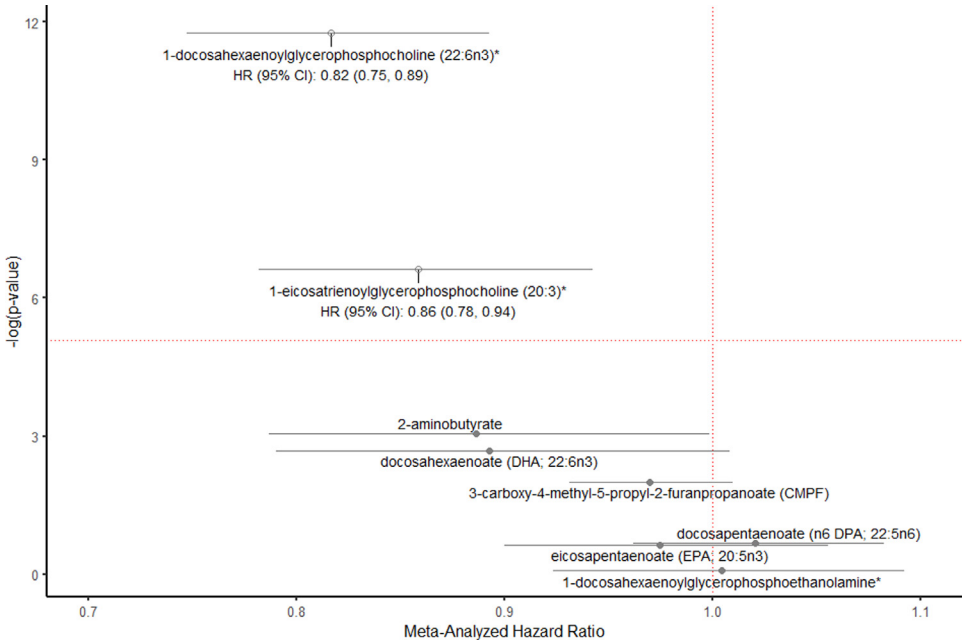

Figure 1. Meta-analyzed associations between fish-related metabolites and incident chronic kidney disease. Cox regression models are adjusted for age, sex, race (in subgroup 2), study center (in subgroup 2), body mass index, total energy intake, and estimated glomerular filtration rate based on creatinine, smoking status, physical activity, education, alcohol consumption, total vegetable intake, total fruit intake, dairy intake, whole grain intake, refined grain intake, diabetes, hypertension, and coronary heart disease. The red dashed vertical line denotes the null value (hazard ratio = 1.0). The red dashed horizontal line denotes the statistical significance threshold, defined using the Bonferroni method as follows: -ln (0.05/8 fish-related metabolites) = 5.08. *Tier 2 metabolites that had no reference standard available but were identified based on physiochemical properties or spectral similarities. CI, confidence interval; HR, hazard ratio.

first 3 years of follow-up produced similar estimates to the main analysis (Table S4).

DISCUSSION

In this study of 3,726 middle-aged US adults, we identified 30 significant associations between serum metabolites and protein-rich foods (fish, n = 8; nuts, n = 5; red and processed meat, n = 5; eggs, n = 3; poultry, n = 9), which are supported by research conducted in humans and animals. Panels of metabolites and many individual metabolites improved discrimination of the dietary intake of fish, nuts, red and processed meat, eggs, and poultry, suggesting that these candidate markers have the potential to contribute to dietary assessment in the future. One metabolite, 1-docosahexaenoylglycerophosphocholine (22:6n3), was positively associated with the dietary intake of fish and inversely associated with incident CKD. These findings have implications for dietary assessment and CKD prevention.

Table 3. Significant Associations Between Protein-Related Metabolites and Incident CKD

	Protein-Rich Food	Meta-Analyzed HR (95% CI)	Meta-Analyzed *P* -Value
Full cohort (N = 3,726)			
1-Eicosatrienoyl-GPC (20:3)[a]	Fish	0.86 (0.78, 0.94)	0.001
1-Docosahexaenoyl-GPC (22:6n3)[a]	Fish	0.82 (0.75, 0.89)	7.81×10^{-6}
Subgroup 2 only (N = 1,957)			
Sulfate[a]	Poultry	1.73 (1.22, 2.46)	0.002
1-Stearoylplasmenylethanolamine[a]	Red and processed meat	0.86 (0.76, 0.97)	0.01

