

# Red blood cell $\delta^{15}\text{N}$ : a novel biomarker of dietary eicosapentaenoic acid and docosahexaenoic acid intake<sup>1-4</sup>

Diane M O'Brien, Alan R Kristal, M Alyssa Jeannet, Michael J Wilkinson, Andrea Bersamin, and Bret Luick

## ABSTRACT

**Background:** The long-chain omega-3 (n-3) fatty acids derived from fish, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are associated with a reduced risk of cardiovascular disease and other chronic diseases. Study of the associations between EPA and DHA intake and disease requires a valid biomarker of dietary intake; however, the direct measurement of tissue fatty acid concentrations is expensive and time consuming.

**Objective:** Because the nitrogen stable isotope ratio ( $^{15}\text{N}/^{14}\text{N}$ , expressed as  $\delta^{15}\text{N}$ ) is elevated in fish, we investigated whether  $\delta^{15}\text{N}$  is a valid alternative biomarker of EPA and DHA intake.

**Design:** We examined the relation between red blood cell (RBC)  $\delta^{15}\text{N}$  and RBC EPA and DHA in a community-based sample of 496 Yup'ik Eskimos with widely varying intake of n-3 fatty acids. We also assessed the correlation between  $\delta^{15}\text{N}$  and dietary EPA and DHA intake based on 24-h dietary recalls and 3-d food records completed by a subset of 221 participants.

**Results:** RBC  $\delta^{15}\text{N}$  was strongly correlated with RBC EPA and DHA ( $r = 0.83$  and  $0.75$ , respectively). These correlations differed only modestly by sex and age class. RBC  $\delta^{15}\text{N}$  also correlated with dietary EPA and DHA intake ( $r = 0.47$  and  $0.46$ , respectively) and did not differ by sex and age.

**Conclusions:** The results strongly support the validity of RBC  $\delta^{15}\text{N}$  as a biomarker of EPA and DHA intake. Because the analysis of RBC  $\delta^{15}\text{N}$  is rapid and inexpensive, this method could facilitate wide-scale assessment of EPA and DHA intake in clinical and epidemiologic studies. *Am J Clin Nutr* 2009;89:913-9.

## INTRODUCTION

The omega-3 (n-3) fatty acids derived from fish, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are associated with a reduced risk of cardiovascular disease and other chronic diseases (1-3). EPA and DHA promote an antiinflammatory state (4) and regulate the expression of genes involved in fatty acid metabolism (5-7). However, our ability to detect associations between EPA and DHA intake, gene variants, and disease is limited by the validity and feasibility of dietary assessment. Both plasma and red blood cell (RBC) fatty acid composition are valid biomarkers of EPA and DHA intake (8-13), but their measurement requires technically challenging, expensive, and time-consuming assays that are impractical in large-scale studies. Simpler and less expensive biomarkers of EPA and DHA intake are clearly needed.

Ratios of naturally occurring stable isotopes have recently gained attention for their potential as accurate, inexpensive dietary biomarkers in nutritional studies (14-18). This approach is useful for foods that are enriched with the heavier isotopes of carbon, nitrogen, oxygen, or hydrogen (14, 18-23). For example, fish has a uniquely high  $^{15}\text{N}/^{14}\text{N}$  (expressed as  $\delta^{15}\text{N}$  as defined in Subjects and Methods), for 2 reasons: 1) marine environments tend to be enriched in  $^{15}\text{N}$  relative to terrestrial environments, particularly those that are fertilized, and 2) fish are typically predatory and  $\delta^{15}\text{N}$  reflects the length of an animal's food chain (24). These isotopic differences in diet are passed on to the tissues with only minor, predictable changes (25-27), and anthropologists have long used  $\delta^{15}\text{N}$  as a marker of the consumption of marine foods in human populations (28-31). Recently,  $\delta^{15}\text{N}$  was shown to be highly elevated in a Greenland Inuit population with a very high dietary intake of marine foods (17). Because fish is also the predominant source of EPA and DHA in human diets, we hypothesized that there would be a strong relation between these n-3 fatty acids and  $\delta^{15}\text{N}$  in human tissues, driven by differences in the dietary intake of fish. If true,  $\delta^{15}\text{N}$  could serve as an alternative biomarker of EPA and DHA intake that is accurate, relatively inexpensive, and highly robust.

We examined the relation between RBC EPA and DHA and RBC  $\delta^{15}\text{N}$  in a community-based sample of 496 Yup'ik Eskimos (32). This population is ideal for testing this relation because they have widely varying fish intake, depending on the degree to which they adhere to a traditional, marine-based diet (33, 34).

<sup>1</sup> From the Center for Alaska Native Health Research, Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK (DMO, MAJ, MJW, AB, and BL), and the Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA (ARK).

<sup>2</sup> The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Research Resources, the National Institutes of Health, or the National Science Foundation.

<sup>3</sup> Supported by a Centers of Biomedical Research Excellence grant from the NIH NCRR (P20 RR16430) and undergraduate research awards to MJW and MAJ from Alaska EPSCOR (NSF 0346770), Alaska INBRE (NIH NCRR RR016466), and the UAF Center for Research Services.

<sup>4</sup> Address reprint requests and correspondence to DM O'Brien, Institute of Arctic Biology, PO Box 757000, University of Alaska Fairbanks, Fairbanks, AK 99775-7000. E-mail: ffd@uaf.edu

Received October 2, 2008. Accepted for publication December 16, 2008. First published online January 28, 2009; doi: 10.3945/ajcn.2008.27054.

