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Serum Nitrogen and Carbon Stable Isotope Ratios Meet Biomarker Criteria for Fish and Animal Protein Intake in a Controlled Feeding Study of a Women's Health Initiative Cohort

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ABSTRACT

Background: Natural abundance stable isotope ratios are candidate biomarkers of dietary intake that have not been evaluated in a controlled feeding study in a US population.

Objectives: Our goals were to evaluate dietary associations with serum carbon (CIR), nitrogen (NIR), and sulfur (SIR) isotope ratios in postmenopausal women, and to evaluate whether statistical models of dietary intake that include multiple isotopes and participant characteristics meet criteria for biomarker evaluation.

Methods: Postmenopausal women from the Women's Health Initiative ($n = 153$) were provided a 2-wk controlled diet that approximated each individual's habitual food intake. Dietary intakes of animal protein, fish/seafood, red meat, poultry, egg, dairy, total sugars, added sugars, sugar-sweetened beverages (SSBs), and corn products were characterized during the feeding period with the use of the Nutrition Data System for Research (NDS-R). The CIR, NIR, and SIR were measured in sera collected from fasting women at the beginning and the end of the feeding period. Linear models based on stable isotope ratios and participant characteristics predicted dietary intake. The criterion used for biomarker evaluation was $R^2 \geq 0.36$, based on the study's power to detect true associations with $R^2 \geq 0.50$.

Results: The NIR was associated with fish/seafood intake and met the criterion for biomarker evaluation ($R^2 = 0.40$). The CIR was moderately associated with intakes of red meat and eggs, but not to the criterion for biomarker evaluation, and was not associated with intake of sugars (total, added, or SSB). A model of animal protein intake based on the NIR, CIR, and participant characteristics met the criterion for biomarker evaluation ($R^2 = 0.40$). Otherwise, multiple isotopes did not improve models of intake, and improvements from including participant characteristics were modest.

Conclusion: Serum stable isotope ratios can, with participant characteristics, meet biomarker criteria as measures of fish/seafood and animal protein intake in a sample of postmenopausal women. This trial was registered at clinicaltrials.gov as NCT00000611. *J Nutr* 2018;148:1931–1937.

Keywords: Nutrition and Physical Activity Assessment Study Feeding Study (NPAAS-FS), nitrogen isotope ratio, carbon isotope ratio, sulfur isotope ratio, dietary biomarkers

Introduction

There is a pressing need for objective biomarkers of diet for use in nutritional epidemiologic research (1–3). Natural abundance stable isotope ratios are a new class of dietary biomarkers that have been relatively little explored in US populations (4). The stable isotope ratios of carbon (CIR), nitrogen (NIR), and sulfur (SIR)—expressed as δ^{13}C, δ^{15}N, and δ^{34}S values—vary naturally among foods, are captured in body proteins and other molecules, and can be measured in a variety of tissues (4). Recent studies support stable isotope ratios as candidate measures for dietary intake of added sugar or sugar-sweetened beverages (SSBs) (5–11), meat (12–14), and fish (13–15); however, they have not been evaluated in the context of a controlled feeding study in a US population.

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The NIR is a candidate biomarker of meat, animal protein, and/or fish intake because it is elevated in animals relative to plants, and is particularly elevated in fish. In populations native to Alaska and Greenland the NIR is strongly associated with fish intake (16–19), and in European studies the NIR has been associated with both meat and fish intake (12–14). The CIR is a candidate biomarker of added sugar/SSB intake because corn and sugar cane, the predominant sources of added sugar in the United States, have elevated CIRs relative to most other plant-based foods (20, 21). The CIR has shown weak to moderate associations with added sugar/SSB intake in several US studies (5, 8, 11, 22, 23). However, the CIR is also a candidate biomarker for meat intake, because livestock in the United States are heavily corn-fed and have elevated CIR relative to many other foods (4, 18). The CIR has shown moderate associations with meat and/or animal protein intake in the United States and Germany, where livestock are also corn-fed (5, 12, 22). In addition, the CIR is elevated in corn-based foods such as corn cereals and chips, potentially confounding associations with added sugar or meat intake. The SIR has been less studied, but is elevated in fish relative to other foods, including other animal protein sources, and has been associated with high fish intakes (19).