Cox regression models are adjusted for age, sex, race (in subgroup 2), study center (in subgroup 2), body mass index, total energy intake, and estimated glomerular filtration rate based on creatinine, smoking status, physical activity, education, alcohol consumption, total vegetable intake, total fruit intake, dairy intake, whole grain intake, refined grain intake, diabetes, hypertension, and coronary heart disease. Bonferroni-adjusted *P* values are as follows. Fish: 0.05/(8 fish-related metabolites in full cohort) = 0.006; poultry: 0.05/(2 poultry-related metabolites in subgroup 2 only) = 0.03; and red and processed meat: 0.05/(1 red and processed meat–related metabolites in subgroup 2 only) = 0.05. The values provided are per doubling of dietary protein-related metabolites.
Abbreviations: CI, confidence interval; CKD, chronic kidney disease; GPC, glycerophosphocholine; HR, hazard ratio.
[a]Tier 2 metabolites that did not have a reference standard available but were identified based on physiochemical properties or spectral similarities.

Several of our metabolomic markers of the dietary intake of protein-rich foods are consistent with prior research. The dietary intake of fish was significantly positively associated with CMPF, DHA, and EPA. EPA and DHA have been directly measured in fish, and in prior human studies, all 3 metabolites have been consistently associated with fish intake.[2,5,28,29] Moreover, we found that EPA and DHA were strongly correlated (r = 0.65), which is coherent with their known metabolism by which EPA can be converted to DPA and then DHA.[30] Nut intake was strongly associated with tryptophan betaine and 4-vinylphenol sulfate. Peanuts have been previously associated with these 2 metabolites in a nested case-control study of 502 US adults,[4] and both metabolites have been identified as peanut constituents.[31,32] Nuts have also been associated with tryptophan betaine in 3 Canadian birth cohorts (N = 900).[33] In our study, poultry intake was significantly associated with 3-methylhistidine, creatine, and ectoine. 3-methylhistidine is a well-recognized biomarker of chicken intake and has also been associated with the total protein intake in the Modification of Diet in Renal Disease study.[33-35] Poultry and a protein-rich dietary pattern have been associated with creatine in prior studies, including the Modification of Diet in Renal Disease study and the OmniHeart trial.[35-38] Creatine is derived from both exogenous (ie, animal proteins) and endogenous (ie, muscle breakdown) sources.[39] Ectoine, a xenobiotic, is synthesized in the gut microbiota of chickens and has been associated with poultry intake in previous human studies.[36,40] Altogether, our study has added evidence that these 8 metabolites (CMPF, EPA, DHA, tryptophan betaine, 4-vinylphenol sulfate, 3-methylhistidine, creatine, and ectoine) are strong markers of specific protein-rich foods.

We also identified many novel associations between protein-rich foods and serum metabolites. Red and processed meat intake was positively associated with an acylcarnitine, decanoylcarnitine. Researchers have previously found that short- and medium-chain acylcarnitines are positively associated with red and processed meat intake.[36,41] Egg intake was positively associated with an n-6 polyunsaturated fatty acid, DPA (22:5 n-6). Eggs are a known source of n-6 polyunsaturated fatty acids, and DPA was previously associated with egg consumption in a smaller subset of ARIC study participants (subgroup 1, n = 1,977).[2,42] Upon replication, these promising candidate metabolites could serve as new markers of protein-rich foods.

Panels of metabolites significantly improved the discrimination of a higher intake of fish, nuts, red and processed meat, eggs, and poultry, although the improvement beyond covariates was marginal for red and processed meat. Panels of multiple metabolites may better reflect the diverse biochemistry of foods and offer a greater discrimination value than a single metabolite for dietary assessment.[43] Selected individual metabolites also largely improved the discrimination of a higher intake of protein-rich foods, that is, CMPF and DHA for fish, tryptophan betaine and 4-vinylphenol sulfate for nuts, 3-methylhistidine for poultry, and DPA (22:5n6) for eggs. These findings help to prioritize candidate diet biomarkers for future research.