We also investigate the relations between  $\delta^{15}\text{N}$  and dietary intake of EPA and DHA and RBC EPA and DHA in a subset of 221 participants.

## SUBJECTS AND METHODS

### Participant recruitment and procedures

Data are from the Center for Alaska Native Health Research I (CANHR I) Study, a cross-sectional, community-based participatory research study of biological, genetic, nutritional, and psychosocial risk factors for obesity and related disease in Yup'ik Eskimos. The CANHR study was approved by the University of Alaska Institutional Review Board, the National and Area Indian Health Service Institutional Review Board, and the Yukon-Kuskokwim Health Corporation Human Subjects Committee. Between 2003 and 2005, 1003 men and women aged  $\geq 14$  y were recruited from 10 communities in southwest Alaska, as described in detail elsewhere (32, 35). At entry into the study, the participants completed extensive interviewer-administered interviews covering demographic characteristics, economic status, ethnicity, and medical history. All participants completed an interviewer-administered 24-h dietary recall and were requested, but not required, to complete an additional 3-d food record (3DFR). Blood was collected into EDTA-containing tubes and processed locally; serum, lymphocytes, and the remaining RBC clot were separated into aliquots and stored at  $-20^\circ\text{C}$ . Within 6 d, samples were shipped to the University of Alaska Fairbanks and stored at  $-80^\circ\text{C}$ . Aliquots of RBCs were removed for fatty acid and stable isotope analyses, as described below.

### Study sample

Analyses of RBC fatty acids were based on a subset of 496 of the 1003 CANHR participants, who were selected after recruitment was completed from 7 of the 10 participating communities, with an effort to balance the sample across age and community. Three communities were very small; therefore, all participants were selected ( $n = 164$ ). For the remaining communities, we defined 3 age groups (14–19, 20–49, and  $\geq 50$  y) and selected a random sample from each to obtain  $\approx 28$  from each age stratum. If 28 participants were not available, we selected all participants in that stratum and adjusted the selection in the remaining age strata to yield  $\approx 84$  per community. Of the 496 participants selected, 221 had completed both a 24-h dietary recall and a 3DFR, and this subsample was used in analyses of dietary intake.

### Stable isotope analyses

RBC aliquots were autoclaved for 20 min at  $121^\circ\text{C}$  to destroy blood-borne pathogens, and the samples were weighed into tin capsules ( $3.5 \times 3.75$  mm) and freeze-dried to a final mass of 0.2–0.4 mg. Neither autoclaving nor the use of EDTA-treated tubes affects RBC nitrogen isotope ratios (22). Samples were analyzed at the Alaska Stable Isotope Facility by continuous-flow isotope ratio mass spectrometry with a Costech ECS4010 Elemental Analyzer (Costech Scientific Inc, Valencia, CA) interfaced to a Finnigan Delta Plus XP isotope ratio mass spectrometer via the Conflo III interface (Thermo-Finnigan Inc, Bremen, Germany). Data are presented in standard delta values as  $\delta X = (R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}} \cdot 1000\text{‰}$ , where R is the ratio of heavy to

light isotope ( $^{15}\text{N}/^{14}\text{N}$ ) and the standard is atmospheric nitrogen.  $\delta^{15}\text{N}$  values are hereafter abbreviated as  $\delta^{15}\text{N}$ . We concurrently prepared and ran multiple peptone standards ( $\delta^{15}\text{N} = 7.00$ ) to assess analytic accuracy and precision, which were analyzed after every fifth sample and gave values of  $\delta^{15}\text{N} = 7.01 \pm 0.22\text{‰}$  (SD).

### RBC fatty acid measurements

The RBC fatty acids were analyzed at the Fred Hutchinson Cancer Research Center in Seattle, WA. Fatty acids were extracted from washed RBCs in a total lipid fraction with a combination of organic solvents. Briefly, 250  $\mu\text{L}$  RBCs were mixed with an equivalent volume of distilled water, and lipids were extracted with 2-propanol and chloroform according to Rose and Oklander (36); 5 mg BHT/100 mL of 2-propanol was added as an antioxidant. The lipid extract was transesterified in 5 mL acetyl chloride reagent and processed according to Lepage and Roy (37). After transesterification, fatty acid methyl esters were recovered in hexane, dried under nitrogen ( $40^\circ\text{C}$ ), and redissolved in 100  $\mu\text{L}$  hexane for gas chromatography.

Fatty acid methyl esters were injected in a split mode (1:50) and were separated by using an SP-2560 (Supelco, Bellefonte, PA) capillary column ( $100 \text{ m} \times 0.25 \text{ mm} \times 0.2 \mu\text{m}$ ) on a Hewlett-Packard (model 5890B) gas chromatograph (GC) (now Agilent, Santa Clara, CA). The GC system was equipped with a flame ionization detector, electronic pressure control, Chemstation software (Hewlett-Packard), and automatic sampler (HP7673). This method allows the resolution of 46 different membrane fatty acids. The accuracy of the GC system was monitored by using commercial standards (GLC-87, NIH-D, and NIH-F; Nu-Chek, Elysian, MN). The precision of the RBC fatty acid measurements was monitored with repeat analysis of an in-house RBC quality-control pool that was included in each batch of 23 study samples. The CV for EPA was 2.7% and for DHA was 2%. Fatty acid composition is reported as the percentage by weight of total RBC fatty acids.

