DEVELOPING STABLE ISOTOPE BIOMARKERS OF YUP'IK TRADITIONAL AND MARKET FOODS TO DETECT ASSOCIATIONS WITH CHRONIC DISEASE RISK

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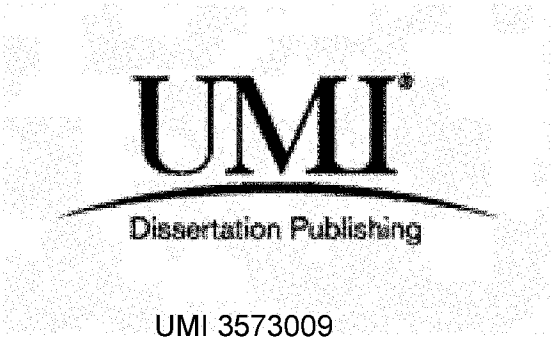

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ABSTRACT

This dissertation addresses the need for valid measures of dietary intake for use in studies of chronic disease risk in the Yup'ik population of Southwest Alaska. The Yup'ik people have experienced dietary changes over the past century, as consumption of traditional foods has been increasingly supplemented or replaced by market-purchased foods. Determining whether this dietary change is associated with increases in chronic disease risk is important for making nutritional recommendations for disease prevention. However, monitoring dietary change is challenging, in part due to the limitations of self-reported methods of dietary assessment. Dietary biomarkers are promising alternatives to self-reported methods, because they can provide unbiased, reliable estimates of intake. In this dissertation, I present evidence towards the validation of stable isotope dietary biomarkers. Stable isotope ratios vary among foods that are important in Yup'ik diets, and are incorporated into tissues, including several commonly collected biological sample types. They are simple, inexpensive and reliable measures that would be powerful tools for dietary assessment if they could be validated as biomarkers of certain foods. This work was conducted with two Yup'ik study populations that participated in studies conducted by the Center for Alaska Native Health Research. I begin by showing that the nitrogen isotope ratio is a marker of the marine component of traditional food intake, and the carbon isotope ratio is a marker of market food intake. I then calibrate a model of sugar intake based on both the carbon and nitrogen isotope ratios. I focus specifically on sugars because intake of sugary foods and beverages has been linked to obesity-related disease risk in other US populations. Finally, I use this dual isotope model to assess associations of sugar intake with chronic disease risk factors. I find that sugar intake is associated with blood pressure, blood lipids, leptin and adiponectin, suggesting a potential adverse effect of sugar intake on Yup'ik health. The findings of this dissertation provide substantial evidence to support carbon and nitrogen isotope ratios as markers of Yup'ik dietary intake, and demonstrate their potential to be informative in studies of associations between dietary intake and the health of Yup'ik people.

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My first acknowledgement is to the Yup'ik people who participated in this research, without whom this work would not be possible. I would like to express my sincerest thanks to these study participants and their communities for being so welcoming during our fieldwork visits, and for teaching me so much about the Yup'ik way of life. I would also like to acknowledge the community Tribal Councils and the Yukon-Kuskokwim Health Corporation for their support of both this research and the Center for Alaska Native Health Research (CANHR).

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INTRODUCTION

This dissertation addresses the need for valid and reliable measures of dietary intake for studies of chronic disease risk in Yup'ik people. The Yup'ik people, who are an Alaska Native group that predominantly live in the Yukon-Kuskokwim region of Southwest Alaska, have experienced a diet pattern shift over the past several decades. Diets that were once dominated by traditional foods (1) now contain large proportions of energy derived from market-purchased foods (approximately 40-85% (2, 3)). This shift from a traditional to market-based diet is termed the nutrition transition (4), and has been documented in other circumpolar (5-7) and Native American (8) populations. Importantly, the nutrition transition has been associated with profound changes in culture and health (8-11). In particular, rates of obesity-related chronic diseases have increased in populations that exchange reliance on traditional food intake for a "western" dietary pattern (8, 9).

Although there have been no longitudinal studies which explicitly address the impact of nutritional change on the health of Yup'ik people, it may be that these effects are already being seen. For example, type 2 diabetes was rare in Alaska Native people 50 years ago (12, 13); however, despite prevalence being low relative to other Native populations who have experienced the nutrition transition (14, 15), prevalence in the Yukon-Kuskokwim region increased 38% between 1990 and 2004 (from 16/1000 in 1990 to 22/1000 in 2004 (16)). Similarly, rates of colorectal, breast and stomach cancers are between 1.5 and 3-fold higher today than they were in the 1970's (17). In contrast, while mortality from heart disease more than doubled between 1979 and 1988, rates have remained relatively constant since this time (16). Evidently research is needed to determine whether the nutrition transition has played a role in changing chronic disease risk in the Yukon-Kuskokwim Delta, and, if so, what that role might be.

The fact that dietary change has occurred in the Yup'ik population may be obvious; however, these changes are actually quite difficult to quantify and monitor. This difficulty arises in part due to the lack of baseline and time-series data, and in part

due to the limitations of existing methods of dietary assessment. The most commonly used tools to assess dietary intake rely on self-report. However, self-report instruments that are appropriate for use in larger studies, such as the food frequency questionnaire, suffer large amounts of error and bias that may obscure associations of intake with disease risk (18, 19). Conversely, more reliable methods, such as the repeated 24 hour recall, can be prohibitively expensive, labor intensive and burdensome on both the participant and the researcher (20, 21). Clearly, improved measures of dietary intake are needed to more validly study associations between diet and disease risk. Biochemical measurements of intake, "biomarkers", are promising alternatives to self-reported methods of dietary assessment, because they can provide unbiased, reliable estimates of dietary intake (22-24). This dissertation investigates the potential of naturally occurring variations in stable isotope ratios to indicate dietary intake in the Yup'ik population.

Stable isotope ratios have been widely used in ecological and archaeological studies (25-28). Most commonly employed are the carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively). The nitrogen isotope ratio increases with trophic level (29, 30), so $\delta^{15}N$ values are elevated in animal proteins (meat, dairy, eggs) relative to plant foods. Values of $\delta^{15}N$ are particularly high in large fish and marine mammals, as these animals are high in the marine food chain and the marine environment is slightly ^{15}N enriched relative to the terrestrial environment. In contrast, the carbon isotope ratio has been primarily been used to indicate plant consumption. Values of $\delta^{13}C$ are particularly elevated in plants that use the C_4 (Hatch-slack) photosynthetic pathway, relative to the more common C_3 (Calvin) photosynthetic pathway (31). C_4 plants are typically those which grown in more tropical climates and many grass species, and their most common representatives in the US diet are corn and sugar cane. However, foods deriving from the marine environment also have elevated $\delta^{13}C$ values because oceanic bicarbonate, the source of carbon to marine foodwebs, is enriched in ^{13}C relative to atmospheric CO_2 (32, 33).

The carbon and nitrogen isotope ratios of a variety of human foods were published in the 1980's for the United States (34) and Japan (35), and these studies paved the way for contemporary applications of isotopes to diet. Early studies of dietary effects on human stable isotopes investigated vegetarianism (36, 37) and global travel (38, 39), but sample sizes were small and the studies were not designed to be validations in an epidemiological context. Subsequent studies investigated the use of isotope ratios to indicate intake of animal protein (40) and fish (41); these studies were more akin to epidemiological validations, being population-level comparisons of isotope ratios with self-reported estimates of intake. More recently, stable isotope ratios have piqued the interest of nutritional epidemiologists because of the potential for the carbon isotope ratio to indicate sweetener intake (42, 43), a food group whose role in chronic disease risk has been highly contentious (44).

Because corn- and sugar cane-based sweeteners are ^{13}C enriched, recent studies have proposed the carbon isotope ratio of serum (43) or whole blood (42) as a candidate biomarker of sweetener intake; however, the validity of this marker in those populations is low because of confounding by consumption of other ^{13}C enriched foods, such as other corn products, commercial meats and fish (45). Alternatively, the $\delta^{13}C$ value of postprandial plasma glucose has demonstrated associations with C_4 sweeteners in a controlled setting (45), but this marker is also limited because it provides only a very short-term reflection of intake. Markers of corn- and cane-sugar based sweetener intake are of particular interest because sugar intake, in particular high fructose corn syrup, has been linked to several intermediate risk factors for chronic disease, including excess energy intake, body mass index (BMI), dyslipidemia, insulin resistance and proinflammatory cytokines (46-54).

Carbon and nitrogen isotope ratios have particular potential to be informative about dietary intake in the Yup'ik population because several traditional and market food groups are isotopically distinct. My hypotheses for this dissertation are threefold. First, because the Yup'ik traditional diet is heavily reliant on fish and marine mammals, I

hypothesize that tissue $\delta^{15}N$ values will indicate intake of a traditional diet. Second, , because C_4 plants do not naturally grow in Alaska and are only available in the Yup'ik diet from market-purchased foods, I hypothesize that the carbon isotope ratio will be a valid marker of C_4-based market food intake. I also hypothesize that tissue $\delta^{13}C$ values will be associated with other ^{13}C enriched foods, such as fish and marine mammal intake, as well as intake of corn-fed commercial meats. Finally, if it is possible to remove the influence of these confounders on tissue $\delta^{13}C$ values, perhaps using tissue $\delta^{15}N$, I hypothesize that it might be possible to develop a valid and specific marker of corn- and cane sugar-based sweetener intake for use in studies of Yup'ik health. Together, these isotopic markers will be useful for determining whether increasing prevalence of obesity-related chronic diseases in the Yukon-Kuskokwim Delta are related to changes in intake of both traditional and market foods.

This dissertation focuses on the development and application of carbon and nitrogen isotope markers of dietary intake in the Yup'ik population. The ultimate aim of this research is to validate biomarkers of Yup'ik foods that can be used in population-based studies of how dietary change affects chronic disease risk. I begin this research by demonstrating the ability of red blood cell (RBC) $\delta^{13}C$ and $\delta^{15}N$ values to indicate traditional and market food intake in a sample of 230 Yup'ik participants that completed both a 3-day food record and a 24-hour recall (Chapter 1). I then use these isotope ratios to examine patterns of traditional and market food intake by age, sex, community location, and cultural identification, in a larger sample of 1003 Yup'ik participants.

Chapter 2 focuses specifically on the nitrogen isotope ratio of hair as an indicator of traditional marine food intake. Here, I compare hair $\delta^{15}N$ values to measures of the marine polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are long-chain omega-3 fatty acids known to reduce risk for cardiovascular and other chronic diseases (55,56). These measures included RBC $\delta^{15}N$, an isotope biomarker that has been previously validated for the Yup'ik population

(57), as well as an established biomarker of EPA and DHA intake, % RBC membrane fatty acids (58). Isotope ratios of hair are particularly attractive candidates for assessing dietary intake because sampling is non-invasive and does not require a nurse or phlebotomist, unlike collection of blood samples. This chapter continues by examining associations between RBC and hair nitrogen and carbon isotope ratios; this information will facilitate comparisons with existing and future studies that utilize one or the other of these sample types.

Chapter 3 focuses on developing a model of sweetener intake based on both $\delta^{13}C$ and $\delta^{15}N$ values. While the carbon isotope ratio is naturally elevated in corn and cane sugar-based sweeteners, this isotope ratio alone cannot indicate intake of sweeteners because of confounding by other ^{13}C enriched foods, such as commercial meats and traditional marine foods. Because the nitrogen isotope ratio is elevated Yup'ik people who eat a lot of traditional marine foods, I hypothesized that $\delta^{15}N$ adjustment would improve the carbon isotope biomarker by removing the influence of fish and marine mammals on tissue $\delta^{13}C$. However, $\delta^{15}N$ is very strongly correlated with traditional marine intake in this Yup'ik study population; therefore, I did not know whether we would find an association with commercial meat intake, and whether we would be able to account for the effects of this confounder on tissue $\delta^{13}C$. In this study, I find that a dual isotope model using both $\delta^{13}C$ and $\delta^{15}N$ explains over 5 times the variation in sweetener intake than does a model using $\delta^{13}C$ only, but that we are unable to account for the association of $\delta^{13}C$ and commercial meat intake using $\delta^{15}N$.

Finally, Chapter 4 uses this calibrated dual isotope model of total sugar intake to examine associations with obesity and other chronic disease risk factors in a cross-sectional sample of 1076 Yup'ik people. I find that while sugar intake is not associated with either body mass index or waist circumference in this population, it is positively associated with blood pressure, triglycerides, insulin, and leptin, an adipokine that plays a key role in appetite and metabolism. Sugar intake was inversely associated with total and HDL cholesterol, as well as adiponectin, an adipokine that promotes insulin sensitivity.

This chapter is important in two ways: first, it demonstrates the ability of our isotopic markers to be informative in studies of disease risk in the Yup'ik population, and second, it discovers associations of sugar intake with chronic disease risk factors that were previously unknown for this population. I hope that these findings will provide the basis for longitudinal studies of the association of sugar intake with risk factors for obesity-related chronic diseases.

Determining the effect of dietary change on the health of the Yup'ik population requires valid, objective measurements of intake. The first three chapters of this dissertation present substantial evidence to support the use of the carbon and nitrogen isotope ratios as valid, objective markers of traditional and market food intake. The final chapter demonstrates the utility of these markers in a study of the association between sugar intake and chronic disease risk factors. In summary, this dissertation provides substantial evidence to support the use of stable isotope markers in epidemiologic studies of the association between dietary change and health in the Yup'ik population.

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CHAPTER 1. STABLE NITROGEN AND CARBON ISOTOPE RATIOS INDICATE TRADITIONAL AND MARKET FOOD INTAKE IN AN INDIGENOUS CIRCUMPOLAR POPULATION[1]

1.1. ABSTRACT

The transition of a society from subsistence to market-based diets (termed the nutrition transition) has been associated with profound changes in culture and health. We are developing biomarkers to track the nutrition transition in the Yup'ik population of Southwest Alaska, based on naturally occurring variations in the relative abundances of carbon and nitrogen stable isotopes ($\delta^{15}N$ and $\delta^{13}C$ values). Here, we provide three pieces of evidence toward the validation of these biomarkers. First, we analyzed the $\delta^{15}N$ and $\delta^{13}C$ values of a comprehensive sample of Yup'ik foods. We found that $\delta^{15}N$ values were elevated in fish and marine mammals and that $\delta^{13}C$ values were elevated in market foods containing corn or sugar cane carbon. Second, we evaluated the associations between red blood cell (RBC) $\delta^{15}N$ and $\delta^{13}C$ values and self-reported measures of traditional and market food intake (n = 230). RBC $d^{15}N$ values were correlated with intake of fish and marine mammals (r = 0.52, P <0.0001). RBC $\delta^{13}C$ values were correlated with intake of market foods made from corn and sugar cane (r = 0.46, P <0.0001) and total market food intake (r = 0.46, P <0.0001). Finally, we assessed whether stable isotope ratios captured population-level patterns of traditional and market intake (n = 1003). Isotopic biomarkers of traditional and market intake were associated with age, community location, sex, and cultural identity. Self-report methods showed variations by age and cultural identity only. Thus, stable isotopes show potential as biomarkers for monitoring dietary change in indigenous circumpolar populations.

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1.2. INTRODUCTION

The transition of a society from traditional to market-based diets (termed the nutrition transition) has been associated with profound changes in culture and health (1-4). Many indigenous circumpolar populations are undergoing this transition (5-7), which is associated with increased rates of chronic disease (6, 8). Dietary change can be difficult to monitor, due in part to the lack of baseline data and in part to the limitations of existing methods for dietary assessment. Self-report methods that are feasible to collect from large populations (e.g. food frequency questionnaire) are subject to large error and bias (9, 10), whereas more reliable methods (e.g. repeated 24-hour recall) can be prohibitively expensive (11, 12). Dietary biomarkers provide a promising alternative to self-report methods, because they are unbiased, more reliable, and can be measured from archived samples (13-16). We are developing biomarkers of traditional and market intake for the Yup'ik population of Southwest Alaska, based on the relative abundances of naturally occurring carbon and nitrogen stable isotopes (17, 18). Isotopic markers have been widely used as markers of diet in ecological and anthropological studies (19-22). Furthermore, they are inexpensive to measure, precise, and can be measured in multiple tissue types, including serum, RBC and hair (16-18, 23, 24).