This study evaluates multiple stable isotope biomarkers in the Nutrition and Physical Activity Assessment Study Feeding Study (NPAAS-FS), an ancillary study to the Women's Health Initiative (WHI) designed to evaluate biomarkers of dietary exposure in 153 Seattle-area postmenopausal women (24, 25). The NPAAS-FS is unique in that for 2 wk each participant consumed a controlled, provided diet designed to approximate reported usual intake, to have a high level of dietary control while maintaining baseline biomarker measurements and preserving the dietary variability of the population (24). This study had 2 goals: first, to evaluate whether serum CIR, NIR, and SIR are associated with dietary intake in the NPAAS-FS; and second, to examine whether predictive models of dietary intake based on stable isotope ratios and participant characteristics, such as age, race/ethnicity, or BMI, meet criteria for biomarker evaluation.

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Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

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Abbreviations used: CIR, carbon isotope ratio; HNL, Human Nutrition Laboratory; LPA, leisure physical activity; NDS-R, Nutrition Data System for Research; NIR, nitrogen isotope ratio; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SIR, sulfur isotope ratio; SSB, sugar-sweetened beverage; UAF, University of Alaska Fairbanks; WHI, Women's Health Initiative; 4DFR, 4-day food record.

Methods

Participants

The NPAAS-FS involved 153 postmenopausal women in the Seattle area who were current participants in the WHI Extension Study. Eligibility and recruitment procedures for the NPAAS-FS, including participants' prior WHI enrollment, are described in detail elsewhere (24, 25). The NPAAS-FS was conducted between April 2011 and October 2013. The Fred Hutchinson Institutional Review Board in accordance with the Declaration of Helsinki approved this study. The WHI Observational Study Monitoring Board provides additional oversight to all such ancillary studies in which participants complete study procedures. The WHI program was registered at clinicaltrials.gov as NCT00000611.

Study design and procedures

The study was designed so that each woman's experimental diet approximated her habitual intake, as captured before the study by a 4-d food record (4DFR) with fine-tuning during the review of the 4DFR with participants. By this design, the study aimed to maintain the normal intake distribution of the sample and to minimize the perturbation of biomarker measurements from baseline. Here we provide a brief description of study procedures, reported in detail elsewhere (24, 25). A research dietitian reviewed each participant's 4DFR before the feeding study and conducted an in-depth interview to identify dietary supplement use, food choices, and dietary patterns not captured by the 4DFR. Participant diets were further adjusted for energy requirements based on the Mifflin-St Jeor equation (26) and prior calibration of energy reporting in the WHI (27, 28). Foods were prepared in the Fred Hutchinson Cancer Research Center's Human Nutrition Laboratory (HNL) and all meals were entered into the Nutrition Data System for Research (NDS-R; Nutrition Coordinating Center, version 2010, University of Minnesota). Participants visited the HNL 2–3 times/wk to consume a study meal on site, to have their body weight measured, and to collect foods for the intervening meals. Uneaten foods were returned to the HNL, where they were weighed and recorded. Women also completed a daily menu checklist to record consumption of all study foods and beverages (and nonstudy foods and beverages if applicable).

During the first feeding intervention visit women provided a fasting blood specimen and trained staff measured their height and weight. Participants also completed questionnaires on diet (FFQ), medication use, dietary supplement use, and leisure physical activity (LPA), as described in detail elsewhere (24). Women returned to the HNL at the end of the feeding period to provide a fasting blood sample and have their weight measured. Fasting blood samples were processed to serum aliquots and stored at −80°C. These were subsequently shipped on dry ice to the University of Alaska Fairbanks (UAF) for stable isotope analysis. All procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board and all participants provided written informed consent.