### Dietary assessment

Diet was assessed with an interviewer-administered 24-h dietary recall and a 3DFR. Data from these instruments were combined to achieve a stable estimate of dietary nutrient intake. Data from the 24-h dietary recall were collected from each participant by certified interviewers using a computer-assisted recall [Nutrition Data System for Research (NDS-R) software version 4.06; University of Minnesota, Minneapolis, MN]. Participants were asked to recall all food and beverages consumed over a 24-h period using a multiple pass approach. Although most of the participants were bilingual, a native Yup'ik speaker who was trained in the use of NDS-R software assisted the non-English speakers.

Because of an already high participant burden, the 3DFR was not mandatory, although it was offered to all participants. Participants were instructed to maintain their usual eating habits. A research team member reviewed all 3DFRs for completeness, which were then entered into the NDS-R software package by certified coders. A second researcher reviewed all entries for accuracy.

Nutrient calculations for both the 24-h dietary recall and 3DFR were performed by using the NDS-R Food and Nutrient Database



33, released July 2003. A few Alaska Native foods were missing from the database; these were either substituted for similar food items when appropriate or the food was added to the database. Here we only examined data on dietary intake of EPA and DHA.

### Statistical analyses

All statistical analyses were performed by using JMP IN software (version 5.1.2; SAS Institute, Cary, NC). We used a chi-square test to evaluate differences in the sex, age, and BMI distribution between the complete sample of participants and the subset of participants with dietary intake data. We assessed mean differences in biomarkers between sex, age, and data sets using 2-tailed *t* tests. Associations of  $\delta^{15}\text{N}$  with RBC EPA and DHA were assessed by using both Pearson's product moment and Spearman rank correlation coefficients; however, Spearman rank correlation coefficients were only reported when these differed. We described the relation between RBC  $\delta^{15}\text{N}$  and EPA and DHA using linear regression or nonlinear fitting, depending on the shape of the relation. Exponential relations were described by nonlinear fitting and parameter estimation by using the general model  $y = a(1 - e^{-rx})$ . This procedure generates a best-fit model value for each *y*, and  $R^2$  is calculated as  $1 - (\text{RSS}/\text{TSS})$ , where RSS and TSS are the residual sum of squares and the total sum of squares, respectively. For parametric analyses of both linear and nonlinear relations, the normality of residuals was tested by using the Shapiro-Wilks test. We used 2-tailed *t* tests to determine whether mean EPA, DHA, and  $\delta^{15}\text{N}$  differed between males and females. We used multiple regression analysis to test the effects of age and sex on the associations between fatty acids and  $\delta^{15}\text{N}$ , where associations were linear and met the assumptions for parametric statistical tests. For regression analysis, age was treated as a dichotomous variable (<40 y and  $\geq 40$  y). Dietary intake data were log transformed— $\ln(1 + \text{daily FA intake})$ —and averaged across all days of intake. We tested whether the correlation between RBC FA and dietary FA intake differed from that between RBC  $\delta^{15}\text{N}$  and dietary FA intake according to Wolfe (38).

### RESULTS

The age, sex, and BMI distribution of the study participants are shown in **Table 1**. Of the 496 participants selected for this study, 58% of the participants were female and 42% were male. Fe-

**TABLE 1**

Age, sex, and body weight distribution for the complete study sample ( $n = 496$ ) and the subset of participants with dietary intake data ( $n = 221$ )

	Complete sample ( $n = 496$ )	Subset ( $n = 221$ )
Age (y)	$39.7 \pm 18.1^1$	$35.1 \pm 16.1^2$
14–24 y (%)	29	36
25–39 y (%)	22	24
40–54 y (%)	25	26
$\geq 55$ y (%)	24	14
Male (%)	42	44
Female (%)	58	56
Normal weight (%)	37	38
Overweight (%)	31	35
Obese (%)	32	27

<sup>1</sup> Mean  $\pm$  SD (all such values).

<sup>2</sup> Significantly different from the complete sample,  $P < 0.05$ .

male participants ranged in age from 14 to 94 y and had a mean age of 39 y. Male participants ranged in age from 14 to 83 y and had a mean age of 41 y (Table 1). Forty-three percent of females and 47% of males completed a 3DFR, and the proportion of males and females did not differ between the complete sample and the diet data subset. Younger participants (age: 14–24 y) were more likely to elect to complete a 3DFR and were over-represented in the dietary intake sample, whereas elder participants (age:  $\geq 55$  y) were underrepresented ( $\chi^2 = 12.6$ ,  $P < 0.01$ ) (32). The distribution of BMI was 37% for normal-weight, 31% for overweight, and 31% for obese participants and did not differ significantly in the subset of participants with dietary intake data.

The means and distribution characteristics for  $\delta^{15}\text{N}$ , EPA, and DHA in RBCs are shown in **Table 2**. In the complete sample of 496 participants, the mean  $\delta^{15}\text{N}$  and EPA did not differ between sexes, and the mean DHA was 14% higher in females ( $P < 0.0001$ ). In the subset of 221 participants with dietary intake data, means of all 3 markers were similar but significantly lower than in the complete sample (Table 2).