Stable isotope biomarkers are informative in the Yup'ik population because many commonly consumed traditional and market foods are isotopically distinct (25-29). The nitrogen stable isotope ratio ($^{TM}{}^{15}N$) indicates consumption of marine mammals and fish (25, 28), which are a large component of the traditional Yup'ik diet (30-32). This biomarker has been recently validated for Yup'ik people based on comparisons with the marine fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (17, 18). Thus, we propose that $\delta^{15}N$ will indicate consumption of traditional marine foods in this population. The carbon isotope ratio ($\delta^{13}C$) is elevated in plants using the C_4 (Hatch-Slack) photosynthetic pathway, relative to those using the more common C_3 (Calvin) photosynthetic pathway (33). The most common representatives of these plants in the US agricultural system are corn and sugar cane, which are widely present in the market diet

as sweeteners (29), as ingredients in processed foods, and indirectly via domestic animals raised on corn (34, 35). The carbon isotope ratio has shown moderate associations with reported C_4-based sweetener and sweetened beverage intake in the US population (23, 24, 36). Here, we propose that $\delta^{13}C$ will provide an independent biomarker of market food intake for the Yup'ik population.

The overall objective of this study is to evaluate isotopic biomarkers of market and traditional food intake in a Yup'ik study population. Developing reliable and accurate markers of dietary change for this population could help to predict increases in disease incidence and develop appropriate dietary interventions. The specific aims of this study are threefold. First, we determine expected relationships between dietary intake and RBC isotope ratios by completing a comprehensive analysis of $\delta^{15}N$ and $\delta^{13}C$ values in traditional and market foods important to the Yup'ik population. Second, we evaluate the association between RBC $\delta^{15}N$ and reported fish and marine mammal intake, and RBC $\delta^{13}C$ and reported market food intake, based on four days of diet records from 230 Yup'ik people. Finally, we evaluate whether variations in dietary intake by age, community location and cultural identity that have been previously reported for this population based on self report are also seen using isotopic biomarkers (30, 37-39). The extensive nature of previous dietary assessment in this population provides an ideal framework with which to evaluate the efficacy of these proposed biomarkers.

1.3 METHODOLOGY

1.3.1. Participant recruitment and procedures

Data are from the Center for Alaska Native Health Research (CANHR) study, a cross sectional, community based participatory research study of genetic, nutritional and psychosocial risk factors affecting obesity and related disease in Yup'ik people (40, 41). The CANHR study was approved by the University of Alaska Fairbanks Institutional

Review Board, the National and Area Indian Health Service Institutional Review Boards, and the Yukon-Kuskokwim Health Corporation Human Studies Committee.

Between 2003-2005, 1003 participants aged 14-94 were recruited from 10 communities in Southwest Alaska as described in detail elsewhere (41). We categorize these communities as either coastal (<5 miles from the coast) or inland. At entry into the study, participants completed extensive interviewer-administered demographic questionnaires. A subset of 315 participants from the first seven communities visited completed a single interviewer-administered 24 h dietary recall as well as a 3 d food record. A subset of 767 participants from all 10 communities completed a wellness questionnaire. We use the responses to two questions about cultural identification, which asked how much an individual felt they followed a Yup'ik or Kass'aq (non-native) way of life. Responses to these questions were coded numerically (1= follows the lifestyle a lot, 2 = some, 3= not at all) and were not mutually exclusive (e.g., a person could respond "follows the lifestyle a lot" for both ways of life).

1.3.2. Biological sample collection

Blood was collected into Ethylenediaminetetraacetic acid (EDTA) tubes and processed in rural communities using a portable centrifuge. Serum, lymphocytes and RBC were aliquotted and stored at -20°C in a portable freezer. Within six days, samples were shipped to the University of Alaska Fairbanks and stored at -80°C. Aliquots of RBC were removed for stable isotope analysis, as described below. RBC were chosen for analyses because they reflect dietary intake over a period of 1-3 months (42-44), and thus provide a more stable estimate of dietary intake than serum (45). RBC aliquots were autoclaved for 20 minutes at 121°C to destroy blood-borne pathogens, apportioned into tin capsules (3.5 x 3.75 mm), and oven dried at 60°C to a final weight of 0.2 - 0.4 mg. Neither autoclaving or the use of EDTA treated tubes affects RBC carbon or nitrogen isotope ratios (28).

1.3.3. Food sample collection

We sampled a broad range of traditional and market foods that were known to contribute to this study population's diet (n = 254). Foods were defined based on their NDS-R (Nutrition Data System for Research software version 4.06; University of Minnesota, Minneapolis, MN) food codes. Traditional foods were harvested from the local environment, and samples were donated by residents from three Yup'ik communities. Market foods were purchased from community grocery stores, or grocery stores in Fairbanks, Alaska. We sampled three or more representatives of foods contributing > 5% of energy (based on dietary self-report data), and one or more representatives from foods contributing 1-5% of energy. We sampled more rarely consumed traditional food items (contributing <1% energy) when donated. Marine mammal samples were collected under permit number 932-1905-00IMA-009526, issued by the National Marine Fisheries Service and the U.S. Fish and Wildlife Service, under the authority of the Marine Mammal Protection Act and Endangered Species Act. A list of foods sampled and their sampling frequencies is given in **Supplemental Table 1.S1**.

Food samples were grouped into traditional or market-based food groups. Traditional foods were divided into four groups: Marine (marine mammals, fish, and seal oil), terrestrial animals (birds and mammals), terrestrial plants (berries and wild plants) and waterfowl (ducks, geese, and swans). We define waterfowl separately because these species forage in both marine and terrestrial habitats. Furthermore, we note that the "marine" category contains both marine and freshwater fish species. While freshwater and marine fish are expected to differ slightly in $\delta^{15}N$ and $\delta^{13}C$ values, their values are likely to be more similar to each other than to other classes of foods. Market foods were divided into five groups: market grains and vegetables (foods from C_3 plants, including pasta, wheat, rice, nuts, fruits and vegetables), corn and cane sugar (C_4-based foods, including beverages sweetened with high fructose corn syrup, cane sugar, candy, corn), meat (Chicken, Turkey, Beef, Eggs), dairy (Milk, Cheese), or mixed (containing ingredients from more than one group). For isotope analysis, food samples were oven

dried at 60°C for at least 48 h, ground to a fine powder, and weighed into tin capsules to a final weight of 0.2 -0.4 mg.

1.3.4. Stable isotope analysis

Samples were analyzed at the Alaska Stable Isotope Facility by continuous-flow isotope ratio mass spectrometry, using a Costech ECS4010 Elemental Analyzer (Costech Scientific Inc.) interfaced to a Finnigan Delta Plus XP isotope ratio mass spectrometer via the Conflo III interface (Thermo-Finnigan Inc.). The conventional means of expressing natural abundance isotope ratios is as delta values in permil (‰) relative to international standards, defined as $dX = (R_{sample} - R_{standard})/(R_{standard}) \cdot 1000‰$ (46). Here, R is the ratio of heavy to light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$) and the standards are atmospheric N for nitrogen and PeeDee Belemnite for carbon. Because the foods and RBC samples from this study have a lower $^{13}C/^{12}C$ than the standard, values of $\delta^{13}C$ are negative. We concurrently prepared and ran multiple peptone working standards ($\delta^{15}N = 7.0$, $\delta^{13}C = -15.8‰$, $n = 128$) to assess analytical accuracy and precision, measured as the standard deviation of these analyses. Accuracy was within 0.1‰, and precision was within 0.2‰, for both isotopes.

1.3.5. Assessment of dietary intake

Dietary intake was estimated using an interviewer-administered 24 h dietary recall and a 3 d food record. Data from these instruments were combined to achieve a stable estimate of dietary intake. The 24HR was collected from each participant by certified interviewers using a computer-assisted recall (Nutrition Data System for Research (NDS-R) version 4.06). Participants were asked to recall all food and beverages consumed over the 24 h period covering the day prior to interview using a multiple pass

approach to minimize recall bias. Although most participants were bilingual, a native Yup'ik speaker trained in the use of NDS-R was available for non-English speakers.

When completing the 3 d food record, participants were instructed to maintain their usual eating habits. A research team member reviewed all records for completeness, which were then entered into the NDS-R software package by certified coders. A second researcher reviewed all entries for accuracy. Records were considered unreliable and excluded from analysis if daily energy intake was greater than 5000 kcal or less than 500 kcal (38 participants had one day excluded, 4 had two days excluded). Individuals who had >2 days considered unreliable (n = 2) or whose 3-day food record or 24-hour recall was incomplete (n= 83) were excluded from self-report analyses; 230 individuals remained.

1.3.6. Dietary analysis

The contributions of traditional and market foods to an individual's diet were assessed as follows: all food items were assigned to traditional and market food groups (as defined above and given in Supplemental Table 1.S1) based on their food codes from the NDS-R Food and Nutrient Database 33 (July 2003). A few Alaska Native foods were missing from the database; these were substituted for similar food items where appropriate or the foods were added to the database. We then summed total energy consumed for three categories of foods: traditional marine, market, and C_4-based market foods, and used these totals for analyses. Foods assigned to traditional and market food groups were mutually exclusive.

As market foods included food groups that were partially C_4 - based (Mixed foods, Meat, and Dairy) as well as entirely C_4-based (Corn and cane sugar), we defined C_4-based market food intake by weighting energy derived from market-based food groups based on their fractional C_4 carbon content, and summing this weighted energy

(kcal). This fractional content (f_{C4}) was calculated using the mean $\delta^{13}C$ of food groups and an isotopic mixing model, as follows:

$$f_{C4} = (\delta^{13}C_{\text{food group mean}} - \delta^{13}C_{C3})/ (\delta^{13}C_{C4} - \delta^{13}C_{C3}) \qquad [1.1]$$

where f_{C4} is the fraction of the food that is C_4-based, $\delta^{13}C_{C4}$ is the mean carbon isotope ratio of C_4-based plant foods (corn and cane sugar, **Table 1.1**), and $\delta^{13}C_{C3}$ is the carbon isotope ratio of C_3–based plant foods (grains and vegetables; 47). We note that this is a highly simplified mixing model that does not take into account differences in macronutrient composition among foods (48), and uses a mean $\delta^{13}C$ for food classes rather than adjusting foods individually. However, the purpose of the calculation is to get a broad estimate of how much of the market diet is derived from C_4 sources rather than to present a highly precise measure.

1.3.7. Correcting RBC $\delta^{13}C$ values for the influence of fish and marine mammal intake

Our aim was to use $\delta^{13}C$ as an index of C_4-based market food intake however both C_4 and marine foods have elevated $\delta^{13}C$ values relative to C_3-based market foods (27, 29). Therefore, we used $\delta^{15}N$ values to adjust for the influence of marine foods on RBC $\delta^{13}C$ as follows:

$$\delta^{13}C_A = \delta^{13}C - [(\delta^{15}N - \delta^{15}N_{\text{no marine}}) \times \Delta\delta^{13}C/\Delta\delta^{15}N_{\text{marine foods}}] \qquad [1.2]$$

where $\delta^{13}C_A$ = Adjusted $\delta^{13}C$ value

$\delta^{13}C$ and $\delta^{15}N$ are measured RBC isotope ratios

$\delta^{15}N_{no\ marine}$ = mean $\delta^{15}N$ value for all members of the population reporting no traditional marine food intake (n = 42). This was measured to be 7.8 ‰

$\Delta\delta^{13}C/\Delta\delta^{15}N_{marine\ foods}$ = the increase in $\delta^{13}C$ for each unit increase in $\delta^{15}N$ across all fish and marine mammal samples. This was measured to be 0.91 (see Results).

We tested the accuracy of this approach by assessing the agreement between prediction of C_4-based market foods based on $\delta^{13}C_A$ values and a multiple linear regression model including $\delta^{13}C$ and $\delta^{15}N$ as independent variables (49). Agreement between the two methods was good (mean difference: 0.0 ± 1.7% of total energy). Although both of these methods account for the influence of marine food intake on $\delta^{13}C$ values, we have chosen to adjust RBC $\delta^{13}C$ values in this study in order to generate a single, independent variable that can be used in multiple analyses.

1.3.8. Statistical analyses

All statistical analyses were performed using JMP version 8 (SAS Institute, Cary, NC). We evaluated differences in the sex and age distributions between the complete sample of participants and those who completed dietary interviews or cultural identity questions using a chi-square test. We used analysis of variance (ANOVA) to compare the isotope ratios of food samples in each market and traditional food group and compared means using Tukey-Kramer's honest significant difference. We assessed the associations between isotope ratios and dietary intake data, as well as between dietary intake variables, using linear regression.

We evaluated the effects of demographic variables on intake variables (isotope and self-report), using analysis of covariance (ANCOVA) models where relationships were linear and met assumptions for parametric statistical tests. Demographic variables (age, sex, community location) were the independent variables in these models. ANOVA was used to assess the effects of cultural identity on dietary intake. Cultural identity variables were the independent variables in these models. The effects of demographic and cultural identity variables on intake were assessed separately, as cultural identity questions were only completed by a subset of the population.

In all analyses that compare dietary self-report data to isotope, demographic, or cultural identity information, intake was expressed as the percentage of total energy represented by each food group. Where dietary self-report information is compared to other dietary self-report information, intake is expressed as total energy reported (kcal). Normality was confirmed using normal probability plots; dietary intake data were log transformed for analyses and results back transformed for ease of interpretation (50). Outliers were identified by using Mahalobnis distance >3 and excluded from analyses (n = 3 for $\delta^{15}N$/ marine food intake, n = 6 for $\delta^{13}C_A/C_4$ intake, n = 3 for $\delta^{15}N$ /total traditional food intake and n = 13 for $\delta^{13}C_A$/total market intake). All means are given ± standard deviation. A significance level of 0.05 is used throughout analyses.

1.4. RESULTS

1.4.1. Stable isotope ratios of food

Nitrogen isotope ratios varied significantly among traditional and market food groups ($\delta^{15}N$: $P < 0.001$, Table 1.1, Supplemental Table 1.S1). Traditional marine foods had substantially higher $\delta^{15}N$ values than any other food group (Table 1.1). Marine fish species had higher $\delta^{15}N$ values than freshwater fish species ($P = 0.0013$, marine $\delta^{15}N$ = 14.3 ± 3.4‰, freshwater $\delta^{15}N$ = 11.5 ± 2.7‰); however, their values overlapped and were both significantly higher than all other food groups. Thus, we continued to group

these species together as "marine foods" for further analyses. Excluding marine foods, animal-based food groups had higher mean $\delta^{15}N$ values than plant-based food groups ($P < 0.0037$). We found a strong positive relationship between $\delta^{13}C$ and $\delta^{15}N$ values of traditional foods (Figure 1.1A, $\beta = 1.86$, $r = 0.82$, $P < 0.0001$) and marine foods only ($\beta = 0.91$, $r = 0.71$, $P < 0.0001$), but not market foods (Figure 1.1B, $\beta = 0.14$, $r = 0.38$, $P = 0.08$).

Carbon isotope ratios differed significantly between food groups ($\delta^{13}C$: $P < 0.001$, Table 1.1, Figure 1.1), with a clear distinction between C_3 and C_4-based foods. Corn and cane sugar (C_4) had elevated $\delta^{13}C$ values (Table 1.1) relative to grains and vegetables (C_3, Table 1.1). The mean isotopic difference between these groups was almost 13‰ and was consistent with reported $\delta^{13}C$ values for C_3 and C_4 plants generally (51). Market meats, dairy and mixed foods had $\delta^{13}C$ values intermediate between C_3 and C_4-based market foods, reflecting corn-based feeds or ingredients. Marine fish species had significantly higher $\delta^{13}C$ values compared to freshwater species ($P < 0.0001$, marine $\delta^{13}C = -20.4 \pm 1.8$‰, freshwater $\delta^{13}C = -24.3 \pm 3.3$).