We also found that one metabolite that was positively associated with fish intake, 1-docosahexaenoyl-glycerophosphocholine (1-docosahexaenoyl-GPC) (22:6n3), a lipid, was associated with a reduced risk of incident CKD. In a prior ARIC study (n = 3,799), docosahexaenoyl-GPC (22:6n3) was inversely associated with kidney failure, as was a metabolite cluster composed mainly of GPC lipids.[21] Previous results have also identified a closely related lipid, 1-docosapentaenoyl-GPC (22:5n3), as a marker of progressive nephrotic syndrome.[44] Our findings on 1-docosahexaenoyl-GPC (22:6n3), within the context of these other findings, suggest that fish consumption could be beneficial for reducing CKD risk through a favorable impact on serum lipids. In the main analysis, we identified inverse associations between 1-eicosatrienoyl-GPC (20:3) and fish intake as well as CKD, which is inconsistent with what we would have expected, given that a higher fish intake has been associated with a reduced risk of CKD.[7] This metabolite was also individually related to a lower risk of kidney failure in a prior ARIC study.[21] In the secondary analysis, 1-stearoylplasmenylethanolamine was positively associated with red and processed meat, yet inversely related to CKD. Plasmalogens were positively associated with red and processed meat in the Chronic Renal Insufficiency Cohort study, so our observed diet-metabolite association is plausible.[45] More work is needed to elucidate how 1-eicosatrienoyl-GPC (20:3) and 1-stearoylplasmenylethanolamine mechanistically affect CKD development.

Several limitations warrant discussion. Given that the ARIC study is an observational study, residual confounding is possible, though we adjusted for 16 covariates that were rigorously collected. The long storage duration of serum specimens before metabolomic profiling could have affected metabolite values. However, 3 compounds (ie, urea, glucose, and cholesterol) that were measured using clinical assays in 1989 were moderately correlated with metabolite values.[46] We did not identify any metabolites significantly associated with legume consumption. Additional research on metabolites of protein-rich foods, and the dietary intake of legumes in particular, and incident CKD in independent study populations is necessary to provide greater validity of our findings.

This study also had several strengths. Our sample of African American and White participants was large (n = 3,726) and representative of multiple geographic regions. In both the cross-sectional and prospective analyses, we studied associations within each ARIC subgroup before doing a meta-analysis. This approach mitigated the possibility of batch effects, which is important considering metabolomic analyses were performed at different points in time. We were able to report unique metabolites associated with 5 distinct protein-rich foods, which highlights

the diverse metabolic implications of protein sources of food.

These findings have several implications for dietary assessment and kidney disease prevention. Our findings on metabolomic markers of protein-rich foods help to prioritize candidate diet biomarkers for quantitative assay development, and eventually for integration with self-reported data to improve dietary assessment. Furthermore, our finding of the positive association between 1-docosahexaenoylglycerophosphocholine (22:6n3) and fish and its inverse association with incident CKD provides support for the 2020 Kidney Disease Outcomes Quality Initiative guideline for nutrition, which suggests prescribing a Mediterranean diet, which emphasizes fish as the main dietary protein source to adults with CKD stages 1-5, to improve plasma lipid profiles.[47]

In conclusion, we identified 30 associations between protein-rich foods and serum metabolites (fish, n = 8; nuts, n = 5; red and processed meat, n = 5; eggs, n = 3; poultry, n = 9), including 8 associations that were substantiated by prior studies (CMPF, EPA, DHA, tryptophan betaine, 4-vinylphenol sulfate, 3-methylhistidine, creatine, and ectoine). Metabolites collectively and individually had a strong discrimination value for the dietary intake of protein-rich foods, especially CMPF, DHA, tryptophan betaine, 4-vinylphenol sulfate, 3-methylhistidine, and DPA. These metabolites improved the discrimination of the dietary intake of proteins beyond covariates (demographics, clinical factors, health behaviors, and intake of nonprotein food groups). One fish-related metabolite (1-docosahexaenoyl-GPC [22:6n3]) was inversely associated with the risk of incident CKD. These metabolites are candidate biomarkers of dietary intake, and 1-docosahexaenoyl-GPC (22:6n3), as a marker of fish intake, may be important for the primary prevention of CKD.

SUPPLEMENTARY MATERIAL

Supplementary File 1 (PDF)

Figure S1: A flow chart of study participant selection in the Atherosclerosis Risk in Communities (ARIC) Study.

Figure S2: Correlations for metabolites related to fish intake.

Figure S3: Correlations for metabolites related to nut intake.

Figure S4: Correlations for metabolites related to red and processed meat intake.

Figure S5: Correlations for metabolites related to egg intake.

Figure S6: Correlations for metabolites related to poultry intake.

Table S1: Subgroup Estimates for 30 Significant Associations Between Protein-Rich Foods and Serum Metabolites.

Table S2: Eleven Significant Associations Between Protein-Rich Foods and Serum Metabolites in Subgroup 2 Only.

Table S3: Ability of Metabolites to Discriminate Highest Versus Lowest Three Quartiles of Protein-Rich Food.

Table S4: Sensitivity Analysis for Metabolite-Chronic Kidney Disease Associations.

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