Assessment of dietary intake

The following dietary intake variables were extracted from the NPAAS-FS "consumed" NDS-R files over the period of dietary intake: total energy (kcal/d), carbohydrate (g/d), fat (g/d), protein (g/d), animal protein (g/d), total sugar (g/d), and added sugar (g/d). In addition, specific categories of animal-based food intake, corn product intake, and SSB intake were created from the NPAAS-FS "consumed" NDS-R food components output file. These categories were selected based on prior studies of stable isotope ratios in foods (18, 20, 29–32). There were 101,314 distinct food components in this output file, representing every food component consumed by ≥1 of the 153 participants over the 14-d feeding period. These were coded as 0 or 1 for each of 7 new categories of intake: fish/seafood, red meat, poultry, eggs, dairy, corn products, and SSB. Detailed coding inclusion/exclusion criteria are presented in **Supplemental Table 1**; briefly, "red meat" included beef, pork, lamb, and liver, "fish/seafood" included fish and shellfish, and "corn products" included whole corn, popcorn, corn chips, corn tortillas, corn cereals, and corn oil. If a category was the second or third ingredient listed in a commercial food component it was coded as 0.5.

These instances were infrequent and are documented in Supplemental Table 1. Coding variables were multiplied by grams of intake (or kcal), summed over each participant, and divided by the number of days of intake to generate daily intake for each participant for each new food category. For components that included beverages (dairy and SSB), we expressed intake in kcal rather than grams to exclude the weight of the water.

Stable isotope analyses

Measurement of serum CIR, NIR, and SIR. Serum CIR and NIR were analyzed at the Alaska Stable Isotope Facility at UAF. Samples were prepared for measurement of serum SIR at UAF and sent to the Stable Isotope Facility at the University of California Davis for analysis. Analysis of CIR and NIR required a single, 5-μL aliquot of serum; analysis of SIR required a 20-μL aliquot of serum. These were pipetted into tin capsules (Elemental Microanalysis, IsoMass Scientific, Inc.), which were subsequently autoclaved, dried, weighed to the nearest 0.001 mg on a microbalance, crushed, and introduced into an autosampler for combustion and isotope analysis (4). Serum CIR and NIR were measured with the use of a Costech Elemental Analyzer (ECS 4010) interfaced to a Delta V Plus isotope ratio mass spectrometer via the Conflo IV interface (Thermo Scientific, Inc.). Serum SIR was measured with the use of an Elementar vario ISOTOPE cube interfaced to a SerCon 20–22 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, United Kingdom). Isotope ratios are presented in permil (‰) abundance of heavy isotope relative to reference values as follows: $\delta X = (R_{sample} - R_{reference})/(R_{reference}) \times 1000$ (‰), where X is the heavy isotope, R is the ratio of heavy to light isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{34}S/^{32}S$), and the reference values are internationally recognized standards calibrated to Vienna Pee Dee Belemnite ($^{13}C/^{12}C = 0.01124$), atmospheric nitrogen ($^{15}N/^{14}N_{atm\text{-}N} = 0.003677$), and Canyon Diablo Troilite ($^{34}S/^{32}S = 0.0450$). Analytic precision was assessed as the SD of laboratory working standards calibrated to the above reference materials that were measured after every 10th sample; these were within 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, and within 0.4‰ for $\delta^{34}S$.

Statistical analysis. All statistical analyses were performed with the use of JMP version 11 (SAS Institute). Because stable isotope ratios reflect diet over periods longer than 2 wk, we excluded participants ($n = 6$) from dietary analyses whose serum isotope ratios were not stable over the 2 wk of dietary control, suggesting that the intervention diet did not adequately reflect usual intake. Our criterion for exclusion was a difference in CIR, NIR, or SIR measured between pre- and postintervention serum samples that exceeded the mean difference by ≥3 IQR widths (<4% of 153 participants). We examined associations between dietary intake variables and stable isotope variables measured in serum samples collected after the feeding period through the use of Pearson correlation coefficients. We used a significance level of $P = 0.005$, based on the Bonferroni-Holm adjustment to account for the number of comparisons made with each potential biomarker, i.e., $k = 10$, where k refers to the number of dietary comparisons made for each biomarker (33). Stable isotope biomarkers were evaluated for their associations with animal protein (g/d), fish/seafood (g/d), red meat (g/d), poultry (g/d), eggs (g/d), dairy (kcal/d), total sugar (g/d), added sugar (g/d), SSB (kcal/d), and corn products (g/d). Where >1 dietary intake variable was associated with a stable isotope ratio, biomarker associations were adjusted by the other intake variables to determine if associations were independent of dietary pattern.