1.4.2. Sample population

Females were slightly over-represented relative to males in the whole study population (54%), the subset of participants with dietary self-report data (59 %), and the subset of participants reporting cultural identification (56%, **Table 1.2**, all $P < 0.01$). The age distribution of participants with dietary self-report data differed significantly from the complete study sample ($P < 0.01$), with reduced participation by those who were > 60 y. The age distribution of participants reporting cultural identification did not differ from the complete study sample.

1.4.3. Associations between stable isotope ratios and reported dietary intakes

Nitrogen isotope ratios were significantly correlated with intake of traditional marine foods based on dietary self-report (**Table 1.3**). Reported intake of total traditional and traditional marine foods was highly correlated (Table 1.3). Marine foods accounted for 77% energy from traditional sources.

Adjusted carbon isotope ratios were positively correlated with C_4-market food intake (Table 1.3), as well as total market food intake. Intake of C_4-based market foods was positively associated with total market food intake (Table 1.3). C_4 sources accounted for 40% of total energy from market foods.

1.4.4. Population level patterns of traditional marine food intake

Mean RBC $\delta^{15}N$ values reflected a high and variable intake of marine foods (Table 2). Marine foods were reported by 82% of people with dietary self-report data (n = 188), and mean intake was 210 ± 245 kcal.

We found strong associations between RBC $\delta^{15}N$ values and sex, community location and age (Table 1.2). Values of RBC $\delta^{15}N$ increased with age (β = 0.052, CI = 0.048 – 0.056, $P < 0.0001$), and were higher in coastal communities ($P < 0.0001$) and females ($P < 0.0001$). There was a community location by age interaction ($P < 0.0001$), as differences in $\delta^{15}N$ values between coastal and inland locations increased with age. In the self-report data, we found an increase in intake of marine foods with age (β = 1.003, CI = 1.002 – 1.004, $P < 0.0001$), but not community location (P = 0.11) or sex (P =0.09).

Intake of marine foods was positively associated with following a Yup'ik way of life, as indicated by nitrogen isotope ratios (P <0.0001) and self-report data (P = 0.016). Following a Kass'aq way of life was inversely associated with nitrogen isotope ratios (P = 0.0004) and self-reported intake of marine foods (P = 0.45, Table 1.2).

1.4.5. Population level patterns of C_4 - based market food intake

Adjusted RBC carbon isotope ratios reflected a mixed diet of C_3 and C_4 foods (Table 1.2). All participants for whom we have dietary self-report information reported consuming market foods (n = 230), and mean intake of C_4-based market foods was 543 ± 301 kcal.

We found strong associations between sex, age and community location and RBC $\delta^{13}C_A$ values (Table 1.2). Values of RBC $\delta^{13}C_A$ decreased with age (β = -0.057, CI = -0.062 – -0.053, $P < 0.0001$), and were higher in upriver communities ($P < 0.0001$) and males ($P < 0.0011$). There was a community location by age interaction ($P < 0.0223$). In the self-report data, we did not find any differences in market food intake by sex or community location, although there was a significant decrease in intake of market foods with age (β = 0.98, $P < 0.0001$, Table 1.2).

RBC $\delta^{13}C_A$ values were positively associated with following a Kass'aq way of life ($P < 0.0001$, Table 1.2), and inversely associated with following a Yup'ik way of life ($P < 0.0001$). Self-reported intake of C_4-based market foods was inversely associated with a Yup'ik way of life (P = 0.0006, Table 1.2), but was not associated with a Kass'aq way of life (P = 0.26).

1.5. Discussion

Carbon and nitrogen stable isotope biomarkers capture patterns of traditional marine and market food intake in a Yup'ik study population. Foods commonly consumed by the Yup'ik population exhibited highly distinct patterns of carbon and nitrogen isotope ratios, with elevated $\delta^{15}N$ and $\delta^{13}C$ values found in key traditional and market foods, respectively. Self-reported intake of these food groups was associated with RBC $\delta^{15}N$ and $\delta^{13}C_A$ values. These isotope ratios detected demographic and cultural variations in both traditional and market food intake known to exist within this population (30), as well as some that were undetected by self-reported measures. Stable

isotope markers have the potential to be useful in assessing the health impacts of dietary change in indigenous circumpolar populations, as they impose low participant burden, and can be measured with precise, high throughput and inexpensive methods.

Traditional marine foods had significantly elevated $\delta^{15}N$ values relative to all other food groups, causing a 1‰ increase in RBC $\delta^{15}N$ for each 5% increase in energy intake from marine foods. RBC $\delta^{15}N$ measurements showed that marine food consumption increased with age, reflecting trends in total traditional food intake described for this study population (30), and other circumpolar populations (38, 52, 53). RBC $\delta^{15}N$ values also captured differences in traditional marine food intake between coastal and inland communities, an effect seen primarily in elders, and a slight increase of marine food intake in women relative to men. Self-reported intake of traditional marine (this study) or total traditional foods (30) does not vary with community location or sex in this population. Thus, stable isotope biomarkers were able to identify patterns of traditional food intake that were not evident in dietary self-report data.

Corn and cane sugar-based market foods exhibited uniquely high $\delta^{13}C$ values relative to all other foods. Animal-based market foods (beef, pork, poultry, eggs and dairy) also had elevated $\delta^{13}C$ values, reflecting corn in the diet of commercially raised animals (34, 35). We refer collectively to these foods as "C_4-based market foods". RBC $\delta^{13}C_A$ values were associated with intake of C_4-based market foods, as well as total market food intake, with each 8% increase in energy from market foods causing a 1‰ increase in $\delta^{13}C_A$. The carbon isotope ratio has been moderately associated with sweetener intake in other US populations (23, 24, 36), however the development of this marker is complicated by its concurrent association with animal protein intake (20, 54). In our study, the fact that multiple market foods are influenced by corn likely strengthens the association between $\delta^{13}C$ and market food intake.

While market foods are known to be a significant source of energy to all age groups in this study population (39), both self-report and isotope data showed that

consumption of market foods decreased with age. Adjusted $\delta^{13}C$ values also showed that coastal communities consumed slightly less market foods than those inland, and men consumed slightly more market foods than women. Both RBC $\delta^{13}C_A$ and self-reported measures of market food intake were positively associated with a non-native (Kass'aq) way of life. As expected, these patterns are the reverse of those found for traditional food intake. However, the use of $\delta^{13}C_A$ as an independent biomarker for market food intake based on sugar cane and corn provides an alternative way to assess the nutrition transition that, unlike $\delta^{15}N$, does not require traditional food intake to be marine. Such a biomarker could be particularly useful for assessing dietary change in Alaska Native populations relying more heavily on traditional foods such as moose and caribou (55, 56), which are not distinct from market foods in $\delta^{15}N$.

The primary limitation of this study was that we compared stable isotope ratios to self-reported estimates of traditional and market food intake, which are subject to error and biases associated with age, sex and other individual characteristics. These errors may have obscured the true relationships between isotopic markers and diet in our study population. For example, relationships between $\delta^{15}N$ and EPA were very strong for this population ($r > 0.8$; 17, 18), when compared with the relationship between $\delta^{15}N$ and reported marine food intake presented here ($r = 0.52$). However, this study had several unique strengths. The Yup'ik population of Southwest Alaska is culturally and linguistically among the most intact of Alaska Native peoples and traditional food use is still common (41); therefore, this is an ideal population in which to test markers of both traditional and market foods. Furthermore, the extensive nature of dietary research in this population provided an ideal opportunity to identify relevant foods for isotopic sampling and to test stable isotope biomarkers within the context of known dietary patterns.

In summary, we have demonstrated that stable nitrogen and carbon isotope ratios in RBC indicate use of traditional and market foods in a Yup'ik study population in Southwest Alaska. Isotopic biomarkers have great potential due to their affordability, their reliability, and their ability to be measured noninvasively and with low burden (17,

18). Furthermore, because these markers can be measured in stored specimens they have the potential to provide baseline data for studies of dietary change over time (16). The development of reliable biomarkers of traditional and market-based food intake will help in evaluation of the overall health impacts of dietary change in circumpolar populations

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Table 1.1. Nitrogen and carbon isotope ratios of market and traditional foods[1].

Food Group	$\delta^{15}N$ (‰)	$\delta^{13}C$[2] (‰)
Subsistence foods		
Marine[3]	14.2 ± 3.2^{a}	$-21.1 \pm 3.4^{b,c}$
Waterfowl	7.3 ± 2.1^{b}	$-23.8 \pm 3.6^{c,d}$
Terrestrial Animals	$3.7 \pm 4.1^{b,c}$	$-24.5 \pm 4.2^{c,d}$
Terrestrial Plants	-0.3 ± 2.2^{c}	-27.4 ± 1.8^{d}
Market foods		
Corn and Cane Sugar	$4.0 \pm 0.6^{b,c}$	-12.4 ± 1.3^{a}
Meat	$3.7 \pm 1.4^{b,c}$	-17.2 ± 1.2^{b}
Mixed	1.9 ± 2.3^{c}	$-21.2 \pm 2.2^{b,c}$
Dairy	$4.5 \pm 0.5^{b,c}$	$-21.4 \pm 1.3^{b,c,d}$
Grains and Vegetables	2.1 ± 1.9^{c}	-26.6 ± 2.0^{d}

[1]Values are mean ± standard deviation, $n = 254$. Means in a column without a common letter differ, $P < 0.05$. Means were calculated using the mean isotope ratio for each food within the group, given in Supplemental Table 1.

[2]Carbon isotope ratios are negative, as all samples have less ^{13}C than the standard against which they are measured

[3] Includes both marine and freshwater fish species

Table 1.2. Effect of sex, age and community location on $\delta^{15}N$, $\delta^{13}C_A$ values and self-reported intake of traditional marine and C_4-based market foods in a community based sample of Yup'ik people participating in the Center for Alaska Native Health Research study[1].

	Isotope measures			Self-report measures		
	n	$\delta^{15}N$, ‰	$\delta^{13}C_A$[2], ‰	*n*	Traditional marine foods, *% total energy*	C_4–based market foods, *% total energy*
Whole population						
Total	1003	9.0 ± 1.5	-20.8 ± 1.6	230	13 ± 14	33 ± 14
Sex						
Male	460	8.8 ± 1.5	-20.7 ± 1.6	95	12 ± 14	33 ± 15
Female	543	9.1 ± 1.5	-21.0 ± 1.6	135	14 ± 14	33 ± 13
Age, *y*						
14 – < 20	200	7.8 ± 0.7	-19.6 ± 0.8	59	6 ± 9	43 ± 13
21 – < 40	374	8.6 ± 1.1	-20.4 ± 1.2	81	13 ± 12	35 ± 13
41 – < 60	303	9.5 ± 1.4	-21.4 ± 1.4	78	18 ± 15	25 ± 11
>60	126	10.7 ± 1.7	-22.8 ± 1.4	12	21 ± 20	21 ± 10

Table 1.2 continued

Location,%						
Coastal	402	9.6 ± 1.8	-21.2 ± 1.7	88	15 ± 16	34 ± 14
Upriver	601	8.6 ± 1.1	-20.6 ± 1.4	142	12 ± 12	32 ± 14
Cultural identification subset						
Total	767	9.0 ± 1.5	-20.8 ± 1.5	216	13 ± 14	33 ± 14
Yup'ik						
High	349	9.3 ± 1.6	-21.2 ± 1.6	92	16 ± 17	29 ± 13
Medium	399	8.7 ± 1.4	-20.5 ± 1.4	118	11 ± 11	35 ± 13
Low	19	7.8 ± 0.9	-19.6 ± 1.3	6	2 ± 4	48 ± 21
Kass'aq						
High	129	8.3 ± 1.2	-20.0 ± 1.3	44	8 ± 9	36 ± 13
Medium	575	9.0 ± 1.5	-20.9 ± 1.5	159	14 ± 15	33 ± 14
Low	63	9.7 ± 1.8	-21.5 ± 1.7	13	18 ± 14	30 ± 15

[1]Values are mean ± standard deviation

[2] Carbon isotope ratios are negative, as all samples have less ^{13}C than the standard against which they are measured

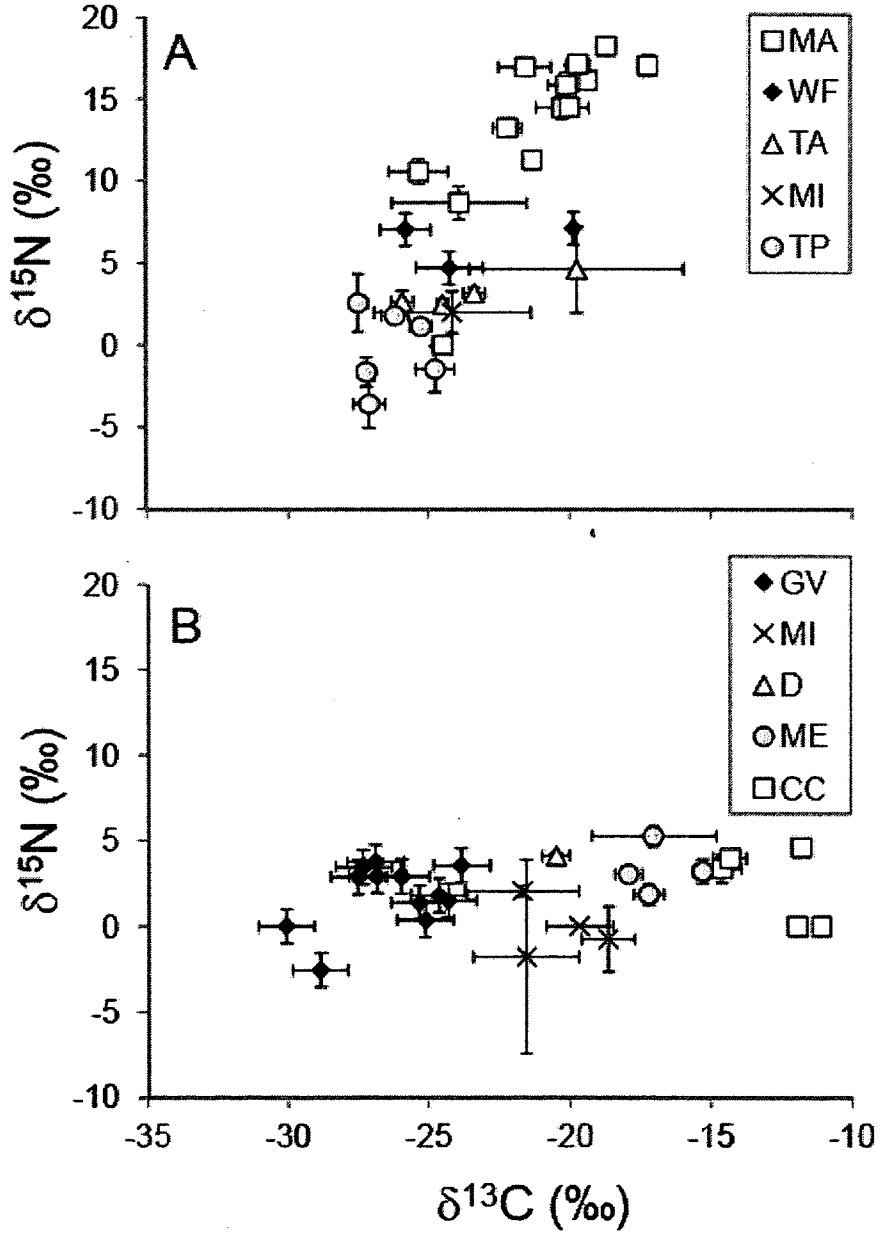
Table 1.3. Relationships between stable isotope biomarkers and self-reported measures of traditional marine and C_4-based market food intake (n = 230).

