AGE-RELATED VARIATION IN RED BLOOD CELL STABLE ISOTOPE RATIOS (δ\(^{13}\)C AND δ\(^{15}\)N) FROM TWO YUPIK VILLAGES IN SOUTHWEST ALASKA: A PILOT STUDY

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ABSTRACT

Objectives. A significant fraction of the Alaska Native population appears to be shifting from a primarily subsistence-based diet to a market-based diet; therefore, the ability to link diet pattern to disease risk has become increasingly important to predicting public health needs. Our research aims to develop the use of stable isotope ratios as diet pattern biomarkers, based on naturally-occurring isotopic differences in the elemental composition of subsistence and non-subsistence foods. These differences are reflected in human blood, hair and fingernail isotope signatures.

Study design. In this preliminary study, we investigate the potential for \(^{13}\)C and \(^{15}\)N to serve as dietary biomarkers for age-related dietary differences in a subset of participants involved with a long-term study initiated by the Center for Alaska Native Health Research (CANHR) at the University of Alaska Fairbanks (UAF).

Methods. We measured δ\(^{13}\)C and δ\(^{15}\)N in red blood cells collected from 12 “elder” participants (age 60+ yrs) and 14 younger participants (age 14-19 yrs). Samples were evenly divided between males and females, and between two villages sampled in 2004. We also sampled market and subsistence foods in Fairbanks, AK, as an indicator of the isotopic differences likely to be observed in village foods.

Results. Elders were significantly enriched in \(^{15}\)N, but depleted in \(^{13}\)C, relative to younger participants. These differences are consistent with increased intake of marine subsistence in elders, and of certain market foods in younger participants. However, elders were considerably more variable in δ\(^{15}\)N, suggesting greater differences among individuals in their usual intake.

Conclusions. Overall we find that RBC stable isotope signatures exhibit variation consistent with previously documented dietary patterns in Alaska Natives, and we describe future directions for developing these biomarkers for diet pattern monitoring.

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Keywords: Yup’ik, diet assessment, stable isotopes, marine subsistence
STABLE ISOTOPES AND NUTRITION IN ALASKA NATIVES

INTRODUCTION

The Yup’ik Eskimos of Southwest Alaska are a population in which the prevalence of obesity is equivalent to that observed in the general NHANES III study, yet rates of obesity-related chronic disease, such as diabetes, are relatively low (< 3%). The genetic and dietary factors contributing to obesity-related health risk in this population are the subject of ongoing research by the Center for Alaska Native Health Research (CAHNR) at the University of Alaska Fairbanks (UAF). The marine subsistence-based diet of the Yup’ik people may historically have served to protect them from chronic, obesity-related diseases, via high levels of n-3 polyunsaturated fatty acids (PUFAs) (1-4). However, many Alaska Native populations are transitioning from subsistence to market-based diets and lifestyles, with associated increases in non-insulin dependent diabetes mellitus, hypertension, and cancer (5-9). If subsistence-based diets are protective, then a diet shift toward market foods may accelerate the development of chronic disease in this population. Young people may be particularly at risk, as their diet is the highest in carbohydrates and sugars, and lowest in subsistence intake (4, 5). If these diet patterns are maintained into adulthood, they may have significant consequences for public health.

Establishing links between diet pattern and health risk is challenging, due to the multitude of correlated factors contributing to risk of chronic obesity-related disease. Dietary methods based on self-reporting are essential to understanding the complex mix of subsistence and market diets in these populations (1, 4, 10-12). However, these methods can be problematic due to bias, cost, and the time investment and compliance required of participants (13-15). While biomarker-based methods of diet assessment typically yield less information, data obtained by these techniques are often easier to collect and are relatively free of bias (16). The development of precise instruments for quantifying dietary exposure is essential for relating diet pattern to risk of complex disease (17).