To evaluate whether multiple-isotope models were better predictors of dietary intake than single-isotope models, we performed linear regression analyses ($P = 0.05$) with 3 serum isotope ratios as independent variables. To evaluate whether including participant characteristics improved biomarker associations, the regression analyses were extended by race/ethnicity, BMI, dietary supplement use (yes or no), and LPA (metabolic equivalent task hours per week). A backward-selection procedure ($P = 0.10$) was applied to exclude noncontributing isotope ratios and participant characteristics from final models. Outliers were identified by Mahalobnis Distance > 4.0 and excluded from analyses ($n = 2$ for animal protein model only). Normality of residuals was confirmed with the use of the Shapiro-Wilks test.

We used $R^2 \geq 0.36$ as the criterion for biomarker evaluation, following the parent study (24). With a sample size of 153, the NPAAS-FS had >88% power to detect a true diet-biomarker association of $R^2 \geq 0.5$ (correlation = 0.7) with a sample $R^2 \geq 0.36$ (24). Well-characterized recovery biomarkers measured in the NPAAS-FS study had dietary associations in that range; for example, regression of total protein intake (g/d) on the urinary nitrogen biomarker had an $R^2 = 0.37$, which improved to 0.43 when adjusted for participant characteristics (24). Similarly, total energy intake (kcal/d) regressed on biomarker-estimated E_{in} (doubly labeled water) had an $R^2 = 0.51$, which improved to 0.53 when adjusted for participant characteristics (24).

We examined the stability of serum CIR, NIR, and SIR over the duration of the feeding study by examining mean differences and Pearson's correlation coefficients between serum samples collected at baseline and after the 14-d feeding period.

Results

An overview of the demographic and lifestyle characteristics of NPAAS-FS participants is presented in **Table 1**, as well as intake of energy, macronutrients, and dietary variables specific to this study. More detailed demographic and lifestyle information is published elsewhere (24). Participants were mostly Caucasian

TABLE 1 Baseline demographic, lifestyle, and dietary characteristics of the postmenopausal women in the NPAAS-FS included in this study[1]

Variable	Value
n	147
Age, y	75 ± 4
BMI, kg/m^2	26 ± 4
Race/ethnicity, *n* (%)	
Caucasian	140 (95.2)
Non-Caucasian[2]	7 (4.8)
LPA[3] (MET-h/wk), *n* (%)	
0–5.0	37 (25.3)
5.1–12.0	36 (24.5)
12.1–23.0	37 (25.3)
>23	37 (25.3)
Dietary supplement use[3], *n* (%)	
Yes	126 (85.7)
No	21 (14.3)
Macronutrient intake	
Total energy, kcal/d	1920 ± 287
Carbohydrate, % kcal/d	45 ± 7
Fat, % kcal/d	38 ± 5
Protein, % kcal/d	17 ± 3
Total animal protein, g/d	51 ± 15
Fish/seafood, g/d	33 (18, 49)
Red meat, g/d	43 (23, 63)
Poultry, g/d	34 (21, 52)
Eggs, g/d	20 (13, 34)
Dairy, kcal/d	326 (215, 436)
Total sugar, g/d	98 (80, 117)
Added sugar, g/d	48 (35, 64)
SSBs, kcal/d	17 (15, 41)
Corn products, g/d	1.5 (0.1, 13.4)

[1]Values are means ± SDs or medians (IQRs). LPA, leisure physical activity; MET-h, metabolic equivalent task hour; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SSBs, sugar-sweetened beverages.
[2]Included 3 African Americans, 2 Hispanics, 1 American Indian/Alaska Native, and 1 Asian/Pacific Islander.
[3]Measured at time of enrollment in the NPAAS-FS.