	β^2	CI	Intercept	r	*P*
$\delta^{15}N$[1] vs Total marine (%)	0.06	0.05 – 0.07	- 0.4	0.52	<0.0001
Total marine (kcal) vs Total traditional (kcal)[2]	0.05	0.04 – 0.06	56.7	0.93	<0.0001
$\delta^{13}C_A$ vs C_4 (%)	0.05	0.0.4 – 0.06	1.3	0.46	<0.0001
$\delta^{13}C_A$ vs Total market (%)	0.05	0.04 – 0.07	1.9	0.46	<0.0001
C_4 (kcal) vs Total market (kcal)	1.55	1.39 – 1.71	594.6	0.78	<0.0001

[1] Independent variables are listed first

[2]Indicates that the dependent variables have been transformed; estimates of β are back-transformed for ease of interpretation, and are interpreted as proportional increase in the dependent variable for each 1‰ increase in the isotopic independent variable

Figure 1.1. Carbon and nitrogen isotope ratios of a representative sample of A) traditional and B) market food items reportedly consumed by this Yup'ik study population. Only foods with $n > 2$ are represented in this figure. Foods are assigned to groups, which are abbreviated in the legend as follows: MA, Marine; WF, Waterfowl; TA, Terrestrial animals; MI, Mixed; TP, Terrestrial plants; GV, Grains and vegetables; D, Dairy; ME, Non-traditional meats; CC, corn and cane sugar-based.



APPENDIX

Supplemental Table 1.S1. Stable carbon and nitrogen isotope ratios of Yup'ik traditional and market foods, by isotopic food group[1].

Food Name	Food Group	*n*	$\delta^{15}N$	$\delta^{13}C$
Traditional foods				
Akutaq[2]	Mixed	3	2.0 ± 1.8	-24.2 ± 4.8
Blackfish[3]	Marine	1	8.5	-27.8
Dolly varden[3]	Marine	3	16.1 ± 0.4	-19.4 ± 0.2
Flounder	Marine	2	14.5 ± 1.0	-20.3 ± 1.3
Halibut	Marine	4	17.0 ± 1.3	-17.2 ± 0.3
Herring	Marine	12	14.5 ± 2.1	-20.0 ± 1.0
Herring eggs	Marine	5	16.0 ± 2.3	-20.0 ± 0.8
King salmon	Marine	8	13.2 ± 0.6	-22.2 ± 1.4
Burbot[3]	Marine	2	16.9 ± 0.1	-21.6 ± 1.3
Needle fish[3]	Marine	1	10.2	-25.9
Pike[3]	Marine	8	10.6 ± 2.1	-25.3 ± 2.9
Salmon[4]	Marine	15	11.3 ± 1.0	-21.3 ± 1.1
Seal	Marine	9	15.7 ± 2.6	-20.0 ± 2.2
Seal oil[5]	Marine	3	-	-24.5 ± 0.6
Seaweed	Marine	1	11.5	-16.0

Supplemental Table 1.S1 continued

Smelt	Marine	4	17.1 ± 0.4	-19.7 ± 0.8
Stink fish[6]	Marine	1	13.1	-23.2
Tomcod	Marine	4	18.2 ± 1.0	-18.7 ± 0.6
Trout[3]	Marine	1	16.5	-18.7
Whitefish[3]	Marine	2	8.7 ± 1.4	-23.9 ± 3.4
Bear	Terrestrial Animals	1	10.8	-21.6
Caribou[4]	Terrestrial Animals	11	3.2 ± 1.0	-23.4 ± 1.3
Moose[4]	Terrestrial Animals	6	2.6 ± 1.7	-25.9 ± 0.9
Moose fat	Terrestrial Animals	1	-1.7	-31.9
Muskox	Terrestrial Animals	2	2.5 ± 0.4	-24.5 ± 0.3
Ptarmigan	Terrestrial Animals	2	4.6 ± 3.8	-19.8 ± 5.3
Beach greens	Terrestrial Plants	4	2.6 ± 3.5	-27.5 ± 0.7
Blackberries	Terrestrial Plants	3	-1.5 ± 2.4	-24.8 ± 1.2
Buttercup	Terrestrial Plants	4	1.8 ± 1.0	-26.2 ± 0.9
Crowberry[5]	Terrestrial Plants	1	-	-26.2
Mouse food[7]	Terrestrial Plants	2	-1.6 ± 1.2	-27.2 ± 0.4
Pond plant	Terrestrial Plants	1	-0.6	-31.0
Salmonberries	Terrestrial Plants	5	1.1 ± 1.0	-25.3 ± 0.9
Sourdock	Terrestrial Plants	1	2.8	-28.9

Supplemental Table 1.S1 continued

Stinkweed	Terrestrial Plants	1	-2.7	-29.3
Tundra tea	Terrestrial Plants	3	-3.6 ± 2.5	-27.1 ± 1.0
Unknown fruits	Terrestrial Plants	1	-0.8	-28.1
Crane	Waterfowl	1	6.8	-22.9
Duck	Waterfowl	1	11.1	-20.5
Goose	Waterfowl	8	7.0 ± 4.2	-25.8 ± 2.5
Goose gizzard	Waterfowl	2	4.7 ± 3.1	-24.2 ± 1.7
Swan	Waterfowl	3	7.1 ± 0.4	-19.9 ± 0.2
Waterfowl (unknown)	Waterfowl	1	7.3	-29.5
Market foods				
Corn chips	Corn and Cane Sugar	3	3.4 ± 1.4	-14.6 ± 1.2
Diet Soda[5]	Corn and Cane Sugar	1	-	-11.4
Ketchup	Corn and Cane Sugar	3	4.0	-14.3 ± 1.0
Pancake syrup[5]	Corn and Cane Sugar	1	-	-11.2
Preserves[5]	Corn and Cane Sugar	1	-	-12.8
Soda[4,5]	Corn and Cane Sugar	9	-	-11.1 ± 0.5
Sugar	Corn and Cane Sugar	3	4.6	-11.8 ± 0.1

Supplemental Table 1.S1 continued

Sugar-based candy[5]	Corn and Cane Sugar	1	-	-12.1
Tang[4,5]	Corn and Cane Sugar	4	-	-12.0 ± 0.4
Cheese	Dairy	1	4.9	-22.3
Milk	Dairy	6	4.1 ± 0.3	-20.5 ± 1.2
Bread	Grains and Vegetables	2	1.5 ± 1.9	-24.3 ± 0.4
Cakes and cookies	Grains and Vegetables	2	2.9 ± 1.4	-26.0 ± 1.7
Cereals	Grains and Vegetables	3	1.4 ± 2.6	-25.3 ± 0.5
Condiments[8]	Grains and Vegetables	3	2.9 ± 2.7	-27.5 ± 2.5
Honey	Grains and Vegetables	1	5.0	-25.8
Margarine	Grains and Vegetables	1	1.6	-30.8
Fruits	Grains and Vegetables	4	3.5 ± 0.6	-23.8 ± 2.9
Vegetables	Grains and Vegetables	4	0.4 ± 2.5	-25.1 ± 0.9
Nuts	Grains and Vegetables	1	-0.5	-27.7
Oils	Grains and Vegetables	3	-2.6	-28.8 ± 1.4
Pasta[4]	Grains and Vegetables	3	1.8 ± 0.2	-24.6 ± 0.5
Pilot cracker[4]	Grains and Vegetables	3	3.4 ± 0.3	-27.3 ± 0.6
Potato chips	Grains and Vegetables	3	3.7 ± 1.7	-26.9 ± 1.3
Rice[4]	Grains and Vegetables	6	2.9 ± 2.1	-26.8 ± 0.4

Supplemental Table 1.S1 continued

Vegetable shortening[4,5]	Grains and Vegetables	3	-	-30.1 ± 0.1
Beef[4]	Meats	4	5.2 ± 1.2	-17.0 ± 4.4
Chicken[4]	Meats	2	1.9 ± 0.9	-17.2 ± 0.8
Eggs	Meats	1	5.0	-18.5
Pork	Meats	3	3.2 ± 1.2	-15.3 ± 0.5
Turkey[4]	Meats	2	3.0 ± 0.3	-17.9 ± 0.7
Candy	Mixed	2	-1.8 ± 8.0	-21.6 ± 2.6
Canned fruits	Mixed	1	3.9	-22.8
Creamer[5]	Mixed	3	-	-19.7 ± 2.0
Ice cream	Mixed	1	4.6	-20.9
Kool aid	Mixed	1	3.4	-24.7
Mixed dish	Mixed	2	2.0 ± 0.4	-21.7 ± 2.8
Sweetened/corn cereals	Mixed	3	0.6 ± 3.0	-17.3 ± 2.5

[1]Values are mean ± standard deviation

[2] "Akutaq" is a traditional food, which is now commonly made with a mixture of market and traditional ingredients (vegetable shortening, sugar, and traditional or market fruits), and has therefore been classed as a "mixed" food item. It was traditionally made with seal oil or blubber.

[3]These fish species are known to live and feed completely, or partly in freshwater habitats.

[4]Isotope ratios for 42 of these food samples have been previously reported (28).

[5] Samples did not contain nitrogen, or contained too little nitrogen for accurate measurement.

[6]"Stink fish" is fermented fish.

[7] "Mouse food" are rodent caches of edible seeds and roots that are harvested from the tundra.

[8] "Condiments" includes items such as mayonnaise, mustard and soy sauce that do not contain large amounts of corn or cane sugar

CHAPTER 2. RELATION BETWEEN STABLE ISOTOPE RATIOS IN HUMAN RED BLOOD CELLS AND HAIR: IMPLICATIONS FOR USING HAIR ISOTOPIC VALUES AS A BIOMARKER OF EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID[1]

2.1. ABSTRACT

Background: The nitrogen isotope ratio (expressed as $\delta^{15}N$) of red blood cells (RBC) is highly correlated with RBC long chain omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in Yup'ik people. Because $\delta^{15}N$ can also be measured in hair samples, it could provide a non-invasive, retrospective biomarker for EPA and DHA intake.

Objective: We investigated the agreement between $\delta^{15}N$ in hair and RBC, and then evaluated the relationships between hair $\delta^{15}N$ and RBC EPA and DHA. We also evaluated the agreement in carbon isotope ratios ($\delta^{13}C$) between hair and RBC, as $\delta^{13}C$ has also been proposed as a dietary biomarker in certain populations.

Design: We assessed relationships between hair and RBC $\delta^{15}N$ and $\delta^{13}C$ in a community-based sample of 144 Yup'ik participants, and examined the correlations between $\delta^{15}N$ and RBC EPA and DHA in a subset of these participants (n =44).

Results: We demonstrated a 1:1 relationship with good agreement between hair and RBC $\delta^{15}N$ ($r = 0.91$) and $\delta^{13}C$ ($r = 0.87$). Hair isotope ratios were elevated over RBC by 1.5‰ for $\delta^{15}N$ and 2.3‰ for $\delta^{13}C$. There were strong correlations between hair $\delta^{15}N$ and RBC EPA and DHA ($r = 0.83$ and 0.84).

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[1] **Nash, S.H.**, Kristal, A.R., Boyer, B.B., King, I.B., Metzgar, J.S., and O'Brien, D.M. 2009. Relation between stable isotope ratios in human red blood cells and hair: implications for using the nitrogen isotope ratio of hair as a biomarker of eicosapentaenoic acid and docosahexaenoic acid. *The American Journal of Clinical Nutrition* 90:1642-1647

Conclusions: These results support the use of hair $\delta^{15}N$ values as a biomarker of EPA and DHA intake. Because hair collection is non-invasive and the samples require no special processing, studies of EPA and DHA intake in large populations could use biomarkers rather than self-report to assess these fatty acids.

2.2. INTRODUCTION

Naturally occurring variations in stable isotope ratios are gaining attention for their potential to serve as unbiased biomarkers of diet (1-4). Recently, the nitrogen isotope ratio (expressed as $\delta^{15}N$) of red blood cells (RBC) was shown to be highly correlated with RBC omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and validated as a biomarker of EPA and DHA intake in a Yup'ik study population (4). Values of $\delta^{15}N$ act as a biomarker for these omega-3 fatty acids in this population because both are elevated in marine subsistence foods (3), and these foods are an important component of the traditional diet (5). Because measurement of RBC $\delta^{15}N$ is inexpensive and highly accurate, it is ideal for large-scale epidemiological studies of EPA/DHA intake and disease in Alaska Native people. However, sampling blood is invasive and processing and storing samples is expensive. Here we investigate whether $\delta^{15}N$ in hair is equally valid as a biomarker of RBC EPA/DHA, thus allowing measurement to be entirely non-invasive. Isotopic markers in hair have an additional advantage over blood: as hair is grown continuously, hair provides a continuous record of the biomarker back through time, facilitating analysis of dietary seasonality or annual change.

Stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) have also been used as markers of animal protein intake (1, 6, 7) and traditional food intake in Greenland Eskimos (3), Amazonian Indians (8) and Gidra-speaking Papuans (9). In these studies, isotope ratios were measured in either hair or fingernails, and the isotopic relationship between these tissues is well understood (7, 10). Yet several recent studies report stable

isotope ratios in blood only (4, 11, 12), and the relationship between isotope ratios in human blood and hair has not been characterized. Thus, an understanding of how these markers are related will facilitate comparison of isotopic markers among these and future studies, especially where investigations take advantage of blood collected from clinical or epidemiological studies (11). However, we note that $\delta^{13}C$ is not related either to animal protein intake or intake of marine foods in our population (4).

Here we examine the relationship and agreement between stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) in hair and RBC from 144 participants in the Center for Alaska Native Health Research I (CANHR I) study (13). We also investigate the relationships between hair $\delta^{15}N$ and RBC EPA and DHA in a subset of 44 participants. RBC EPA and DHA vary with EPA and DHA intake, and are validated biomarkers for these important omega-3 fatty acids (14-16). The CANHR I study population is ideal for testing the relationship between hair $\delta^{15}N$ and because participants have widely varying levels of EPA and DHA intake, depending on the degree to which individuals adhere to a traditional, marine-based diet (5, 17).

2.3. Subjects and methods

2.3.1. The CANHR population

Data are from the 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 people. Between 2003 to 2005, 1003 men and women, ages 14 and older, were recruited from 10 communities in Southwest Alaska, as described in detail elsewhere (13, 18). At entry into the study, participants completed an extensive interviewer-administered questionnaire covering demographic characteristics, economic status, ethnicity, and medical history. Diet interviews, body measurements, blood pressure and biological samples were also collected. The

CANHR I 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 Studies Committee.

2.3.2. Study sample

Our study sample was drawn from the last three communities to participate in the CANHR I study, because these were the only CANHR I participants from whom both hair and blood samples were available. Of 210 participants from these three communities, 144 were included in this study based on having hair >3 cm in length. Blood samples were available for all 144 of these participants. Analyses of RBC fatty acids were conducted for study participants from the first of these three communities, n = 44. Comparisons between hair $\delta^{15}N$ and RBC EPA and DHA were based on that sample.

2.3.3. Specimen collection

Blood samples were collected into 10 ml K3 Ethylenediaminetetraacetic acid (EDTA)-treated Vacutainer® Whole Blood tubes (15% solution, 0.117 ml, 17.55 mg), and centrifuged for 15 minutes at 1000 rpm. The RBC portion was frozen at -12°C, transported to the University of Alaska Fairbanks, and placed in an ultra-low freezer at -80°C. Aliquots were removed for both stable isotope and fatty acid analysis (below).

Three hairs were collected by either pulling or cutting three hairs from the back of the head, and most participants elected to pull their own hair samples. Samples were taped with the follicle end labeled and stored in plastic bags. The follicle was removed with a razor blade and the 2 cm of hair closest to the scalp was selected for analysis. The length of the hair sample was chosen to correspond with average age of RBC in the blood, and therefore the dietary inputs recorded by the RBC. Hair grows at a rate of approximately 1 cm per month (19-21) and thus our samples were expected to be

reflective of the last two months of intake. RBC have a lifespan of approximately 90 - 120 days (22-24) and a mean age of ~50 days (23). Therefore, the hair sample bracketed the time when the majority of RBC were synthesized.