This research aims to develop practical, unbiased diet pattern biomarkers, based on naturally-occurring isotopic differences in the elemental composition of subsistence and non-subsistence foods (18). These isotopic signatures (\(^{13}C/^{12}C\), \(^{15}N/^{14}N\), \(^{18}O/^{16}O\), D/H, and \(^{34}S/^{32}S\)) are incorporated into tissues, including blood, nail and hair, and reflect diet at the time the tissue was formed (19, 20). Unique isotopic signatures distinguish marine from terrestrial foods (\(^{15}N/^{14}N\), \(^{34}S/^{32}S\)) (21, 22), food and water deriving from higher and lower latitudes (\(^{18}O/^{16}O\), D/H) (19), foods deriving from different trophic levels (\(^{15}N/^{14}N\)), and foods deriving from C3 plants (most herbaceous plants and shrubs) or C4 plants (typically grasses) (\(^{13}C/^{12}C\)) (23, 24). The difference in carbon isotope signatures among plant types is manifested in the human food supply by strong \(^{13}C\) enrichment in corn and sugar cane, both being C4 plants. This signature is also passed to commercial meat, most of which is fed on corn for a large portion of its lifetime (25). Soda sweetened with high fructose corn syrup is also \(^{12}C\) enriched (this study). \(^{15}N\) is enriched in marine subsistence for two reasons: the N cycle in marine ecosystems produces enriched \(^{15}N\), and because \(^{15}N\) bioaccumulates and marine subsistence foods are typically high on the food chain (21, 22).
Stable isotope ratios have been used to assess paleohuman diets for over two decades (26, 27); for example, to date the introduction of agriculture to prehistoric human societies (28, 29), and to assess the importance of marine foods to the diet of a prehistoric population (30-32). Stable isotope analyses are also gaining attention for their potential as dietary biomarkers in modern nutritional studies (18, 33-35), and in human forensics (36). Because Yup’ik Eskimos are a high-latitude population which relies on a mix of local marine and terrestrial subsistence and market foods, they present an ideal population for testing the utility of isotopic dietary biomarkers for diet pattern assessment in nutritional studies.

This paper introduces our approach and presents isotope data from a preliminary subset of our study population. Here we investigate the potential for $^{13}$C and $^{15}$N to serve as dietary biomarkers for age-related dietary differences. We present isotope data from foods commonly consumed in Southwest Alaska, to establish the patterns of isotopic variation expected to affect study participants. We test whether different aspects of sample handling affect blood $\delta^{13}$C and $\delta^{15}$N, to establish a sampling protocol. Finally, we compare isotope signatures in red blood cells from two groups of study participants, elders (> 60 yrs) and teenagers (14-19 yrs), taking advantage of blood samples collected for ongoing research by CANHR. We select these two age classes because we expect they will have the greatest differences in diet (4), and thus be the most likely to exhibit significant differences in isotopic signatures. Because $^{15}$N tends to be enriched in marine foods, and elders consume more subsistence than teens, we predict that elders will be comparatively enriched in $^{15}$N. Because teens consume significant quantities of sweetened foods like soda (4, 5), we predict that they will be enriched in $^{13}$C, which is particularly high in cane sugar and corn syrup. It is important to evaluate whether isotopic methods can capture these important dietary differences before stable isotope methods for diet assessment in this population are developed further. The ability to track such changes using isotopic analysis may prove to be a significant tool in efforts to monitor the health of this population and to associate dietary differences with disease.