### Associations between RBC $\delta^{15}\text{N}$ and RBC EPA and DHA

RBC  $\delta^{15}\text{N}$  was strongly correlated with the percentages of EPA ( $r = 0.84$ ) and DHA ( $r = 0.75$ ) in RBC membranes (**Table 3**,

**TABLE 2**

Distribution of biomarker variables for the complete study sample ( $n = 496$ ) and the subset of participants with dietary intake data ( $n = 221$ )<sup>1</sup>

	Total	Male	Female
Complete sample			
No. of subjects	496	208	288
$\delta^{15}\text{N}$			
Mean $\pm$ SD (‰)	$8.8 \pm 1.4$	$8.7 \pm 1.5$	$8.9 \pm 1.4$
Range (‰)	6.4–13.8	6.4–13.5	6.4–13.8
CV (%)	16.2	16.7	15.7
EPA (%)			
Mean $\pm$ SD	$2.4 \pm 1.8$	$2.3 \pm 1.9$	$2.6 \pm 1.7$
Range	0.2–9.7	0.2–9.7	0.2–7.8
CV	72.4	82.7	65.3
DHA (%)			
Mean $\pm$ SD	$6.4 \pm 1.9$	$5.9 \pm 1.9$	$6.8 \pm 1.8^2$
Range	1.6–10.3	1.6–9.4	1.6–10.3
CV	29.2	31.8	26.4
Subset <sup>3</sup>			
No. of subjects	221	97	124
$\delta^{15}\text{N}$			
Mean $\pm$ SD (‰)	$8.5 \pm 1.2$	$8.4 \pm 1.1$	$8.7 \pm 1.2$
Range (‰)	6.4–12.1	6.4–11.6	6.7–12.1
CV (%)	13.9	13.6	14.1
EPA (%)			
Mean $\pm$ SD	$2.0 \pm 1.5$	$1.8 \pm 1.4$	$2.1 \pm 1.6$
Range	0.2–6.0	0.2–6.0	0.2–6.0
CV	74.3	73.5	74.3
DHA (%)			
Mean $\pm$ SD	$6.0 \pm 1.9$	$5.6 \pm 1.8^4$	$6.3 \pm 1.9^4$
Range	1.6–9.5	1.6–9.2	2.1–9.5
CV	30.9	31.7	29.4

<sup>1</sup> EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

<sup>2,4</sup> Significantly different from males: <sup>2</sup> $P < 0.0001$ , <sup>4</sup> $P = 0.0051$ .

<sup>3</sup> All 3 mean biomarker values were slightly but significantly lower in the subset,  $P < 0.006$  (*t* test).

**TABLE 3**

Pearson correlations between red blood cell (RBC)  $\delta^{15}\text{N}$  and RBC fatty acids for the complete sample of participants ( $n = 496$ ) and the subset of participants with dietary intake data ( $n = 221$ )<sup>1</sup>

	Sex			Age	
	Total	Male	Female	<40 y	≥40 y
Complete sample ( $n = 496$ )					
$\delta^{15}\text{N}$ vs EPA	0.84	0.89	0.79	0.70	0.82
$\delta^{15}\text{N}$ vs DHA <sup>2</sup>	0.75	0.79	0.72	0.69	0.69
Subset ( $n = 221$ )					
$\delta^{15}\text{N}$ vs EPA	0.80	0.83	0.79	0.66	0.82
$\delta^{15}\text{N}$ vs DHA <sup>3</sup>	0.76	0.80	0.72	0.68	0.74

<sup>1</sup> EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.  $P < 0.0001$  for all coefficients.

<sup>2</sup> Spearman rank ( $\rho_s$ ) correlations were 0.82, 0.85, and 0.80 for the total, male, and female groups, respectively, and 0.70 and 0.74 for the age groups <40 and ≥40 y, respectively.

<sup>3</sup> Spearman rank ( $\rho_s$ ) correlations were 0.79, 0.82, and 0.77 for the total, male, and female groups, respectively, and 0.68 and 0.79 for the age groups <40 and ≥40 y, respectively.

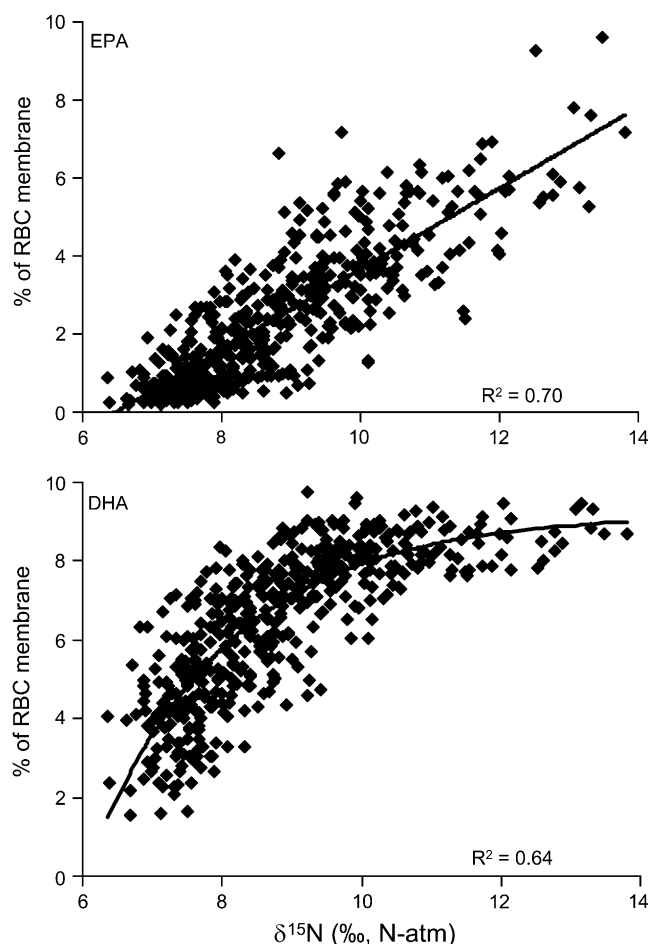
**Figure 1).** For EPA, the relation was positive and linear across the range of  $\delta^{15}\text{N}$  (Figure 1A;  $R^2 = 0.70$ ). The relation between RBC  $\delta^{15}\text{N}$  and DHA was exponential and approached an asymptote at DHA = 9.2% of RBC membrane fatty acids (upper and lower 95% CIs: 8.8, 9.6; Figure 1B). The predicted best-fit values of the exponential model are shown as a line in Figure 1B ( $R^2 = 0.64$ ). These associations were similarly strong when analyzed within the subset of participants for whom we also have dietary intake data (Table 3). Because the relation was nonlinear, the correlation between  $\delta^{15}\text{N}$  and DHA improved to  $\rho = 0.82$  when assessed by using Spearman rank correlation (Table 3). The association of  $\delta^{15}\text{N}$  with EPA differed significantly by sex (slope = 1.15 for males and 0.95 for females;  $P_{\text{interaction}} = 0.0014$ ) and age (slope = 0.97 for those aged <40 y and 0.82 for those aged > 40 y;  $P_{\text{interaction}} < 0.04$ ); the 3-factor interaction ( $\delta^{15}\text{N} \times \text{sex} \times \text{age}$ ) was not significant. Because of these differences, correlation coefficients are presented both for the entire sample and stratified by sex and age category (Table 3).