2.3.4. Stable isotope analyses

Aliquots of RBC (250 µl) were autoclaved for 20 minutes at 121°C to destroy blood-borne pathogens. Samples were then apportioned into 3.5 x 3.75 mm tin capsules and dried to a final mass of 0.2 - 0.4 mg. Hair samples were cleaned with triplicate 30 minute rinses in 2:1 methanol chloroform. Hairs were chopped into small pieces, placed into 11 x 8 mm tin capsules and oven dried at 50°C for 24 hours. The resulting sample masses ranged from 0.1 to 0.6 mg. Capsules were crushed into balls and loaded into an autosampler for isotope analysis.

Blood and hair samples were analyzed for carbon and nitrogen isotope ratios at the Alaska Stable Isotope Facility, using continuous-flow isotope ratio mass spectrometry. A Costech ECS4010 Elemental Analyzer (Costech Scientific Inc., Valencia, CA) combusted samples to CO_2 and N_2 gas, which were carried in a constant flow of helium to a Finnigan Delta Plus XP isotope ratio mass spectrometer via the Conflo III interface (Thermo-Finnigan Inc., Bremen, Germany). Data are presented in the accepted delta notation as $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \cdot 1000‰$, where R is the ratio of heavy to light isotope (for both nitrogen and carbon) and the internationally recognized standards are atmospheric nitrogen and Pee Dee Belemnite (PDB) for carbon (25). We concurrently weighed and ran multiple peptone standards ($\delta^{15}N = 7.0‰$, $\delta^{13}C = -15.8‰$) to assess analytical accuracy and precision; these gave values of $\delta^{15}N = 7.1 \pm 0.3‰$ (SD) and $\delta^{13}C = -15.7 \pm 0.2‰$ (SD). The purity of the samples was assessed through the molar C: N ratios, which were 3.0 ± 0.1 for hair and 3.3 ± 0.1 for blood. The

C: N ratios for hair were consistent with pure keratin values from other published studies (7, 10, 26). The C: N ratios for RBC have not been previously published; however, the small deviations of these values reflected a lack of contamination.

2.3.5. RBC fatty acid measurements

The RBC fatty acids were analyzed at the Fred Hutchinson Cancer Research Center in Seattle, WA, as described in detail elsewhere (4). Fatty acids were extracted from RBCs using modified methods of Rose and Oklander (27). The lipid extract was transesterified and processed according to Lepage and Roy (28). Fatty acid methyl esters (FAMEs) were recovered in hexane, dried under nitrogen (40 °C) and re-dissolved in 100 ml hexane for gas chromatography.

FAMEs were injected in a split mode (1:50) and were separated using an SP-2560 capillary column (100 m x 0.25 mm x 0.2 mm) (Supelco, Bellefonte, PA) on a Hewlett-Packard, Model 5890B gas chromatograph (GC-FID) (now Agilent, Santa Clara, CA). This method allowed the resolution of 46 different membrane fatty acids. The accuracy of the chromatographic system was monitored using commercial standards (GLC-87, NIH-D, and NIH-F, NU-CHEK, Elysian, MN). The precision of the RBC fatty acids was monitored with repeat analysis of an in-house RBC quality control pool that was included in each batch of 23 study samples. The coefficient of variation for EPA (20:5n-3) was 2.7% and for DHA (22:6n-3) was 2.0%. Fatty acid composition is reported as a weight percent of the total RBC fatty acids.

2.3.6. Statistical analyses

All statistical analyses were performed using JMP version 8 (SAS Institute, Cary, NC). Differences between sex and age strata were assessed using analysis of variance models (ANOVA) using the Tukey Kramer HSD test for individual comparisons.

Relationships between isotope signatures in hair and blood, and between isotope signatures and fatty acid biomarkers were assessed using correlation analysis (presented as Pearson's r). Agreement between hair and blood isotope ratios was evaluated as the mean and standard deviation of their differences, following Bland and Altman (29). Where parametric assumptions were met, we tested the effect of age and sex on the relationship between $\delta^{15}N$ and fatty acids using a factorial linear model, and described the slope of the isotopic relationships between blood and hair with linear regression. Normality of residuals was confirmed using the Shapiro-Wilks test, and outliers were identified using Mahalobnis Distance > 3. A significance level of 0.05 was used throughout analyses.

2.4. RESULTS

Table 2.1 presents the demographic characteristics of our study population, including age, sex and body mass index (BMI) distribution. Because we only collected hair samples when hair was > 3 cm in length, women are over-represented in our study sample (n = 144) when compared to all CANHR 1 participants from the communities presented here (76% vs. 53%, $X^2 = 30.3$, $P < 0.0001$, Table 2.1). We also present age, sex and BMI distributions for samples from the single community for which we also measured RBC fatty acids ("fatty acid sub-sample", n = 44, Table 2.1). The sex, age and BMI distributions did not differ between the full isotope dataset and the fatty acid sub-sample ($P > 0.05$ in all cases).

Table 2.2 gives descriptive statistics for the $\delta^{15}N$ and $\delta^{13}C$ values of RBC and hair, and EPA and DHA in RBC. Mean $\delta^{15}N$ and $\delta^{13}C$ values did not differ between males and females for either RBC or hair in the full isotope data set. For the fatty acid subsample females were slightly but significantly higher in RBC $\delta^{13}C$ values than males (0.4‰; $P = 0.006$). The mean EPA and DHA values did not differ by sex (EPA, P = 0.77; DHA, $P = 0.17$ in the fatty acid subsample).

2.4.1. Relationship between hair and blood isotope values

RBC isotope ratios were very strongly correlated with hair isotope ratios for the 2 cm of hair closest to the scalp ($r = 0.93$ for $\delta^{15}N$ and $r = 0.81$ for $\delta^{13}C$, **Table 2.3**, **Figure 2.1**). Hair values were consistently elevated over RBC; however, agreement between the measures was very good once this bias was accounted for (mean difference = 1.5 ± 0.6‰ for $\delta^{15}N$ and 2.3 ± 0.4‰ for $\delta^{13}C$). Linear regression of isotope values in hair against blood gave slopes that were not statistically different from 1.0 (slopes = 1.01 for $\delta^{15}N$ and 0.96 for $\delta^{13}C$); however, residuals were non-normal due to the influence of several outliers (2 outliers from the relationship between blood and hair $\delta^{15}N$, and 6 outliers from the relationship between blood and hair $\delta^{13}C$). Removal of these outliers normalized the residuals and altered slopes to 1.05 for $\delta^{15}N$ and 1.00 for $\delta^{13}C$. Neither age nor sex had any effect on the relationship between isotope ratios in hair and blood, nor were any interactions significant.

2.4.2. Relationship between isotope values and fatty acids

Both hair and RBC $\delta^{15}N$ values correlated strongly with percentage of EPA and DHA in RBC (all $r > 0.8$ and $P < 0.0001$; Table 2.3). For both fatty acids, the correlation with RBC $\delta^{15}N$ was stronger than with hair $\delta^{15}N$; however, the 95% confidence intervals indicate that this difference was only significant for EPA (Table 2.3). The relationship between RBC $\delta^{15}N$ and EPA was linear (slope = 1.03, $P < 0.0001$; **Figure 2.2a**), and did not differ when controlled for sex and age. The relationship between hair $\delta^{15}N$ with EPA was also linear (slope = 0.87, $P < 0.0001$, **Figure 2.2b**), and was significantly stronger in older participants (slope = 0.42 for those <40 years, 0.96 for those >40 years, $P_{age} = 0.04$, $P_{age*hair\ \delta 15N} = 0.001$). No other interactions were significant. As previously observed (4), the relationship between RBC DHA and hair and RBC $\delta^{15}N$ was non-linear (**Figures 2.2c, 2.2d**).

2.5. DISCUSSION

Hair and RBC isotope ratios were tightly correlated in our study population (r = 0.93 for $\delta^{15}N$ and 0.81 for $\delta^{13}C$, $n = 144$), and showed good agreement. Hair $\delta^{15}N$ was strongly correlated with RBC polyunsaturated fatty acids EPA and DHA in a subset of these participants (r = 0.83 and 0.84 respectively, $n = 44$). The relationships of fatty acids with RBC $\delta^{15}N$ were stronger than with hair $\delta^{15}N$, although the difference was only significant for EPA. Because RBC $\delta^{15}N$, EPA and DHA are established biomarkers for EPA and DHA intakes (4, 30-33) this demonstrates the validity of hair $\delta^{15}N$ to also serve as an intake biomarker for EPA and DHA in this population.

The finding that hair $\delta^{15}N$ is significantly correlated with RBC EPA and DHA is an important development for investigators interested in the impact of EPA and DHA intake on health outcomes, especially in Alaska Native people. Hair can be collected easily and non-invasively, which makes it an ideal tissue to sample for the large-scale epidemiological studies needed to investigate the health consequences of dietary change in this population. An additional advantage of hair is that it grows continuously and does not remodel after growth; therefore, it provides a dietary record over the length of the participant's hair (approximately 1 month / cm of hair). High resolution sampling along the length of hair provides the opportunity to assess seasonal variation in EPA and DHA intake, something that has not been addressed in this population and which is likely to show important variation. Omega-3 fatty acids and EPA/DHA in particular, have been long suspected to protect against diabetes and other chronic disease in northern indigenous populations (30-33). However, until recently there have been no longitudinal studies to clearly link diet to disease incidence in Alaska Native people. This biomarker will greatly enhance our ability to detect these associations.

Stable isotope analysis of hair has been a widespread tool for detecting dietary patterns in anthropological and archaeological studies (26, 34, 35) and has been increasingly applied to modern populations (1, 3, 6-8, 10, 36, 37). However, specimens

collected for medical or epidemiological studies are likely to include blood but not hair (4, 11). This study is the first to measure isotope ratios in both blood and hair and describe the relationship between $\delta^{15}N$ and $\delta^{13}C$ values in the two tissues. For both isotopes, the relationship between hair and blood was 1:1, with hair enriched relative to blood by 1.5 ± 0.6 ‰ for $\delta^{15}N$ and 2.3 ± 0.4‰ for $\delta^{13}C$. Animal studies have also demonstrated isotopic enrichment of hair over blood (38-40); but the magnitude of these enrichments can vary taxonomically. Having this relationship determined for a human population provides a basis for comparison between human studies based on one or the other tissue. However, further work is necessary to evaluate how universal these relationships are among other human populations.

Although correlations between blood and hair stable isotope ratios were strong and followed the expected 1:1 relationship, we found outliers in both the relationships between blood and hair $\delta^{15}N$ and $\delta^{13}C$ (particularly for carbon, for which approximately 4% of observations were classified as outliers). It is possible that these outliers represent analytical errors, although we consider this unlikely given the accuracy and precision of the isotope analyses. Alternatively, the comparatively poor isotopic match between hair and blood for a small number of samples may result from the fact that approximately 10-15% of head hairs are in the telogen phase and not growing (41), and we only sampled 2-3 hairs in our analysis. The diet reflected by non-growing hairs would not be synchronized with that of the blood if a participant's diet varied over time, as might be expected for participants relying on seasonal subsistence foods. We recommend that future studies homogenize at least ten head hairs to minimize potential error caused by non-growing hairs.

There are several limitations to this study. The study sample was not representative of the underlying population because hair length >3cm was required. The sample size for comparisons with fatty acids was small; however, there was still sufficient power to detect a modest difference in correlation between hair and blood $\delta^{15}N$ and EPA. Finally, the range of DHA and EPA intakes in Yup'ik people is far broader

than most populations, and thus findings may differ in populations with a more restricted intake of marine foods.

In summary, we found that hair $\delta^{15}N$ correlated strongly with the omega-3 fatty acids EPA and DHA measured in RBC. We propose that hair $d^{15}N$ can be used a non-invasive, inexpensive, high throughput biomarker to estimate EPA and DHA intake. Non-invasive sampling methods decrease participant burden and facilitate inclusion of biomarker data in a broad range of health studies. We also demonstrated very tight correlation and good agreement between hair and RBC $\delta^{15}N$ and $\delta^{13}C$. These findings will enable meaningful comparisons to be made between studies sampling these different tissues. There have been recent calls for the development of biomarker-based methods of diet investigation (42-44). This study helps advance the application of stable isotope measurements to dietary assessment.

2.6. ACKNOWLEDGEMENTS

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Table 2.1. Age, sex and BMI distribution of all participants recruited by CANHR in the three study communities (n = 210), the set of participants with hair isotopic measurements (n = 144) and the sub-sample of participants with fatty acid data (n = 44).

	All CANHR participants (n = 210)[1]	Hair isotope dataset (n = 144)	Fatty acid sub-sample (n = 44)
Age (y)	37.3 ± 17.7[2]	39.4 ± 18.0	37.3 ± 17.2
14- 24 yr (%)	17	29	36
25- 39 yr (%)	43	26	21
40-54 yr (%)	29	27	27
55+ yr (%)	11	18	16
Sex			
Male (%)	47	24	32
Female (%)	53	76	68
Body Mass Index (kg/m^2)			
<18.5 (%)	1	1	-
20-25 (%)	38	35	43
25-30 (%)	33	34	25
> 30(%)	28	30	32

[1] From the 3 communities sampled for this study

[2] Mean ± SD (all such values)

[3] The distribution of BMI, age and sex is not significantly different between the two study subsamples using the Chi-squared test (in all cases $P > 0.05$), although both include significantly more women than the recruited study population ($P < 0.0001$).

Table 2.2. Means and distributions of biomarker variables for the hair isotope dataset of participants (n = 144) and the fatty acid sub-sample (n = 44).

		Hair	Blood
Hair isotope dataset	*n = 144*		
$\delta^{15}N$ (‰)	Mean ±SD	10.8 ± 1.9	9.3 ± 1.7
	Range	8.5	7.0
$\delta^{13}C$ (‰)	Mean ±SD	-17.5 ± 0.7	-19.8 ± 0.6
	Range	3.5	3.2
Fatty acid sub-sample	*n = 44*		
$\delta^{15}N$ (‰)	Mean ±SD	10.4 ± 1.8	9.1 ± 1.7
	Range	7.2	6.4
$\delta^{13}C$ (‰)	Mean ±SD	-17.7 ± 0.6	-19.9 ± 0.6
	Range	2.7	2.6
EPA (%)	Mean ±SD	-	2.7 ± 2.0
	Range	-	6.3
DHA (%)	Mean ±SD	-	6.1 ± 1.6
	Range	-	5.8

Table 2.3. Pearson product-moment correlations between isotope values in blood and hair (n = 144), and between fatty acids and $\delta^{15}N$ values in hair and RBC (n = 44)[1].

	Pearson's r	Lower 95% CI	Upper 95% CI
Hair isotope dataset (n = 144)			
RBC $\delta^{15}N$ vs. hair $\delta^{15}N$	0.93	0.91	0.95
RBC $\delta^{13}C$ vs. hair $\delta^{13}C$	0.81	0.75	0.86
Fatty acid sub-sample (n = 44)			
RBC $\delta^{15}N$ vs. hair $\delta^{15}N$	0.91	0.85	0.95
EPA vs. hair $\delta^{15}N$	0.83	0.71	0.91
EPA vs. RBC $\delta^{15}N$	0.92	0.87	0.96
DHA vs. hair $\delta^{15}N$	0.84	0.73	0.91
DHA vs. RBC $\delta^{15}N$	0.90	0.82	0.94

[1] $P < 0.0001$ for all coefficients

Figure 2.1. The relationships between isotope ratios in RBC and hair, for (A) $\delta^{15}N$ and (B) $\delta^{13}C$, $n = 144$. Open symbols denote samples identified as outliers by Mahalobnis Distance > 3.0; these were removed for linear regression and calculation of slope. Nitrogen and carbon isotope ratios are presented in delta notation as $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \cdot 1000‰$, where R is the ratio of heavy to light isotope and standards are Air N for nitrogen and PDB for carbon. Carbon isotope ratios are negative, as they contained less ^{13}C than the standard.