TABLE 2 Serum stable isotope ratios at baseline and postfeeding of the postmenopausal women in the NPAAS-FS included in this study[1]

	Baseline	Postfeeding	Difference
CIR, ‰	−19.9 ± 0.6 (−21.4, −17.6)	−20.0 ± 0.5 (−21.7, −18.0)	−0.03 ± 0.34 (−1.1, 1.2)
NIR, ‰	8.8 ± 0.4 (8.0, 9.9)	8.9 ± 0.5 (7.8, 10.0)	0.02 ± 0.26 (−0.7, 0.7)
SIR, ‰	1.6 ± 0.9 (−0.2, 4.2)	1.6 ± 0.9 (−0.7, 4.2)	0.03 ± 0.74 (−2.5, 1.9)

[1]Values are means ± SDs (ranges), $n = 147$. CIR, carbon isotope ratio; NIR, nitrogen isotope ratio; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SIR, sulfur isotope ratio.

(95%), with a mean age of 75 ± 4 y, and well educated, with 83% having a college degree or higher. Sixty percent of the participants were overweight [BMI (kg/m^2) ≥ 25] or obese (BMI ≥ 30). On average, diets were relatively low in carbohydrate (45%) and high in fat (38%). Sixty-four percent of dietary protein was from animal protein sources, including fish/seafood (17%), red meat (23%), poultry (21%), egg (6%), and dairy (30%) (Table 1). Added sugars comprised 49% of total sugar intake, yielding a median 48 g/d across participants. SSB intake among study participants was very low, 17 kcal/d, with 71% consuming ≤1 serving/wk (Table 1).

The mean, SD, and range of serum stable isotope ratios collected before the feeding period (baseline), after the feeding period, and their differences are presented in Table 2. Stable isotope means and distributions did not differ between baseline measurements and those taken after the feeding period. Serum CIR, NIR, and SIR measured after the feeding period were significantly associated with baseline (Pearson's $r = 0.83$, 0.81, 0.66, respectively, all $P < 0.0001$). Mean ± SD differences were all within 0.03‰, and SDs of differences were within 0.4‰ for CIR and NIR, and within 0.8‰ for differences in the SIR, as expected based on assay precision.

Table 3 presents the associations of serum CIR, NIR, and SIR with dietary intake. The NIR was associated with fish/seafood ($r = 0.61$, $P < 0.0001$) and egg intake ($r = 0.25$, $P < 0.005$); however, the association of the NIR with egg intake was attenuated and became nonsignificant when adjusted by fish intake. The CIR was moderately associated with intakes of red meat ($r = 0.39$, $P < 0.0001$), eggs ($r = 0.27$, $P < 0.005$), and dairy ($r = 0.27$, $P < 0.005$); however, the association of the CIR with dairy intake was attenuated and became nonsignificant when adjusted by either red meat or egg intake. The CIR was not associated with SSB, added sugar, or total sugar intake. The serum NIR and CIR were both associated with total animal protein intake ($r = 0.43$ and 0.41, respectively, $P < 0.0001$). The SIR was weakly associated with fish/seafood intake ($r = 0.20$), but the trend was not significant after Bonferroni correction.

Table 4 compares predictive models of dietary intake based on single isotopes, multiple isotopes, and multiple isotopes + participant characteristics. A predictive model for fish/seafood intake based on the NIR was slightly improved by including participant characteristics (race/ethnicity, $R^2 = 0.40$), but not additional isotopes. A predictive model for animal protein intake based on the NIR was significantly improved by including the CIR, BMI, and LPA (model $R^2 = 0.40$). A predictive model for red meat intake based on the CIR was slightly improved by including BMI, but not additional isotopes (model $R^2 = 0.18$). Similarly, a predictive model for egg intake was slightly improved by including BMI and LPA, but not additional isotopes (model $R^2 = 0.08$).