MATERIALS AND METHODS

Food sampling
In the spring of 2005, a variety of foods representing known sources of nutrition for individuals in Yup’ik villages were selected for isotopic analysis. Subsistence foods were donated by local hunters and fishers in the Fairbanks area and included moose, caribou, salmon, and halibut. Non-subsistence food was bought from a regional chain grocery store in Fairbanks (Fred Meyer) and included Crisco™, pilot bread, two types of rice (jasmine and pearl), pasta, two types of chicken, turkey, three types of beef, Coke™, and Tang™. A single muscle sample was taken from each of five different caribou and four moose contributions, whereas three muscle samples were taken from a single halibut and two salmon. Triplicate samples were taken from each bought item. All samples were dried at 50°C for 48 hours and ground to a fine powder using a Wig-L-Bug ball mill. Sub-samples of 0.2 - 0.4 mg were weighed into tin capsules for isotope analysis.
Sample handling test
Blood samples from two Fairbanks residents were drawn to test the effects of EDTA treated tubes and autoclaving on blood δ¹³C and δ¹⁵N. Blood from each participant was collected into three 10 ml Vacutainer® Whole Blood Tubes: one K3 EDTA-treated tube (15% Solution, 0.117 ml, 17.55 mg) and two untreated glass tubes, as described below. Samples were centrifuged for 15 minutes at 1000 rpm, and plasma was removed. Each sample was then further divided into two portions, one of which was autoclaved for 20 minutes at 121°C. Samples were then freeze dried, powdered with a mortar and pestle, and weighed out for isotope analysis. Each sample was analyzed in triplicate to test the effects of tube type and autoclaving on blood isotopic signatures. Each participant handled and prepared their own samples, so that exposure to non-autoclaved samples posed no pathogen risk.

Sample preparation
All blood samples from the Yukon Kuskokwim Delta villages were autoclaved for 20 minutes at 121°C degrees to destroy blood-borne pathogens. Samples were then freeze-dried, powdered with a mortar and pestle or a Wig-L-Bug ball mill, and stored in a dessicator until analysis. Between 0.3 and 0.35 mg of powder was transferred into tin capsules for isotope analysis. Peptone standards ranging 0.25 - 0.3 mg were prepared in an identical fashion.

Blood sampling
Blood samples were collected in 2004 by the Center for Alaska Native Health Research (CANHR) from two Yup’ik villages in Southwest Alaska, as approved by UAF IRB and the Yukon Kuskokwim Health Corporation. Samples were collected into untreated glass tubes, centrifuged for 15 minutes at 1000 rpm, and plasma was removed for separate analyses. The remaining clots of red blood cells (RBCs) were stored at -80°C in the laboratory. A total of 89 clot samples were collected, and among these 26 samples were selected for isotope analysis. These included all participants ≥ 60 years of age (= 12 samples, 6 female, 6 male), regardless of cancer (1) or diabetes (1 “unknown”) status. We matched these samples with 14 samples (7 female, 7 male) drawn from participants 14-19 years of age (from 29 available samples of this age class). These were selected using a random number generator (academics.hws.edu/bio/oldsite/pages/random.html). Unlike elders, younger members of this population were excluded from consideration if cancer, diabetes, or medications were self-reported. Body mass index was allowed to fluctuate as a random variable.

Sample analysis
Food and blood samples were analyzed for carbon and nitrogen isotopes at the Alaska Stable Isotope Facility. Briefly, δ¹³C and δ¹⁵N were determined from 0.3 - 0.35 mg samples with an Elemental Analyzer (Costech Scientific Inc.) interfaced with a Delta Plus XL Isotope Ratio Mass Spectrometer (IRMS) via the Conflo III interface (Thermo-Finnigan) (EA-IRMS). Data are presented in delta notation as δX = (Rsample − Rstandard)/Rstandard • 1000‰, where X = ¹⁵N or ¹³C, R = the ratio of heavy to light isotope, and international standards are VPDB for carbon and N₈ atm for nitrogen. Analytical precision was evaluated via the standard deviations of peptone samples prepared and run concurrently with blood samples. This was 0.08 ‰ for δ¹³C and 0.2 ‰ for δ¹⁵N.
Data analysis
All data were initially analyzed via full factorial ANOVA, with non-significant interaction terms excluded from the final model. Normality of residuals was evaluated with the Shapiro-Wilks test. Outliers were identified via two criteria, first if their residual fell more than 3 standard deviations from the average residual for the model tested, and second by the method of Hoaglin et al (37). All analyses were performed using JMP IN version 5.1.2 (SAS Institute Inc.).