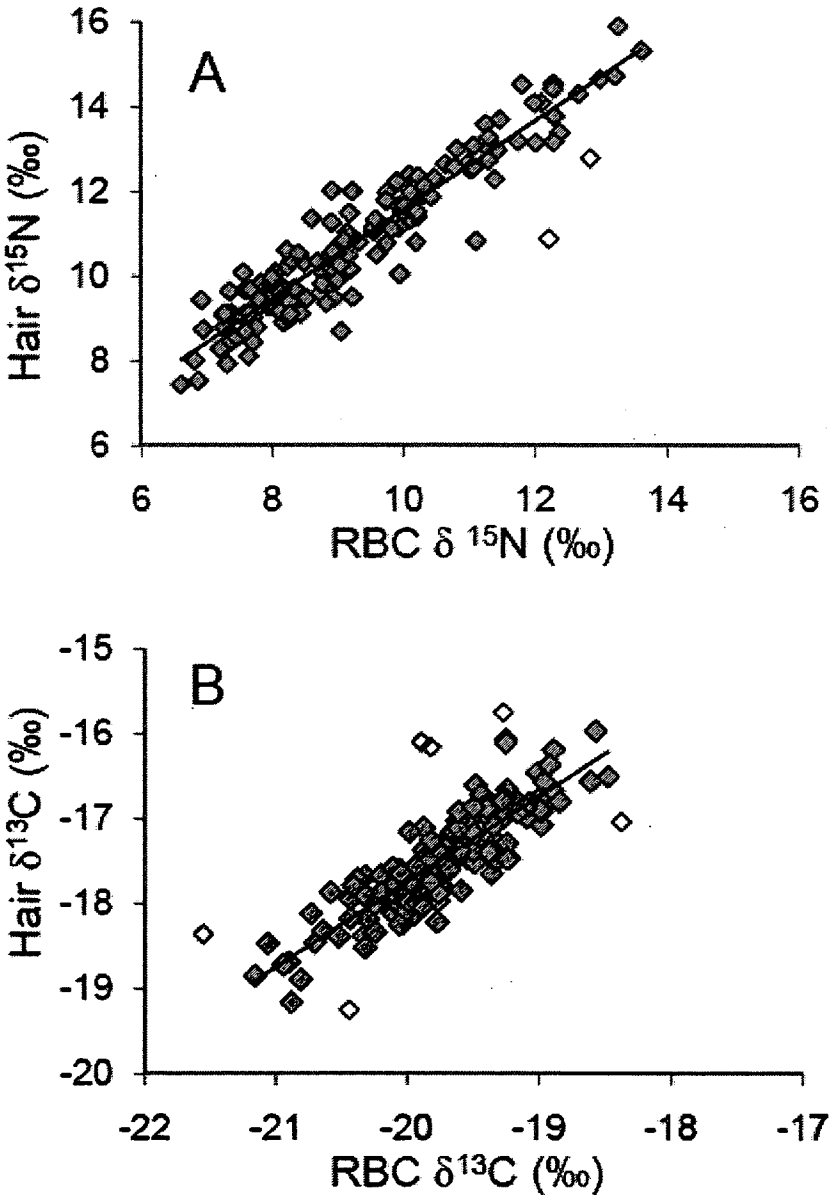
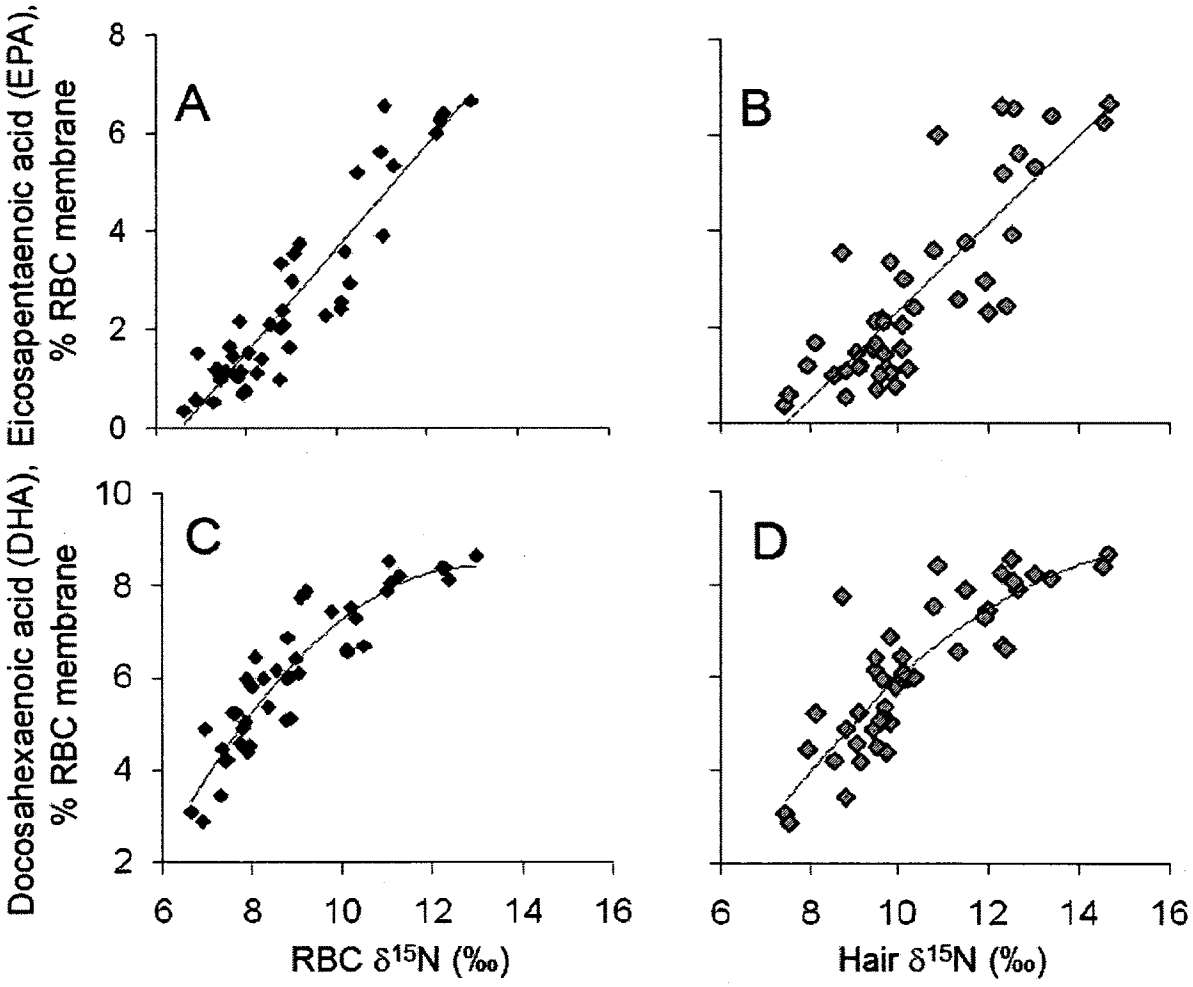


Figure 2.2. RBC and hair $\delta^{15}N$ were significantly correlated with RBC fatty acids EPA and DHA, $n = 44$, all $P < 0.0001$. Correlation coefficients are given in Table 2.3.



CHAPTER 3. CARBON AND NITROGEN ISOTOPE RATIOS PREDICT INTAKE OF SWEETENERS IN A YUP'IK STUDY POPULATION[1]

3.1. ABSTRACT

The carbon isotope ratio ($\delta^{13}C$) is elevated in corn- and cane sugar-based foods, and has recently shown associations with sweetener intake in multiple US populations. However, a high carbon isotope ratio is not specific to corn- and sugar cane-based sweeteners, as other foods, including meats and fish, also have elevated $\delta^{13}C$. This study examines whether the inclusion of a second marker, the nitrogen isotope ratio ($\delta^{15}N$), can control for confounding dietary effects on $\delta^{13}C$ and improve the validity of isotopic markers of sweetener intake. The study participants are from the Yup'ik population of Southwest Alaska and consume large and variable amounts of fish and marine mammals, known to have elevated carbon and nitrogen isotope ratios. Sixty eight participants completed four, weekly 24-h recalls followed by a blood draw. Red blood cell (RBC) $\delta^{13}C$ and $\delta^{15}N$ were used to predict sweetener intake, including total sugars, added sugars and sugar-sweetened beverages. A model including both $\delta^{13}C$ and $\delta^{15}N$ explained more than three times as much of the variation in sweetener intake than did a model using $\delta^{13}C$ only. Because the carbon and nitrogen isotope ratios are determined simultaneously in a single, high-throughput analysis, this dual isotope marker provides a simple method to improve the validity of stable isotope markers of sweetener intake with no additional cost. We anticipate that this multi-isotope approach will have utility in any population where a stable isotope biomarker is elevated in several food groups, and there are appropriate "covariate" isotopes to control for intake of foods not of research interest.

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3.2. INTRODUCTION

There is growing consensus that alternatives to self-reported food intake based on objective biomarkers are needed to more validly study associations of diet and chronic disease risk (1-3). Although most dietary biomarkers are based on concentrations of micronutrients in blood or other tissues (4), we and others have shown that naturally occurring variations in stable isotope ratios can also be used as objective measures of diet (5-9). We are developing stable isotope biomarkers to study associations of diet with chronic disease risk in the Yup'ik population of Southwest Alaska. Our previous work with this population has focused on the nitrogen isotope ratio ($\delta^{15}N$) as an indicator of traditional marine food intake (8, 10); however, we have also shown associations between the carbon isotope ratio ($\delta^{13}C$) and intake of non-traditional (market) foods (7). Here, we consider whether isotope ratios can be used to indicate intake of sweeteners in this population.

Previous studies in other US populations have shown positive associations of the carbon isotope ratio with reported sweetener intake (6, 9), based on the elevated $\delta^{13}C$ values of corn- and cane sugar-based sweeteners (11). However, the carbon isotope marker is not specific to sweetener intake because $\delta^{13}C$ values are also elevated in other foods. For example, commercial meat products have elevated $\delta^{13}C$ values because livestock in the US agricultural system are commonly raised on corn-based feed (12, 13). Furthermore, foods deriving from the marine environment have elevated $\delta^{13}C$ values because oceanic bicarbonate, the source of carbon to marine foodwebs, is enriched in ^{13}C relative to atmospheric CO_2 (14, 15). In the Yup'ik population, fish and marine mammals are an important contributor to the traditional diet (16), and one of the primary contributors of elevated ^{13}C (7). Intake of traditional marine foods can be measured using the nitrogen isotope ratio ($\delta^{15}N$) (8), because fish and marine mammals also have elevated $\delta^{15}N$ values (7). Therefore, we hypothesize that the validity of the carbon isotope biomarker of sweetener intake will be increased by using a multivariable model that controls for marine food intake using $\delta^{15}N$. Here, we test this hypothesis in a

community-based sample of 68 Yup'ik people that completed four, weekly 24-h recalls followed by a blood draw. Carbon and nitrogen isotope ratios are determined simultaneously from a single sample; therefore, this method could provide a simple and inexpensive improvement to isotopic biomarkers of sweetener intake.

3.3. MATERIALS AND METHODS

3.3.1. Participant recruitment and procedures

Data are from the Center for Alaska Native Health Research *Negem Nallunailkutaa* ("Foods' Marker") study. This study was approved by the University of Alaska Fairbanks Institutional Review Board, and the Yukon-Kuskokwim Health Corporation Human Studies Committee.

Between 2008-2009, a community-based sample of 68 participants aged 14-79 were recruited from two coastal communities in Southwest Alaska. At entry into the study, participants completed a demographic questionnaire and the first of four 24 h recall dietary interviews (24HR). Three more dietary interviews were conducted over the next 4 wk. Biological samples were collected at least 2 wk after the completion of the final dietary interview, so that the average age of RBC would match the period during which dietary interviews were conducted (17-19).

3.3.2. Assessment of dietary intake

24HR were collected from each participant by certified interviewers using algorithm-driven, computer assisted software [Nutrition Data System for Research (NDSR) software 2008; University of Minnesota, Minneapolis, MN]. The majority of interviews were completed in person (93%, $n = 261$); some participants completed either one ($n = 15$) or two ($n = 2$) interviews over the telephone. Participants were asked to recall all food and beverages consumed the day prior to interview using a multiple pass

approach. For accuracy, all participants were given portion estimation tools (measuring cups, rulers, and food models or portion estimation guides [Fred Hutchinson Cancer Research Center, Seattle, WA]). Although most participants were bilingual, a native Yup'ik speaker conducted interviews for participants who did not speak English. Dietary interviews were, on average, 9 ± 5 d apart, with a minimum of two days between recalls. Most participants (93%) had three weekday recalls and one weekend recall. No recalls were excluded due to unreasonable intake (20).

The NDSR food and nutrient database (21) was used to calculate food and nutrient intake. In this study, sweetener intake is measured in three ways: as total sugars, added sugars, and sugar sweetened beverages. Total sugar intake (g/d) is defined as the total sum of all mono- and di-saccharides consumed, and includes primarily fructose, glucose and sucrose. Added sugars (g/d) were calculated as the sum of sugars and syrups added to foods during food preparation or commercial food processing. Sugar sweetened beverage intake was calculated as the sum of servings of sweetened soft drinks and sweetened fruit drinks (servings per d, 8 fl oz (237 ml) per serving).

We also give data on intake of other food items that have elevated $\delta^{13}C$ values, including commercial meats (% energy), fish and marine mammals (% energy), and corn products (g/d). Commercial meats were those purchased from local grocery stores, and were distinct from intake of traditional meats and fish and marine mammals. We use these terms to refer specifically to traditional foods harvested from the local environment. Consumption of market purchased fish (i.e., tuna) was minimal: among the nine participants reporting market fish, consumption was on average 36 ± 35 kcal/ d. Corn products included whole corn, and other foods made from whole corn, including popcorn, corn chips, and corn tortillas.

3.3.3. Stable isotope analysis

RBC from fasted blood samples were pipetted into tin capsules, autoclaved and prepared for isotopic analysis as previously described (7). Neither autoclaving nor the use of Ethylenediaminetetraacetic acid (EDTA) tubes affects RBC carbon or nitrogen isotope ratios (22). Samples were analyzed at the Alaska Stable Isotope Facility by continuous-flow isotope ratio mass spectrometry, using 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). The conventional means of expressing natural abundance isotope ratios is as delta values in permil (‰) relative to international standards as $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \cdot 1000‰$ (23). Here, R is the ratio of heavy to light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$). The standards are Vienna PeeDee Belemnite (V-PDB) for carbon and atmospheric nitrogen for nitrogen. To assess analytical precision, an internal working standard was analyzed for every ten samples; precision was measured as the standard deviation of these analyses (0.2‰). Because biological samples from this study have a lower $^{13}C/^{12}C$ than V-PDB, $\delta^{13}C$ values are negative. $\delta^{13}C$ values are hereafter abbreviated as $\delta^{13}C$, and $\delta^{15}N$ values are abbreviated $\delta^{15}N$.

3.3.4. Statistical analyses

The following dietary intake variables were log transformed for analyses: total sugars (g/d), added sugars (g/d), sugar sweetened beverages (servings/d+1), and corn products (g/d+1). Because of known relations between age and dietary patterns (7, 24) we tested whether sex, body mass index (BMI) and dietary intakes of sweeteners, fish and marine mammals, commercial meats and corn products differed by age, using chi-squared and one-way analysis of variance. We examined whether foods with elevated isotope ratios were independently associated with RBC $\delta^{13}C$ and $\delta^{15}N$ using multiple regression models where the isotope ratios were the dependent variables. We report both standardized and

unstandardized beta coefficients for these models. To test whether a model using both $\delta^{13}C$ and $\delta^{15}N$ was a better predictor of sweetener intake than a model using $\delta^{13}C$ only, we used linear regression models. Because the dietary dependent variables were log-transformed for analyses, the beta-coefficients of these models were back transformed for ease of interpretation; these are interpreted as percentage change in the dietary variable for every 1‰ change in isotope ratio. Means are presented ± standard deviation (SD), and statistical significance was set at two-sided $\alpha = 0.05$. Statistical tests were performed using JMP version 8 (SAS Institute, Cary, NC).