### Associations between RBC biomarkers and dietary intake

Dietary intake of EPA, calculated from a combined 24-h dietary recall and 3DFR, was strongly and significantly correlated with both RBC  $\delta^{15}\text{N}$  ( $r = 0.47$ ) and RBC EPA ( $r = 0.64$ ); dietary intake of DHA was also strongly and significantly correlated with both RBC  $\delta^{15}\text{N}$  ( $r = 0.46$ ) and RBC DHA ( $r = 0.54$ ) (all  $P < 0.001$ ). The correlations of dietary intake of both DHA and EPA with  $\delta^{15}\text{N}$  were significantly weaker than those of dietary intake of both DHA and EPA and each RBC FA ( $P < 0.0001$ ). The association between  $\delta^{15}\text{N}$  and dietary intake of EPA and DHA did not differ by sex or age.

### DISCUSSION

In this population, RBC  $\delta^{15}\text{N}$  correlated very strongly ( $r \approx 0.8$ ) with RBC polyunsaturated fatty acids EPA and DHA, which are well-established and validated biomarkers of EPA and DHA intake (9–13). These relations differed little by sex and age. Dietary EPA and DHA intake, as measured by a combination of 24-h dietary



**FIGURE 1.** Relation between red blood cell (RBC)  $\delta^{15}\text{N}$  and RBC eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) concentrations ( $n = 496$ ). The relation between  $\delta^{15}\text{N}$  and EPA was linear and highly significant:  $\text{EPA} = 1.04 (\delta^{15}\text{N}) - 6.7$  ( $R^2 = 0.70$ ,  $P < 0.0001$ ). The relation between  $\delta^{15}\text{N}$  and DHA was nonlinear and fitted as  $y = a(1 - e^{-rx})$  ( $R^2 = 0.64$ ). Nitrogen isotope ratios are presented in delta notation relative to international standards:  $[\delta^{15}\text{N} = (R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 1000\text{‰}$ , where R is the ratio of heavy to light isotope, and the standard is atmospheric nitrogen (N-atm).

recall and 3DFR, were also strongly correlated with RBC  $\delta^{15}\text{N}$ ; however, the correlations between biomarkers were stronger.

The correlation between dietary fatty acids and RBC fatty acids was slightly but significantly stronger than the correlation between dietary fatty acids and RBC  $\delta^{15}\text{N}$ . The difference in correlation strength may be driven by the greater CV of RBC fatty acids (72% and 29% for EPA and DHA, respectively) than of RBC  $\delta^{15}\text{N}$  (19%). Alternatively, RBC fatty acids may better capture recent diet than RBC  $\delta^{15}\text{N}$ , thus more closely matching the diet records (collected within 1–2 wk of the blood sample). Whereas turnover of RBC nitrogen matches that of the cells, which live  $\approx 120$  d, plasma fatty acids can be incorporated into RBC membranes in a shorter time frame (39). EPA, which is preferentially distributed in the outer leaflet of the cell membrane, turns over more rapidly than DHA, which is distributed in the inner leaflet (40, 41). Thus, EPA, DHA, and  $\delta^{15}\text{N}$  in RBC may provide dietary information over different time frames. It is important to note that, although both  $\delta^{15}\text{N}$  and fatty acids were measured in RBCs, they are independent markers reflecting different cellular components. Thus, the strong correlation

between these markers can only derive from their having the same dietary sources.

The relation between  $\delta^{15}\text{N}$  and RBC DHA was nonlinear; RBC DHA composition leveled off at  $\approx 9\%$  of total membrane fatty acids. Other authors have documented this effect (9, 42, 43) and have suggested that either that EPA displaces DHA or DHA is converted to EPA at high dietary intake. Our data set allows the form of the relation and the value of the asymptote to be clearly established because dietary intake is so high.

From a practical standpoint, measurement of  $\delta^{15}\text{N}$  has many advantages over measurement of EPA and DHA in RBC membranes. Analysis of  $\delta^{15}\text{N}$  is inexpensive, has a high throughput, is highly accurate, and requires no specialized sample handling. It continues to increase linearly as dietary intake increases and does not approach detection limits at either its highest or lowest values. Thus,  $\delta^{15}\text{N}$  may be a useful tool for dietary assessment that provides the advantages of a biomarker (measurement accuracy, lack of bias, and low participant burden) and that is feasible for use in large-scale studies.