TABLE 3 Pearson correlations between dietary intake and serum stable isotope ratios of the postmenopausal women in the NPAAS-FS included in this study[1]

Dietary intake	NIR, ‰	CIR, ‰	SIR, ‰
Total animal protein, g/d	0.43***	0.41***	0.03
Fish/seafood, g/d	0.61***	0.11	0.20
Red meat, g/d	0.06	0.39***	−0.14
Poultry, g/d	−0.02	0.01	0.09
Eggs, g/d	0.25[2]**	0.27**	−0.06
Dairy, kcal/d	0.21	0.27[3]**	−0.03
Total sugar, g/d	−0.08	−0.08	−0.01
Added sugar, g/d	−0.14	0.02	0.10
SSBs, kcal/d	−0.11	0.01	−0.06
Corn products, g/d	−0.05	0.10	−0.12

[1]Values are Pearson correlation coefficients, $n = 147$. Significance level of 0.005 is used based on Bonferroni-Holm adjustment for 10 multiple comparisons. **$P_{corrected} < 0.005$, ***$P_{corrected} < 0.0001$. CIR, carbon isotope ratio; NIR, nitrogen isotope ratio; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SIR, sulfur isotope ratio; SSB, sugar-sweetened beverage.
[2]Association disappeared when adjusted for fish/seafood intake.
[3]Association disappeared when adjusted for either red meat or egg intake.

Discussion

This study examined dietary associations with stable isotope ratios in a controlled feeding study of 147 postmenopausal women enrolled in the WHI, and evaluated predictive models of dietary intake based on multiple stable isotopes and participant characteristics (race/ethnicity, BMI, and LPA). The study demonstrates that models of dietary intake based on stable isotope ratios and participant characteristics can meet the criterion for biomarker evaluation used by the NPAAS-FS ($R^2 \geq 0.36$) (24). The NIR met the biomarker criterion for fish/seafood intake ($R^2 = 0.38$, strengthening to $R^2 = 0.40$ with inclusion of participant characteristics). A predictive model of total animal protein intake based on the NIR, the CIR, and participant characteristics also met the biomarker criterion ($R^2 = 0.40$). In addition, the CIR was highly significantly associated with red meat intake and significantly associated with egg intake; however, these associations did not meet the criterion for biomarker evaluation used here. In contrast to previous studies, the CIR was not associated with intakes of total sugar, added sugar, or SSB; however, intakes of added sugar and SSB were low in this population. These predictive relations for fish/seafood and animal protein intake may be useful for further evaluation of diet-disease associations in the WHI and possibly other similar populations of postmenopausal women.

This study is the first to our knowledge to demonstrate an association of the NIR with fish/seafood intake in a US population that consumes low to moderate amounts of fish; however, these findings are consistent with the strong association previously reported for an Alaska Native cohort consuming moderate to high amounts of fish [~15% of energy (16, 18, 34, 35)]. Our findings are also consistent with the European Prospective Investigation into Cancer and Nutrition (EPIC)—Norfolk cohort study, which found a modest association of serum NIR with fish protein intake as reported

TABLE 4 Linear models of dietary intake of the postmenopausal women in the NPAAS-FS included in this study, based on single isotopes, multiple isotopes, and final models with participant characteristics[1]