3.4. RESULTS

Table 3.1 gives distributions of sex, and means of BMI, isotope ratios, and dietary intake measures by age. The study sample ranged in age from 14 to 79 y (mean = 40 y ± 18) and was evenly divided between men and women. BMI and diet differed substantially by age. Mean BMI and intake of fish and marine mammals, protein and fat increased with age. Intake of total sugar, added sugars, sugar sweetened beverages, commercial meats and carbohydrates decreased with age. There was no association of age with intake of corn products. There was a positive association of $\delta^{15}N$ with age, but no association with $\delta^{13}C$. There was no association between RBC $\delta^{13}C$ and $\delta^{15}N$ ($r = 0.12$, $P = 0.35$).

Table 3.2 gives associations of $\delta^{13}C$ and $\delta^{15}N$ with foods that are known to have elevated isotope ratios. RBC $\delta^{13}C$ was independently associated with total sugar, fish and marine mammals, and commercial meat intake. RBC $\delta^{13}C$ was not associated with intake of corn products, which was low in this population. There were similar results when total sugar was replaced with either added sugars or SSB in the model. RBC $\delta^{15}N$ was strongly associated with intake of fish and marine mammals, but not commercial meats.

Table 3.3 compares two models to predict total sugar, added sugar and SSB intake. The first model is based on $\delta^{13}C$ alone, as has been proposed elsewhere (6, 9). The second model is based upon $\delta^{13}C$ and $\delta^{15}N$, to account for the contribution of elevated $\delta^{13}C$ from fish and marine mammal intake. For all models predicting sweetener intake based on $\delta^{13}C$, the amount of variance explained and the beta-coefficient for $\delta^{13}C$ were increased markedly with the addition of $\delta^{15}N$ as a covariate. The effect of adding $\delta^{15}N$ was most striking for the model to predict total sugar intake, in which the amount of variance explained by the model (R^2) increased from 6% to 48%, and the beta-coefficient increased from 28% to 39% change in total sugar intake (g/d) per 1 ‰ change in the carbon isotope ratio. **Figure 3.1** shows the relationship between reported total sugar intake and the predicted values from a regression model using both $\delta^{13}C$ and $\delta^{15}N$.

3.5. DISCUSSION

This study evaluated a new approach for using isotope ratios to assess intake of corn- and cane sugar-based sweeteners in a Yup'ik study population. It expanded upon a previously proposed method based on $\delta^{13}C$ alone (5, 6, 9, 11), which is subject to confounding due to the elevated $\delta^{13}C$ of foods other than sweeteners. By using $\delta^{15}N$ as a covariate to control for fish and marine mammal intake, the variability in sweetener intake explained by this marker increased substantially, to a maximum of 48% for total sugars. This improvement likely has two causes in this Yup'ik study population: first, by factoring out the significant effect of high fish and marine mammal intakes on $\delta^{13}C$, and second, because of a strong, age-related diet pattern in this population, in which intakes of fish and marine mammals and sweeteners are inversely correlated. However, because $\delta^{15}N$ was not associated with commercial meat intake in this study population, this adjustment is unable to account for the effect of commercial meat intake on $\delta^{13}C$. These results support the use of a dual isotope model using both $\delta^{13}C$ and $\delta^{15}N$ as a valid measure of sweetener intake in the Yup'ik population, and suggest a candidate marker of

sweetener intake based on both $\delta^{13}C$ and $\delta^{15}N$ for further evaluation in the general US population.

The dual isotope model of sweetener intake presented in this study is an example of a more generalizable approach to using isotopic signatures as dietary biomarkers. Where a stable isotope biomarker is elevated in several food groups, it may be possible to use one or more different "covariate" isotopes to control for intake of foods not of research interest. These multiple isotope models can then be used to generate a predictive equation for estimating dietary intake. However, these equations will require calibration in each target population, because both the isotopic ecology of the diet and the underlying dietary patterns driving tissue isotope ratios will differ by population. For example, in the Yup'ik population $\delta^{15}N$ is associated with intake of fish and marine mammals but not commercial meats, because fish and marine mammals have higher $\delta^{15}N$ than commercial meats and their intakes are inversely associated. In populations that consume relatively little fish, $\delta^{15}N$ is positively associated with meat intake (25, 26), because meats have elevated $\delta^{15}N$ relative to other commercial foods (27, 28). Thus, in the general US population we anticipate that $\delta^{15}N$ will improve $\delta^{13}C$-based biomarkers of sweetener intake by controlling for the significant effect of commercial meat intake on tissue $\delta^{13}C$ (9). Testing this hypothesis to validate the dual isotope biomarker of sweetener intake will be the next step in the development of stable isotope biomarkers in the general US population.

Existing predictive biomarkers of sweetener intake, 24-h urinary sugars, provide valid and reliable measures of absolute sweetener intake (29); however, the isotopic biomarkers presented in this study have several practical advantages over these measures. Stable isotope ratios can be inexpensively measured in many biological samples, including RBC (7, 8), serum (9, 30), hair (10, 31), nails (31, 32), and urine (33). These tissues incorporate dietary information over the period of time when they were synthesized; therefore stable isotope ratios can be informative about intake over the past several weeks or months, depending on the tissue analyzed. In contrast, because 24-hr

urinary measurements reflect only a single day's intake, several collections are required to estimate usual intake, which carries substantial participant burden (4). Validation studies that compare the performance of our proposed biomarker with these predictive markers in a controlled setting are warranted to determine the relative validity of these measures.

The primary limitation of this study is that we evaluated the performance of our proposed biomarkers against self-reported intakes, which are known to be subject to substantial error. Although little is known about factors that may bias dietary self-report in the Yup'ik population, the primary factors identified in other populations are sex and obesity (34, 35). Neither sex nor BMI were associated with $\delta^{13}C$ in this study; therefore these biases could not explain our results. One limitation of the $\delta^{13}C$ biomarker more generally is that it is not associated with intake of sweeteners that are not ^{13}C enriched, such as beet sugar, honey and intrinsic sugars found in fruit and dairy products (11). In the Yup'ik population, intake of these sugars is low (24); therefore, we demonstrate similar associations of $\delta^{13}C$ with total and added sugars. In the general US population, intake of sugars which are not ^{13}C enriched is higher; for this reason, we anticipate that associations of $\delta^{13}C$ values with total sugar intake will be attenuated, as has been shown for serum $\delta^{13}C$ (9). Furthermore, in Europe, where sweeteners are based primarily on beet sugar, isotopic markers will not indicate sweetener use.

In summary, this study evaluated a model for estimating sweetener intake based on RBC $\delta^{13}C$ and $\delta^{15}N$ in a Yup'ik study population. This approach requires evaluation in other US populations, but we expect its validity to be similar or improved in populations with lower fish intake. In addition, controlled feeding studies are needed to further validate this biomarker and calibrate how changes in sweetener intake modify isotopic ratios. This combined isotopic biomarker will increase our ability to discern the role played by sweetener intake in the development of chronic disease, and to monitor the effectiveness of interventions designed to reduce their consumption.

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Table 3.1. Associations of sex, body mass index and measures of dietary intake with age [1, 2].

		Age, *y*				*P* for
	Total	<20	20 < 40	40 < 60	≥ 60	trend
n	68	11	22	27	8	
Sex, *% Female*	50	45	54	52	38	0.95
Body mass index, kg/m^2	27.1 ± 6.4	23.0 ± 2.5	25.4 ± 5.6	29.2 ± 6.2	31.4 ± 9.4	0.0045
$\delta^{13}C$, ‰	-19.8 ± 0.6	-19.9 ± 0.7	-19.7 ± 0.7	-19.8 ± 0.5	-20.0 ± 0.5	0.52
$\delta^{15}N$, ‰	9.3 ± 1.8	7.5 ± 0.6	8.4 ± 1.1	9.8 ± 1.4	12.3 ± 0.8	<0.0001
Macronutrient intake						
Carbohydrate, *% energy*	44 ± 14	54 ± 6	53 ± 10	40 ± 10	22 ± 6	<0.0001
Protein, *% energy*	18 ± 6	14 ± 3	15 ± 4	21 ± 6	25 ± 5	<0.0001
Fat, *% energy*	38 ± 9	33 ± 5	33 ± 7	40 ± 7	52 ± 6	<0.0001
Sweetener intake						
Total sugar, *g/d*	89 (76, 103)	136 (109, 171)	120 (100, 145)	76 (61, 95)	36 (22, 58)	<0.0001
Added sugar, *g/d*	74 (62, 88)	115 (87, 151)	104 (86, 125)	60 (46, 80)	30 (18, 51)	<0.0001
SSB, *servings/d*	1.4 (1.1, 1.8)	2.3 (1.4, 3.4)	2.2 (1.6, 2.9)	1.0 (0.7, 1.4)	0.3 (0.0, 0.6)	<0.0001

Table 3.1 continued

Other foods with elevated ^{13}C						
Corn products, *g/d*	11 (8, 16)	28 (17, 45)	12 (7, 21)	9 (5, 16)	4 (2, 10)	0.50
Commercial meats, *% energy*	11 ± 8	13 ± 5	11 ± 7	12 ± 8	3 ±3	0.0126
Fish and marine mammals, *% energy*	18 ± 18	5 ± 9	10 ± 12	21 ± 16	46 ± 13	<0.0001

[1]Data are presented as mean ± SD, or as geometric means (95% CI) for log transformed variables

[2]SSB: Sugar sweetened beverages

Table 3.2. Independent effects of foods which have elevated isotope ratios on RBC $\delta^{13}C$ and $\delta^{15}N$[1,2,3].

Stable isotope ratio	Dietary Variable	β_s	β (95% CI)	R^2
$\delta^{13}C$, ‰	Total sugar, *g/d*	0.36**	0.35 (0.09, 0.61)	0.26
	Fish and marine mammals, *% energy*	0.46**	1.54 (0.52, 2.57)	
	Commercial meats, *% energy*	0.49**	3.94 (1.80, 6.09)	
	Corn products, *g/d*	0.07	0.03 (-0.08, 0.15)	
$\delta^{15}N$, ‰	Fish and marine mammals, *% energy*	0.72***	7.24 (5.13, 9.35)	0.50
	Commercial meats, *% energy*	0.02	0.49 (-4.55, 5.54)	

[1] In these models, $\delta^{15}N$ and $\delta^{13}C$ were the dependent variables and dietary intake measures the independent variables

[2] Significance of association given as *** P<0.0001; ** P <0.01; * P <0.05

[3] Both standardized (β_s) and unstandardized (β) beta-coefficients are presented

Table 3.3. Multiple regression analyses comparing prediction of dietary sweetener intake from individual and combined isotopic measures[1,2,3].

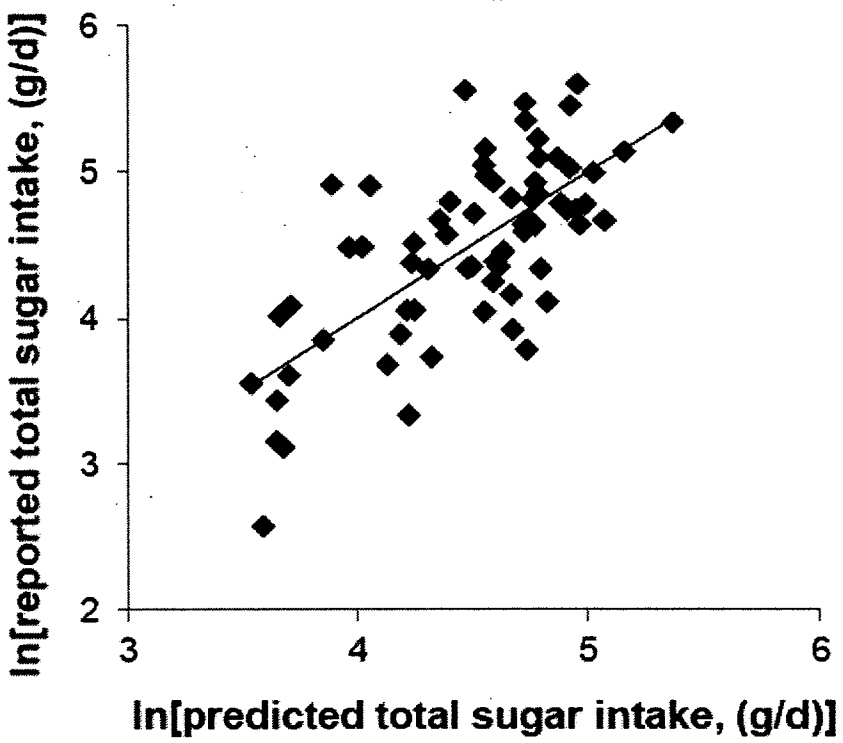
Model	Isotope ratios,	Total sugars, *g/d*			Added sugars, *g/d*			SSB, *servings/d*		
	‰	β	95% CI	R^2	β	95% CI	R^2	β	95% CI	R^2
(1)	$\delta^{13}C$	0.25*	0.00, 0.50	0.06	0.20	-0.08, 0.48	0.03	0.21*	0.00, 0.42	0.05
(2)	$\delta^{13}C$	0.33**	0.14, 0.51	0.48	0.28*	0.04, 0.67	0.33	0.26**	0.08, 0.44	0.31
	$\delta^{15}N$	-0.23***	-0.29, -0.16		-0.21***	-0.29, -0.14		-0.15***	-0.21, -0.09	

[1]In these models, $\delta^{15}N$ and $\delta^{13}C$ were the independent variables and measures of sweetener intake the dependent variables

[2]Slopes have been back transformed for ease of interpretation, and are interpreted as percentage change in sweetener intake for every 1‰ change in isotope ratio

[3]Significance of association given as *** P<0.0001; ** P <0.01; * P <0.05

Figure 3.1. Associations between reported total sugar intake and predicted total sugar intake. Total sugar intake was predicted using the formula ln(Predicted total sugars) = 13.1 + 0.33(δ^{13}C) -0.23(δ^{15}N). This formula was generated based on a model of reported sugar intake using δ^{13}C and δ^{15}N as predictors (Table 3.3).



CHAPTER 4. RELATIONSHIP BETWEEN SUGAR INTAKE AND CHRONIC DISEASE RISK FACTORS IN AN ALASKA NATIVE STUDY POPULATION[1]

4.1. ABSTRACT

Background: Sugar and sugar sweetened beverage intake may be causally associated with chronic disease risk, either directly or by contributing to obesity. However, evidence from observational studies is mixed, which is at least in part due to the error and bias inherent in self-reported measures of sugar intake. Objective biomarkers may clarify the relationship between sugar intake and chronic disease risk.

Objective: This study tested associations of biomarker-based estimates of sugar intake with body mass index (BMI), waist circumference (WC), and a broad array of other physiological and biochemical measures of chronic disease risk in an Alaska Native (Yup'ik) study population. Because obesity is associated with many chronic disease risk factors, we investigated whether associations with sugar intake were independent of BMI.

Design: Sugar intake was estimated using a previously calibrated model based on red blood cell carbon and nitrogen stable isotope ratios. We used linear regression models to test associations of sugar intake with BM, WC, and other chronic disease risk factors in a cross-sectional, community-based sample of 1076 Yup'ik participants.

Results: Sugar intake was not associated with BMI or WC. Sugar intake was positively associated with blood pressure, triglycerides, insulin, homeostasis model of insulin resistance (HOMA-IR) and leptin and inversely associated with total, HDL cholesterol and LDL cholesterol and adiponectin.