Accurate information on polyunsaturated fatty acid intake is of great importance in studies of diet and health generally, but particularly in Alaska Natives and other indigenous Arctic populations. Similarly to many other populations that have undergone a shift toward a Westernized diet, Alaska Natives are experiencing a rapid increase in the rates of diabetes and other chronic diseases (44–46). Many researchers have suspected that a diet high in polyunsaturated fatty acids, characteristic of the indigenous diet, may protect against chronic disease in these and other Arctic populations (47–51). The ability to accurately measure EPA and DHA intake without reliance on self-reporting is critical to understanding the rising rates of obesity and chronic disease in the Yup'ik people and to developing effective interventions for this population.

Many of the Yup'ik participants in the CANHR study consume a large fraction of their total energy from fish, which is a major source of EPA and DHA in their diet (33, 34). An obvious question for further study is whether  $\delta^{15}\text{N}$  will predict EPA and DHA intake in populations with lower levels of fish consumption. Although  $\delta^{15}\text{N}$  is widely used as a marker of marine inputs into ancient human diets (28–31) and modern ecosystem studies (52, 53), relatively little is known about the relation between  $\delta^{15}\text{N}$  and fish intake in modern human populations. Hair  $\delta^{15}\text{N}$  was positively correlated ( $r = 0.39$ ) with the reported frequency of eating fish in 110 community-dwelling older people (mean age: 74 y) in Oxford, United Kingdom (16). This relation is suggestive, but not definitive, and further study is required to better understand the broader utility of  $\delta^{15}\text{N}$  as a marker of fish, EPA, and DHA intake.

Hair  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  have also been proposed as markers for dietary intake of animal protein (15).  $\delta^{15}\text{N}$  is typically  $\approx 3\%$  higher in animals relative to their diets; thus, vegetarians can be identified by low hair  $\delta^{15}\text{N}$  (18, 54, 55). Because corn is enriched in  $^{13}\text{C}$  relative to nearly all other food plants, livestock that are corn-fed also have high  $\delta^{13}\text{C}$  values.  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were positively correlated with each other and with animal protein intake among German participants in the Nutrition Survey and Risk Factor Analysis (Verbundstudie Ernährungserhebung und Risikofaktoren-Analytik) study (15). In our study, however, there was no relation between either  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  and animal protein intake, and  $\delta^{13}\text{C}$  was negatively cor-

related with  $\delta^{15}\text{N}$ , EPA, and DHA ( $r = -0.16, -0.31, -0.36$ , respectively; all  $P < 0.0004$ ). These negative relations reflect a tradeoff between consumption of traditional subsistence (high  $\delta^{15}\text{N}$ ) and market (high  $\delta^{13}\text{C}$ ) foods, where market foods are largely corn-based (34). In contrast, fingernail  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were strongly correlated in Greenland Inuit, because available market foods were not corn-based, and marine subsistence foods had high values of both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  compared with the rest of the diet (17). These conflicting results indicate how different complements of foods can drive different patterns of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  and why caution is required when applying isotopic biomarkers to nutritional studies.

This study had several limitations. It was not based on a representative random sample of the population, and the population in which it has been tested is fairly unique. Thus, whether the relations can be generalized to the rest of the US population remains to be investigated. Physiologic influences on  $\delta^{15}\text{N}$  are not fully understood in humans, although nitrogen status and severe liver damage both are known to have effects (56–58). The measure of dietary intake of EPA and DHA was based on self-report and is subject to errors in recall and potential biases associated with age, sex, and other individual characteristics; therefore, the magnitude of the observed associations between dietary intake and biomarkers were underestimated. This study also had important and unique strengths. It was based on a large sample of participants with a wide variability in dietary EPA and DHA intake, which made the sample ideal for testing the performance of alternative biomarkers of EPA and DHA. It was also the first study to compare the performance of natural abundance stable isotope values against validated nutritional biomarkers in a human population.

In summary, we found that RBC  $\delta^{15}\text{N}$  is highly correlated with RBC EPA and DHA and that both isotopic and fatty acid biomarkers show similar correlations with dietary intake, ranging from 0.46 to 0.65. Thus, we propose that the RBC nitrogen stable isotope ratio provides an accurate and inexpensive biomarker of dietary EPA and DHA intake that could make assessment feasible in large-scale studies. Because accurate assessment of individual dietary intake of these fatty acids is of particular interest in Yup'ik Eskimos, we recommend that future studies include measurement of RBC  $\delta^{15}\text{N}$  as a proxy for RBC membrane EPA and DHA. We also suggest that  $\delta^{15}\text{N}$  is likely an effective and accurate biomarker of EPA and DHA intake in other populations with various levels of fish consumption.

We gratefully acknowledge our participants in the Y-K Delta and the CANHR field teams, especially Scarlett Hopkins. We thank Tim Howe and Norma Haubenstock at the Alaska Stable Isotope Facility for assistance with the stable isotope analyses and Irena King and her staff at the Fred Hutchinson Cancer Research Center for the fatty acid analyses. This manuscript was improved by comments from Bruce Fowler, Sarah Nash, Kate West, and Jordan Metzgar, and we especially thank Mary Sexton for her thoughtful reviews and input.

The authors' responsibilities were as follows—DMO: designed the study, analyzed the data, and wrote the manuscript; MJW and MAJ: conducted the isotope analyses; BL and AB: collected and compiled the nutritional data; and ARK: analyzed and interpreted the data and wrote the manuscript. All authors contributed to the final draft of the manuscript. None of the authors had any conflicts of interest.