Dietary intake	Model type	Model variables	ß ± SE	*P*	R^2
Fish/seafood	Single isotope	NIR	33.42 ± 3.56	<0.01	0.38
	Multiple isotope	NIR	33.10 ± 3.80	<0.01	0.38
		CIR	−1.45 ± 3.13	0.64	
		SIR	1.58 ± 1.83	0.39	
	Final model[2]	NIR	32.90 ± 3.52	<0.01	0.40
		Ethnicity (Causasian)	−8.74 ± 3.73	0.02	
Total animal protein	Single isotope	NIR	13.95 ± 2.46	<0.01	0.18
	Multiple isotope	NIR	11.31 ± 2.44	<0.01	0.30
		CIR	10.17 ± 2.11	<0.01	
		SIR	−0.55 ± 1.17	0.63	
	Final model[2]	NIR	11.76 ± 2.22	<0.01	0.40
		CIR	10.99 ± 1.98	<0.01	
		BMI	0.85 ± 0.25	<0.01	
		LPA	0.30 ± 0.07	<0.01	
Red meat	Single isotope	CIR	21.41 ± 4.21	<0.01	0.15
	Multiple isotope	CIR	21.16 ± 4.36	<0.01	0.16
		NIR	−0.54 ± 5.30	0.92	
		SIR	−3.74 ± 2.55	0.14	
	Final model[2]	CIR	21.05 ± 4.16	<0.01	0.18
		BMI	1.22 ± 0.55	0.03	
Egg	Single isotope	CIR	6.09 ± 2.36	<0.01	0.04
	Multiple isotope	CIR	5.75 ± 2.43	0.02	0.05
		NIR	2.18 ± 3.04	0.47	
		SIR	0.33 ± 1.44	0.82	
	Final model[2]	CIR	6.05 ± 2.33	0.01	0.08
		BMI	0.58 ± 0.31	0.07	
		LPA	0.17 ± 0.09	0.07	

[1] Values are model ß ± SE, *P*, and R^2, $n = 147$ (total animal protein and red meat), $n = 146$ (fish/seafood), $n = 145$ (egg) due to exclusion of model outliers (Mahalobnis distance > 4). CIR, carbon isotope ratio; LPA, leisure physical activity; NIR, nitrogen isotope ratio; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SIR, sulfur isotope ratio.

[2] Final models included isotope ratios and participant characteristics that remained significant on the basis of a backward-selection process ($P = 0.1$).

by FFQ (13), and a controlled feeding study in the United Kingdom in which the urine NIR was associated with both fish and meat intake (14). Importantly, the serum NIR was not associated with any other dietary variables independent of its association with fish/seafood intake in the NPAAS-FS, including red meat, demonstrating its specificity for fish/seafood intake in this cohort. Previous population studies have found the NIR to be associated with fish/seafood (13, 18) or red meat intake (12) but typically not both, depending on the diet pattern of the study population. Contrary to our hypothesis, the SIR was not informative about fish/seafood intake. A previous study of native Greenlanders (19) found the NIR and SIR to be strongly associated and related to fish and marine mammal intake. However, the results of our study suggest that the NIR may be the better measure for populations consuming lower amounts of fish/seafood.

The CIR was associated with red meat and egg intake, but surprisingly not poultry intake. In 1 US study, red meats had a higher CIR than poultry or eggs (29); however, a more recent study found the CIRs of these foods to be similar (18). The CIR was not associated with dairy intake independently of its associations with red meat and egg intake, despite dairy being the largest contributor to animal protein intake in the NPAAS-FS. Dairy has a lower CIR than other animal-based foods, presumably reflecting less use of corn for feed (18, 29). Predictive models of red meat and egg intake based on the CIR did not reach the criterion for biomarker evaluation; however, the CIR could still potentially provide useful information about red meat or egg intake, especially if used in combination with other biomarkers.

The CIR was not associated with sugars intake (total sugar, added sugar, or SSB), contrary to our predictions and the findings of several recent studies (5, 8, 10, 11, 22, 23). Added-sugar intake by NPAAS-FS participants was very similar to that of US women aged ≥60 y nationally, as measured by the NHANES between 2005 and 2010 (48 compared with 46 g/d) (36, 37). In contrast, SSB consumption by NPAAS-FS participants was low even in comparison with NHANES women aged ≥60 y (17 compared with 58 kcal/d) (38), likely contributing to the lack of an association with the CIR. Intake of corn products was also low in the NPAAS-FS (<2 g/d) and was not associated with the CIR.

Total animal protein intake was moderately associated with both the CIR and the NIR due to their associations with different animal protein sources, and a dual-isotope model of animal protein intake based on the CIR, the NIR, BMI, and LPA met the criterion of $R^2 \geq 0.36$. Biomarker-calibrated FFQ protein data from the WHI have shown that a 20% increase in protein intake was associated with a 32% lower risk of frailty in postmenopausal women, but did not identify specific protein sources (animal or vegetable) that were protective (39). An objective measure of animal protein intake could help to clarify the protective effects of specific dietary protein sources in the WHI.