RESULTS

Isotope ratios of food items
Typical subsistence and market foods available in Fairbanks, AK exhibited large variations in δ¹⁵N and δ¹³C (Fig. 1). As predicted, marine derived foods were highly enriched in δ¹⁵N and intermediate in δ¹³C relative to other foods. Halibut was enriched in both δ¹⁵N and δ¹³C relative to salmon. In contrast, caribou and moose were low in both δ¹⁵N and δ¹³C, exhibited little isotopic variation, and were indistinguishable from plant-based market foods such as pasta, rice, crackers, and vegetable shortening. Market meats (chicken and beef) were similar in δ¹⁵N to terrestrial meats but had a considerably higher δ¹³C. This difference, noted elsewhere, is due to the large amounts of corn fed to US livestock (25, 34, 36, 38). Tang™ and Coke™ were distinct from all other foods, showing highly enriched δ¹³C values that reflect the signature of cane sugar and/or corn syrup (Fig. 1).

Figure 1. Carbon and nitrogen isotope ratios of market and subsistence food items collected in Fairbanks, AK. Dotted lines group food items expected to be isotopically similar and of nutritional importance: marine subsistence foods (salmon, halibut), terrestrial subsistence (caribou, moose), market meat (chicken, beef), market grain/vegetable (Crisco™, rice, pasta, crackers) and corn syrup/sugar cane (Coke™, Tang™). Error bars indicate SD of replicate samples as outlined in methods. Coke™, Crisco™, and Tang™ contained no measurable nitrogen and are assigned δ¹⁵N = 0 for graphical purposes.
Sample handling test
Both δ\(^{13}\)C and δ\(^{15}\)N differed significantly between the two participants in the sample handling test: -20.89 ± 0.05‰ and 7.1 ± 0.2‰ (participant 1) vs. -19.95 ± 0.08‰ and 6.4 ± 0.2‰ (participant 2), n=8 (Table I). However, neither tube treatment (EDTA vs. untreated) nor autoclaving affected blood δ\(^{13}\)C or δ\(^{15}\)N (Table I), nor were there any significant interactions. This result suggests that tube type and autoclaving do not influence the isotope composition of field collected samples, and that all subsequently prepared blood samples should be autoclaved for safety.

Isotope ratios of blood samples
Blood δ\(^{13}\)C and δ\(^{15}\)N from elder (≥ 60 yrs) and younger (14-19 yrs) members of two Yup'ik villages indicates age-related differences in blood isotope composition (Fig. 2). Blood samples from elder members of this population were enriched in \(^{15}\)N (two tailed t-test, \(p=0.0005\)) and depleted in \(^{13}\)C (two-tailed t-test, \(p<0.0001\)) relative to teenagers. Although the range of δ\(^{13}\)C within elder and younger groups was similar (2.3 vs. 2.6‰, respectively), δ\(^{15}\)N varied more widely among the elder participants than among younger participants (4.0 vs. 1.9‰ respectively) (Fig. 2).

Table I. Effects of tube type and autoclaving treatment on the δ\(^{13}\)C and δ\(^{15}\)N of two volunteers, tested with ANOVA. n =24

| Effect          | δ\(^{15}\)N  |      |      |      | δ\(^{13}\)C  |      |      |      |
|-----------------|-------------|------|------|------|-------------|------|------|------|
|                 | SS          | df   | F    | p    | SS          | df   | F    | p    |
| Participant     | 3.06        | 1    | 57.76| < 0.0001 | 5.27        | 1    | 1225.7| < 0.0001 |
| Tube type       | 0.008       | 1    | 0.15 | 0.7070 | 0.01        | 1    | 2.52 | 0.1282 |
| Autoclaving     | 0.002       | 1    | 0.05 | 0.8268 | 0.0003      | 1    | 0.078| 0.7823 |
| Error           | 1.06        | 20   |      |      | 20          |      |      |      |