## REFERENCES

1. Breslow JL. n-3 Fatty acids and cardiovascular disease. *Am J Clin Nutr* 2006;83(suppl):1477S–82S.

2. Wang C, Harris WS, Chung M, et al. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *Am J Clin Nutr* 2006;84:5-17.
3. Carpentier YA, Portois L, Malaisse WJ. n-3 Fatty acids and the metabolic syndrome. *Am J Clin Nutr* 2006;83(suppl):1499S-504S.
4. Calder PC. n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* 2006;83(suppl):1505S-19S.
5. Clarke SD. Polyunsaturated fatty acid regulation of gene transcription: A molecular mechanism to improve the metabolic syndrome. *J Nutr* 2001;131:1129-32.
6. Davidson MH. Mechanisms for the hypotriglyceridemic effect of marine omega-3 fatty acids. *Am J Cardiol* 2006;98:271-331.
7. Wahle KWJ, Rotondo D, Heys SD. Polyunsaturated fatty acids and gene expression in mammalian systems. *Proc Nutr Soc* 2003;62:349-60.
8. Arab L. Biomarkers of fat and fatty acid intake. *J Nutr* 2003;133:925S-32S.
9. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* 2006;83(suppl):1467S-76S.
10. Katan MB, Deslypere JP, van Birgelen AP, Penders M, Zegwaard M. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. *J Lipid Res* 1997;38:2012-22.
11. Sands SA, Reid KJ, Windsor SL, Harris WS. The impact of age, body mass index, and fish intake on the EPA and DHA content of human erythrocytes. *Lipids* 2005;40:343-7.
12. Sun Q, Ma J, Campos H, Hankinson SE, Hu FB. Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. *Am J Clin Nutr* 2007;86:74-81.
13. Vidgren HM, Agren JJ, Schwab U, Rissanen T, Hanninen O, Uusitupa MI. Incorporation of n-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation with fish, fish oil, and docosahexaenoic acid-rich oil among healthy young men. *Lipids* 1997;32:697-705.
14. Jähren AH, Saudek C, Yeung E, Kao W, Kraft R, Caballero B. An isotopic method for quantifying sweeteners derived from corn and sugar cane. *Am J Clin Nutr* 2006;84:1380-4.
15. Petzke KJ, Boeing H, Klaus S, Metges CC. Carbon and nitrogen stable isotopic composition of hair protein and amino acids can be used as biomarkers for animal-derived dietary protein intake in humans. *J Nutr* 2005;135:1515-20.
16. Williams JH, O'Connell TC. Differential relations between cognition and <sup>15</sup>N isotopic content of hair in elderly people with dementia and controls. *J Gerontol A Biol Sci Med Sci* 2002;57:M797-802.
17. Buchardt B, Bunch V, Helin P. Fingernails and diet: stable isotope signatures of a marine hunting community from modern Uummannaq, North Greenland. *Chem Geol* 2007;244:316-29.
18. Nardoto GB, Silva S, Kendall C, et al. Geographical patterns of human diet derived from stable-isotope analysis of fingernails. *Am J Phys Anthropol* 2006;131:137-46.
19. Kelly S, Heaton K, Hoogewerf J. Tracing the geographical origin of food: the application of multi-element and multi-isotope analysis. *Trends Food Sci Technol* 2005;16:555-67.
20. Minagawa M. Reconstruction of human diet from  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in contemporary Japanese hair—a stochastic method for estimating multisource contribution by double isotopic tracers. *Appl Geochem* 1992;7:145-58.
21. Schoeller DA, Minagawa M, Slater R, Kaplan IR. Stable isotopes of carbon; nitrogen and hydrogen in the contemporary North-American human food web. *Ecol Food Nutr* 1986;18:159-70.
22. Wilkinson MJ, Yai Y, O'Brien DM. Age-related variation in red blood cell stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) from two Yupik villages in Southwest Alaska: a pilot study. *Int J Circumpolar Health* 2007;66:31-41.
23. O'Brien DM, Wooller MJ. Tracking human travel using stable oxygen and hydrogen isotope analyses of hair and urine. *Rapid Commun Mass Spectrom* 2007;21:2422-30.
24. Fry B. Stable isotope ecology. New York, NY: Springer, 2006.
25. Gannes LZ, O'Brien DM, Martínez del Río C. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology* 1997;78:1271-6.
26. Kelly JF. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Can J Zool* 2000;78:1-27.
27. McCutchan JH Jr, Lewis WM Jr, Kendall C, McGrath CC. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* 2003;102:378-90.
28. Chisholm BS, Nelson DE, Schwarcz HP. Stable carbon isotope ratios as a measure of marine versus terrestrial protein in ancient diets. *Science* 1982;216:1131-2.
29. Newsome SD, Phillips DL, Culleton B, Guilderson T, Koch PL. Dietary reconstruction of an early to middle Holocene human population from the central California coast: insights from advanced stable isotope mixing models. *J Archaeol Sci* 2004;31:1101-15.
30. Richards MP, Jacobi R, Cook J, Pettitt PB, Stringer CB. Isotope evidence for the intensive use of marine foods by Late Upper Palaeolithic humans. *J Hum Evol* 2005;49:390-4.
31. Müldner G, Richards MP. Diet and diversity at later medieval fishergate: the isotopic evidence. *Am J Phys Anthropol* 2007;134:162-74.
32. Mohatt GV, Plaetke R, Klejka J, et al. The center for Alaska Native Health Research study: a community-based participatory research study of obesity and chronic disease-related protective and risk factors. *Int J Circumpolar Health* 2007;66:8-18.
33. Bersamin A, Zidenberg-Cherr S, Stern JS, Luick BR. Nutrient intakes are associated with adherence to a traditional diet among Yup'ik Eskimos living in remote Alaska Native communities: the CANHR Study. *Int J Circumpolar Health* 2007;66:62-70.
34. Bersamin A, Luick BR, King IB, Stern JS, Zidenberg-Cherr S. Westernizing diets influence fat intake, red blood cell fatty acid composition, and health in remote Alaskan Native communities in the Center for Alaska Native Health study. *J Am Diet Assoc* 2008;108:266-73.
35. Boyer BB, Mohatt GV, Lardon C, et al. Building a community-based participatory research center to investigate obesity and diabetes in Alaska Natives. *Int J Circumpolar Health* 2005;64:300-9.
36. Rose HG, Oklander M. Improved procedure for the extraction of lipids from human erythrocytes. *J Lipid Res* 1965;6:428-31.
37. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res* 1986;27:114-20.
38. Wolfe DA. On testing equality of related correlation coefficients. *Biometrika* 1976;63:214-5.
39. Shohet SB, Nathan D, Karnovsky M. Stages in the incorporation of fatty acids into red blood cells. *J Clin Invest* 1968;47:1096-108.
40. Brown AJ, Pang E, Roberts DC. Persistent changes in the fatty acid composition of erythrocyte membranes after moderate intake of n-3 polyunsaturated fatty acids: study design implications. *Am J Clin Nutr* 1991;54:668-73.
41. Popp Snijders C, Schouten JA, Dejong AP, Vanderveen EA. Effect of dietary cod-liver oil on the lipid-composition of human-erythrocyte membranes. *Scand J Clin Lab Invest* 1984;44:39-46.
42. Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. *Clin Chem* 2006;52:2265-72.
43. Blonk MC, Bilo HJG, Nauta JJP, PoppSnijders C, Mulder C, Donker AJM. Dose-response effects of fish-oil supplementation in healthy-volunteers. *Am J Clin Nutr* 1990;52:120-7.
44. Murphy NJ, Schraer CD, Bulkow LR, Boyko EJ, Lanier AP. Diabetes-mellitus in Alaskan Yupik Eskimos and Athabaskan Indians after 25 yr. *Diabetes Care* 1992;15:1390-2.
45. Murphy NJ, Schraer CD, Thiele MC, et al. Dietary change and obesity associated with glucose-intolerance in Alaska Natives. *J Am Diet Assoc* 1995;95:676-82.
46. Burrows NR, Geiss L, Engelgau M, Acton K. Prevalence of diabetes among Native Americans and Alaska Native, 1990-1997: an increasing burden. *Diabetes Care* 2000;23:1786-90.
47. Adler AI, Boyko EJ, Schraer CD, Murphy NJ. Lower prevalence of impaired glucose-tolerance and diabetes-associated with daily seal oil or salmon consumption among Alaska Natives. *Diabetes Care* 1994;17:1498-501.
48. Nobmann ED, Ponce R, Mattil C, et al. Dietary intakes vary with age among Eskimo adults of northwest Alaska in the GOCADAN study, 2000-2003. *J Nutr* 2005;135:856-62.
49. Dewailly E, Blanchet C, Lemieux S, et al. n-3 Fatty acids and cardiovascular disease risk factors among the Inuit of Nunavik. *Am J Clin Nutr* 2001;74:464-73.
50. Dewailly E, Blanchet C, Gingras S, Lemieux S, Holub BJ. Cardiovascular disease risk factors and n-3 fatty acid status in the adult population of James Bay Cree. *Am J Clin Nutr* 2002;76:85-92.