Multi-isotopic models generally did not improve dietary predictions over single-isotope models, with the exception

of predicting animal protein intake. In an Alaska Native population, a model of sugars intake based on the CIR and NIR was a substantial improvement over a model based on the CIR alone, because the NIR was associated with dietary factors confounding the relation between the CIR and sugars (5). However, in a study population from southwest Virginia the NIR was not associated with animal protein intake, and did not improve models of sugar intake based on the CIR (23). Thus, the utility of multi-isotopic models compared with single-isotope models depends on the dietary patterns of the population to which they are applied. Inclusion of participant characteristics significantly improved the isotopic model of animal protein intake; however, improvements to other isotopic models of intake by the inclusion of participant characteristics were modest.

Strengths of this study include the novel design of the NPAAS-FS, which allowed the dietary control needed for biomarker evaluation while maintaining biomarker baseline values and preserving the dietary intakes typical of the population. The NIR and CIR also have unique strengths as practical biomarkers of fish/seafood and animal protein intake (4). The CIR and NIR are both measured during a single, high-throughput analysis that requires extremely small sample volumes (<5 μL) (4). They are robust to freeze-thaw cycles (4), and can be measured in multiple sample types (4). They can be easily applied to stored specimens, enabling the retrospective collection of high-quality biomarker data that can be used to answer questions about dietary trends through time (40).

This study also has limitations. Limited heterogeneity in diet preference, race/ethnicity, BMI, and LPA reduces generalization to a broader population of postmenopausal women. The incompleteness of the dietary database for certain foods may have affected the reliability of estimated consumption; furthermore, exact ingredients and compositions are proprietary for many foods and thus are not available. Some of the foods thought to be associated with stable isotope biomarkers were consumed in low amounts, specifically SSB and added sugars. Finally, turnover of the carbon and nitrogen reservoirs contributing to the serum CIR and NIR would likely be incomplete over the period of dietary control, which may have attenuated diet-biomarker associations. Thus, where the provided diet did not perfectly match usual intake, a longer period of dietary control might have improved dietary associations with stable isotope ratios. However, the fact that biomarker measurements were stable over the period of controlled feeding suggests that the study generally succeeded in matching diets to usual intake, as does the strength of the association of the serum NIR with fish/seafood intake, which was similar to well-established recovery biomarkers reflecting short-term intake.

The goal of the NPAAS-FS was to explore and develop new biomarker calibration equations for the larger WHI population (24, 25). The criterion of $R^2 \geq 0.36$ used by the NPAAS-FS was based on its power to detect relations having a true $R^2 \geq 0.50$, and was consistent with the performance of well-characterized biomarkers for total energy and protein intakes in that study, doubly labeled water and urinary nitrogen (24). A more liberal criterion might be warranted to further explore potential long-term intake biomarkers identified in this study, under more ideal conditions. The essential requirement for a useful intake biomarker is adherence to a classical measurement model whereby the biomarker equals the targeted dietary variable plus random error that is independent of other exposures relevant to the clinical outcome (41). For this model to hold, the biomarker needs to be responsive to all sources of the targeted dietary variable, and to be unresponsive to other dietary intakes. Within these requirements, specific criteria for association strength are still being discussed in the biomarker/nutritional epidemiology literature.

In summary, our study found that natural abundance stable isotope ratios, in addition to participant characteristics, met biomarker criteria for dietary intakes of fish and animal protein in this sample of postmenopausal women. The strength of biomarker-diet associations was similar to those of well-characterized recovery biomarkers in the same study sample. Dietary associations with stable isotope ratios were consistent with evidence from observational studies in both the United States and Europe, and 1 controlled dietary study in Europe. Biomarker-diet associations were improved modestly, but still usefully, by the inclusion of participant characteristics. Our study approach represents an important methodologic contribution toward the utilization of stable isotope biomarkers to assess dietary intake. Studies in more diverse US cohorts, including longer-term dietary interventions, are needed.

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