\(^{a}\)Sum of Squares

Figure 2. Red blood cell δ\(^{13}\)C and δ\(^{15}\)N in elder (≥ 60 yrs) and younger participants (14-19 yrs). Cross hairs give age group means ± SE.
Table II. ANOVA of the effects of sex, age and village on participant RBC $\delta^{15}$N (n= 25) and RBC $\delta^{13}$C (n=26), including significant interactions.

| Effect       | RBC $\delta^{15}$N | RBC $\delta^{13}$C |
|--------------|---------------------|---------------------|
|              | SS $^a$  df  F  p        | SS $^a$  df  F  p        |
| Age          | 19.58  1  48.24 < 0.0001 | 12.87  1  24.47 < 0.0001 |
| Sex          | 2.58   1  6.35  0.0204 | 0.80    1  1.52  0.2318 |
| Age $\times$ Sex | 3.64  1  8.98  0.0071 | 0.15    1  0.29  0.5980 |
| Village      | 0.01   1  0.01  0.9093 | 2.31    1  4.39  0.0485 |
| Error        | 8.04   20 | 11.04  21 |

$^a$ Sum of Squares
The effects of age, sex and village on both $\delta^{13}C$ and $\delta^{15}N$ were evaluated with ANOVA. As reported above, elders were significantly enriched in $\delta^{15}N$ relative to younger participants (Table II). Females were slightly $\delta^{15}N$ enriched relative to males, however, that effect was observed only in the elders, as reflected by a significant age $\times$ sex interaction (Table II, Fig. 3). Younger males and females did not differ in $\delta^{15}N$ in this population (Fig. 3). One elder, female participant (age 92 yrs, $\delta^{15}N = 7.3 \%$) qualified as a statistical outlier and was excluded from this analysis (Fig. 2). Leaving the outlier in did not affect the age effect, but the age $\times$ sex interaction became non-significant. In contrast, younger participants were enriched in $\delta^{13}C$ relative to elder participants, and there was no difference in $\delta^{13}C$ between males and females of either age. However, the magnitude of the $\delta^{13}C$ difference between elders and teenagers differed between villages, as reflected in a significant age $\times$ village interaction (Table II, Fig. 4).

**DISCUSSION**

Although our sampling of foods is still preliminary, we find large isotopic variation in different classes of food commonly consumed in Alaska, consistent with trends identified in other geographic regions (25, 39). These data suggest that nitrogen isotope ratios may be a useful biomarker of marine subsistence intake, as marine foods were $\delta^{15}N$ enriched beyond any of the other foods in our sample (including both subsistence and market meats). This observation is consistent with a number of other studies in which $\delta^{15}N$ in human bone collagen is used to indicate reliance on marine resources (31, 32, 35). In using $\delta^{15}N$ as a quantitative marker of relative intake, care must be taken to measure the foods typically consumed in a given study population. This is because differences in $\delta^{15}N$ between marine foods (here observed between salmon and halibut samples) may confound the relationship between $\delta^{15}N$ and the extent of marine intake by an individual.

Foods exhibited four distinct groups of carbon isotope signatures. Most depleted in $\delta^{13}C$ were market foods manufactured from C3 plants (rice, pasta, crackers, and vegetable shortening). The carbon isotope signatures of these foods were indistinguishable from our samples of terrestrial subsistence (moose and caribou). Marine foods were enriched relative to these foods but still intermediate in $\delta^{13}C$. Market meats and sweetened beverages containing a significant amount of carbon derived from corn or sugar cane (both C4 plants) were substantially enriched in $\delta^{13}C$ isotope. Significantly depleted $\delta^{13}C$ suggests proportionally higher intake of terrestrial subsistence foods or market foods derived from C3 plants. Enriched $\delta^{13}C$ suggests proportionally higher intake of foods manufactured from corn (livestock, soda sweetened with corn syrup) or sugar cane; however, it could also represent greater marine intake relative to terrestrial intake. In this case, information from nitrogen or other isotopes indicative of marine foods can help to disentangle these explanations.