51. Ebbesson SO, Tejero ME, Nobmann ED, et al. Fatty acid consumption and metabolic syndrome components: the GOCADAN study. *J Cardiometab Syndr* 2007;2:244–9.
52. Hicks BJ, Wipfli MS, Lang DW, Lang ME. Marine-derived nitrogen and carbon in freshwater-riparian food webs of the Copper River Delta, southcentral Alaska. *Oecologia* 2005;144:558–69.
53. Roth JD. Variability in marine resources affects arctic fox population dynamics. *J Anim Ecol* 2003;72:668–76.
54. Bol R, Pflieger C. Stable isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{34}\text{S}$ ) analysis of the hair of modern humans and their domestic animals. *Rapid Commun Mass Spectrom* 2002;16:2195–200.
55. O'Connell TC, Hedges REM. Investigations into the effect of diet on modern human hair isotopic values. *Am J Phys Anthropol* 1999;108:409–25.
56. Fuller BT, Fuller JL, Sage NE, Harris DA, O'Connell TC, Hedges REM. Nitrogen balance and  $\delta^{15}\text{N}$ : why you're not what you eat during pregnancy. *Rapid Commun Mass Spectrom* 2004;18:2889–96.
57. Fuller BT, Fuller JL, Sage NE, Harris DA, O'Connell TC, Hedges REM. Nitrogen balance and  $\delta^{15}\text{N}$ : why you're not what you eat during nutritional stress. *Rapid Commun Mass Spectrom* 2005;19:2497–506.
58. Petzke KJ, Feist T, Fleig WE, Metges CC. Nitrogen isotopic composition in hair protein is different in liver cirrhotic patients. *Rapid Commun Mass Spectrom* 2006;20:2973–8.