The isotopic data from participant red blood cells demonstrate that known dietary differences between age groups in the Yup'ik population (Luick and Bersamin, unpublished) are reflected in isotopic differences among individual participants. Enriched $\delta^{15}N$ in the elder participants of this study suggests higher intake of marine foods relative to younger
participants, as has been noted in several other studies of indigenous Arctic populations (1, 4-6). However, variation in nitrogen signatures was also high among elders (4%), suggesting variation in consumption of $^{15}$N enriched foods among individuals. The one data point excluded as an outlier suggested very little marine intake in one elder female. Interestingly, neither of the villages sampled in this study is directly on the coast, although salmon and other fish of marine origin are available, and freshwater fish also show elevated $\delta^{15}$N. We would expect to see even larger age-related disparities in $^{15}$N in villages more closely connected to marine resources. We also found slightly elevated $\delta^{15}$N in female elders compared to male elders, perhaps suggesting slightly more marine intake. We do not suggest that this pattern is general to the population as a whole, as it is based on a very small number of samples. However, a systematic pattern of isotopic variation suggests that the use of stable isotopes as dietary biomarkers may have considerable power when applied to a larger dataset.

Teenagers were significantly enriched in $^{13}$C relative to elder participants from these study villages. Because $\delta^{15}$N is concomitantly reduced among these participants, it is unlikely that enriched $^{13}$C can be attributed to marine intake. Instead, this difference is consistent with a higher consumption of non-indigenous market foods, particularly those based on corn (beef, chicken, high fructose corn syrup) and/or cane sugar. This pattern is consistent with the findings of age-targeted nutritional studies among Yup’ik (4) and Alaska Native (5) youth. Consumption of sweetened drinks and foods by youth is of great concern due to the risk of obesity and juvenile diabetes (40). Because red blood cells are predominantly composed of protein, they will tend to reflect carbon from dietary protein to a greater extent than carbon from dietary sugars (41). Thus, our finding of only elevated $^{13}$C in teenagers is conservative, and probably underestimates the magnitude of difference in corn syrup consumption between age groups. Further study will address whether we can develop a more precise isotopic marker specific to beverage consumption, based on the combination of carbon and deuterium isotopes. Deuterium ($^2$H) and $^{18}$O become less abundant in surface water with increasing latitude, and could indicate reliance on beverages bottled outside of Alaska when assessed jointly with $^{13}$C.

It is also worth considering whether the patterns of stable isotope signatures documented here may have a non-dietary explanation. Animals (including humans) in negative nitrogen balance have been demonstrated to show enriched $\delta^{15}$N values (42, 43). However, the magnitude of these changes is typically on the order of < 1 %, whereas the differences in $\delta^{15}$N exhibited here range over 4 %. None of the participants in this study were categorized as underweight. Lipid content of the sampled tissue can affect $\delta^{13}$C (44); however, red blood cells have very little lipid associated with them and thus this factor is unlikely to affect participant $\delta^{13}$C.

Our goal with this study was to test whether stable isotope signatures measured in individual RBCs exhibit variation consistent with previously documented dietary patterns in Alaska Natives and food isotopic signatures. Our analysis of RBCs from elder and younger members of two Yup’ik villages indicates that isotopic signatures vary in this population based on age-related dietary differences. However, the observed trends are based upon a very small data-set in which dietary recall data were unavailable. It is therefore our aim to match
blood isotopic signatures in a greater number of participants with information on their usual diet, deriving from self-reported, three day diet records. We are also investigating the correlation between blood and hair isotopic signatures. Should isotope signatures correlate well with aspects of usual diet that are of particular interest in health monitoring, and if hair and blood isotope signatures are equally informative, we have the potential to develop a non-invasive, simple but powerful tool for monitoring diet pattern.

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