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[1] **Nash S.H.**, Kristal A.R., Bersamin A, Choy K, Hopkins S.E., Stanhope K.L, Havel P.J., Boyer B.B., O'Brien D.M. "Relationship between biomarker-based estimates of sugar intake and chronic disease risk factors in an Alaska Native study population". For submission to: *The American Journal of Clinical Nutrition.*

Conclusions: Sugar intake was independently associated with many risk factors which are associated with adverse health effects in this sample of Yup'ik participants. Longitudinal studies are required to better understand associations of sugar intake with chronic disease incidence.

4.2. INTRODUCTION

There has been considerable controversy over whether sugar intake is causally associated with chronic disease risk (1), either directly or by contributing to obesity. Intake of a high sugar diet is associated with elevated plasma triglycerides (2-4), and consumption of high sugar snacks has been experimentally linked with elevated glucose and insulin levels (5). Furthermore, many studies have found that sugars consumed in beverage form (sugar-sweetened beverages; SSB) are associated with increased body mass index (BMI) or body weight, type 2 diabetes and coronary heart disease risk (6-8), as well as chronic disease risk factors including dyslipidemia (4, 7, 9, 10), elevated blood pressure (11, 12), insulin resistance (13, 14) and markers of inflammation (8, 15). However, other studies have shown weak or no associations between either sugar or SSB intake and chronic disease risk factors (16-18). All observational studies of the association between sugar or SSB intake and chronic disease risk factors have relied on self-reported measures of food intake, which are subject to substantial error and bias (19, 20). Therefore, associations are likely attenuated, which may, in part, explain the inconsistency of these findings.

A biomarker of sugar or SSB intake would strengthen inferences from observational studies and help to resolve the role of sugar intake in the development of chronic disease. Measures of 24hr urinary sugars have recently been validated as biomarkers of total and added sugars intake (21-23). However, these measurements require multiple urine collections to reliably estimate intake; therefore, they may be impractical to collect for large study samples. Alternatively, the carbon stable isotope ratio ($\delta^{13}C$) has been proposed as a low-burden, economical and easy to measure

indicator of usual sugar intake in several US populations (24-26). We have recently validated an improved isotopic model of sugar intake in an Alaska Native (Yup'ik) study population (26), which incorporates both the carbon and nitrogen ($\delta^{15}N$) isotope ratios. A model based on both $\delta^{13}C$ and $\delta^{15}N$ is improved because $\delta^{15}N$ accounts for confounding dietary effects on $\delta^{13}C$; this dual isotope model explained 48% of the variability in reported total sugar intake.

The overall objective of this study was to use this dual isotope model to investigate associations of sugar intake with chronic disease risk. In this study, we were interested in sugar intake from both food and beverages; therefore we examined associations of chronic disease risk factors with total sugar intake. Our study sample was a community-based, cross-sectional sample of 1076 Yup'ik people. We were interested in whether sugar intake was linked to chronic disease risk factors in this study population because their intake of high sugar foods has increased substantially over the last several decades (27) and the impact of this increase on Yupik people's health is unknown. Our aims were two-fold. First, we investigated associations of estimated total sugar intake with two measures of obesity: BMI and waist circumference (WC). Our hypothesis was that sugar intake would be positively associated with both BMI and WC. Second, we investigated whether estimated total sugar intake was associated with biomarkers of chronic disease risk, independently of BMI. Based on previous studies, we hypothesized that sugar intake would be positively associated with blood pressure, fasting triglycerides, total cholesterol, C-reactive protein (CRP), fasting glucose and insulin resistance. Determining whether sugar intake is associated with BMI and chronic disease risk factors may influence recommendations for its consumption by Yup'ik people, and will provide additional evidence towards our understanding of the role of sugar intake in the etiology of obesity and related chronic diseases.

4.3. METHODS

4.3.1. Participant recruitment and procedures

Data are from the Center for Alaska Native Health Research (CANHR) study, a cross-sectional, community-based participatory research study of the nutritional, genetic and psychosocial factors affecting obesity and related disease risk in the Yup'ik population. This study was approved by the University of Alaska Fairbanks Institutional Review Board, the National and Area Indian Health Service Institutional Review Boards, and the Yukon-Kuskokwim Health Corporation Human Studies Committee.

Between 2003 and 2012, a community-based sample of 1510 participants aged 14-94 was recruited from ten communities in rural Southwest Alaska, as described elsewhere (28). At entry into the study, participants completed questionnaires to provide information on demographics, medical history and smoking status (current: yes/no). Biological samples and anthropometric measurements were also collected.

4.3.2. Study sample

For comparison of isotope-based estimates of total sugar intake with measures of obesity and chronic disease risk factors, we excluded 341 participants aged < 19 y, 87 participants with missing stable isotope measurements, and 6 participants with missing BMI measurements. This left a study sample of $n = 1076$. However, because data were missing for individual risk factors, the sample size for each analysis varied from 783 – 1039, with the exception of interleukin 6 (IL-6) and insulin-like growth factor 1 (IGF-1),which were available only on a subset of the first seven communities enrolled in the study ($n = 360$ and 363, respectively). From within these communities, samples were balanced across age and sex, as described in detail elsewhere (29, 30).

4.3.3. Anthropometric and biochemical measurements

Anthropometric measurements, including height, weight and blood pressure were measured by trained staff using protocols from the NHANES III Anthropometric Procedures Manual (31), as described by Boyer et al. (32). Blood samples were collected from participants after a minimum 8-hour fast, and biomarkers of chronic disease risk, including triglycerides (TG), total cholesterol, HDL cholesterol (HDL),LDL cholesterol (LDL), adiponectin, blood glucose, Hemoglobin A1c (HbA1c), insulin, leptin, ghrelin, CRP, IGF-I and IL-6 were assayed as previously described (29, 32). Insulin resistance was assessed using the homeostastis model of insulin resistance (HOMA-IR) index: [fasting insulin (mU/ml) x fasting glucose (mg/dl)]/405 (33).

4.3.4. Stable isotope analysis

RBC were pipetted into tin capsules, autoclaved and prepared for isotopic analysis as previously described (34). Neither autoclaving nor the use of EDTA tubes affects RBC carbon or nitrogen isotope ratios (35). Samples were analyzed at the Alaska Stable Isotope Facility by continuous-flow isotope ratio mass spectrometry, using 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). The conventional means of expressing natural abundance isotope ratios is as delta values in permil (‰) relative to international standards as $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \cdot 1000‰$ (36). Here, R is the ratio of heavy to light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$). The standards are Vienna PeeDee Belemnite for carbon and atmospheric nitrogen for nitrogen. To assess analytical precision, an internal standard was analyzed for every ten samples; precision was measured as the coefficient of variation of these analyses (3.3% for $\delta^{15}N$ and 0.6% for $\delta^{13}C$). Because biological samples from this study have a lower $^{13}C/^{12}C$ than V-PDB, $\delta^{13}C$ values are negative. $\delta^{13}C$ values are hereafter abbreviated as $\delta^{13}C$, and $\delta^{15}N$ values are abbreviated $\delta^{15}N$.

4.3.5. Estimating sugar intake using stable isotope ratios

Total sugar intake was estimated using a dual isotope model, which was calibrated in a sample of 68 Yup'ik participants based on self-reported total sugar intake from 4 weekly 24 hr recalls (26):

$$\ln(\text{total sugar}_{\text{estimated}}) = 13.07 + 0.33\,(\delta^{13}C) - 0.23\,(\delta^{15}N) \qquad [4.1]$$

This predictive equation explained 48% of the variation in self-reported total sugar intake in the calibration dataset. Although the calibration population was drawn from two of the 10 Yup'ik communities participating in the present study, it is possible that the calibration equation might differ slightly for the larger population studied here. Furthermore, because our isotopic model of total sugar intake was calibrated against self-reported data, it may have incorporated reporting bias. For these reasons, we tested whether our isotopic model of sugar intake was associated with a second, unbiased marker of total sugar intake recently validated for Yup'ik people: the carbon isotope ratio of RBC alanine ($\delta^{13}C_{ALA}$; 37). We tested this association by measuring $\delta^{13}C_{ALA}$ in a random sample of 50 research participants from the present study. Our estimates of total sugar intake calibrated from self report were significantly correlated with $\delta^{13}C_{ALA}$ (Pearson's r: 0.46), which gives us further confidence that our estimate of sugar intake is objective and valid for use in this study population. Hereafter, total sugar intake estimated using this dual isotope model is referred to as simply "sugar intake".

4.3.6. Statistical analyses

We examined the associations of sugar intake with the following measures: systolic blood pressure (SBP) diastolic blood pressure (DBP), triglycerides, total cholesterol, LDL, HDL, ghrelin, leptin, adiponectin, HbA1c, glucose, insulin, HOMA-IR, IGF-I, IL-6 and CRP. Triglycerides, leptin, insulin, HOMA-IR, IL-6 and CRP were log transformed for analysis. Outlying values of chronic disease risk biomarkers (>4 SD above the mean)

were excluded because they were judged to be physiologically unreasonable. We excluded the following values: SBP (n = 2), triglycerides (n = 7), total cholesterol (n = 1), glucose (n = 4), HbA1c (n = 3), leptin (n = 1), insulin (n = 5), HOMA-IR (n = 5), adiponectin (n = 1), total cholesterol (n = 1), CRP (n = 14), IL-6 (n = 5). For IL-6, values below the limit of detection (LOD; n = 3) were replaced by the LOD divided by the square root of 2 (38). Finally, participants taking blood pressure (n = 142), cholesterol-lowering (n = 43) or diabetes (n = 12) medications were excluded for analyses of associations with blood pressure, blood lipids and blood glucose/insulin, respectively.

We assessed whether sugar intake differed by demographic and health characteristics using one-way analysis of variance models. To determine whether sugar intake and chronic disease risk factors were associated with BMI or WC, we used age- and sex-adjusted multiple regression models. To determine whether sugar intake was associated with biomarkers of chronic disease risk we used BMI-adjusted multiple linear regression models. In addition to BMI adjustment, models were also adjusted for age (continuous), sex, and current smoking status (yes or no). Both linear and quadratic associations were assessed. We used a conservative criterion ($P < 0.01$) for reporting quadratic associations due to the likelihood that multiple contrasts would lead to chance associations. We give the unadjusted P value assessed using a significance level of 0.05, and also indicate which tests remained statistically significant after adjustment using the Bonferroni-Holm method to account for multiple testing (39). All statistical analyses were performed using JMP version 8 (SAS Institute, Cary, NC) or STATA I/C version 12 (StataCorp. 2011, College Station, TX).

4.4. RESULTS

Table 4.1 gives associations of demographic and health related characteristics with sugar intake. The total study population ranged in age from 19 to 94 y (mean = 42 y ± 15); 55% were women and 68% were overweight or obese. Mean sugar intake was 93

g/d, and ranged from 24 to 217 g/d. Sugar intake was 7% higher in men, 22% higher in current smokers and 95% higher in participants aged 19 - 40 y compared with those aged >60 y.

After control for age and sex, neither BMI nor WC was associated with sugar intake (**Table 4.2**). Table 4.2 also gives associations of sugar intake with BMI and WC stratified by age and sex. The only significant association between sugar intake and either measure of obesity was an inverse association with BMI in participants over the age of 60 y.

Table 4.3 gives the linear associations of total sugar intake with chronic disease risk factors. Independent of BMI, sugar intake was positively associated with SBP, DBP, triglycerides, leptin, insulin and HOMA-IR and inversely associated with HDL, LDL, total cholesterol, and adiponectin. The largest increases in chronic disease risk factors were seen with leptin, adiponectin, insulin and HOMA-IR, which increased by 13.8%, 13.0%, 7.4% and 6.7% between quartiles 1 and 4 of sugar intake, respectively. Values of LDL, HDL, and total cholesterol were 11.3%, 9.6% and 7.6% lower in quartile 4 than quartile 1 of sugar intake, respectively There was an inverse quadratic association of sugar intake with triglycerides only; this was reflected in a 22% increase in fasting TG between quartiles 1,2, and 3 of sugar intake, with no additional increase between quartiles 3 and 4. There were no associations of sugar intake with ghrelin, glucose, HbA1c, IGF-I, CRP or IL-6. After Bonferoni-Holm correction, the associations with DBP, TG, total cholesterol, HDL, LDL and leptin remained statistically significant.

4.5. Discussion

There were strong associations of isotopic estimates of sugar intake with chronic disease risk factors in a cross-sectional sample of Yup'ik people. Contrary to our original hypothesis, there were no associations of sugar intake with BMI or WC. However, sugar intake was associated with increased blood pressure, TG, leptin, insulin and HOMA-IR

and decreased HDL, LDL, total cholesterol and adiponectin. These results suggest that although sugar intake is not directly associated with obesity in this Yup'ik study population, it may be associated with higher risk of developing hypertension, dyslipidemia, glucose intolerance and insulin or leptin resistance.

The finding that sugar intake was not associated with measures of obesity (BMI or WC) is consistent with some observational studies (16, 40, 41); however, many other studies have demonstrated significant and positive effects of either sugar or SSB intake on BMI, body weight or risk of obesity (7, 42-44). Although we were unable to assess the association between sugar and total energy intake in this study sample, traditional food intake as measured using $\delta^{15}N$ (34) was positively associated with both BMI (β (95%CI) = 0.40 (0.13,0.67), $P = 0.0041$) and WC (β (95%CI) = 1.81 (1.23,2.39), $P < 0.0001$). The higher fat content of the traditional diet (45) coupled with the negative relationship between intake of sugar and traditional foods (37) may obscure any potential positive relationship between sugar intake and BMI or WC.

Sugar intake was positively associated with insulin and HOMA-IR, but not with glucose or HbA1c. The results of other observational studies that examine associations of sugar or SSB intake with indicators of glucose tolerance or insulin resistance have been inconsistent (7, 13, 45-48). Our results suggest that high sugar intake is associated with increased insulin production in this Yup'ik study population, but likely because of this increased production, glucose homeostasis is maintained. This information is of particular relevance to the health of Yup'ik people, because while diabetes prevalence is low (2010: 27/1000), it increased 38% between 1990 and 2004 (49,50). Longitudinal studies will better address whether this increase in insulin will lead to the development of hyperglycemia over time.

Sugar intake was positively associated with both SBP and DBP, independent of both BMI and current smoking status in this Yup'ik study population, a finding that is consistent with several other observational studies (11, 12, 45, 47, 51). Several proposed mechanisms for this relationship have been postulated, including changes to the uric acid pathway induced by fructose consumption (52) or increased sodium retention (53).

Although our findings are not informative regarding the mechanism by which this association may occur, they are suggestive that decreased sugar intake may be beneficial for the blood pressure of Yup'ik people.

Also in agreement with findings from several prospective studies (46, 47, 54) was the strong association of sugar intake with decreased HDL and increased triglycerides. This association may be due to high intake of fructose (either directly, or as high fructose corn syrup or sucrose) (9, 55), which is known to increase triglyceride levels through increased hepatic de novo lipogenesis (3, 56) and decreased rate of peripheral triglyceride clearance (3, 57). Alternatively, high sugar intake may contribute to higher intake of carbohydrates, which is also associated with increased triglyceride levels (58, 59). Finally, it is also possible that the inverse association between sugar intake and traditional food intake contributed to the strength of these relationships (37). Traditional foods are high in the marine polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which show strong and positive associations with HDL, LDL and total cholesterol, and strong and negative associations with TG in this study population (29).

We found that sugar intake was not associated with C-reactive protein in this Yup'ik study population, which contrasts with the positive associations reported by the few studies which examined sugar or SSB intake and inflammation (8, 10, 15). Again, this finding may be related to the overall high intakes of EPA and DHA in the Yup'ik population (60). EPA and DHA promote an anti-inflammatory state (61), and are inversely associated with CRP in this study population (29). Furthermore, Makhoul et al. (62) also showed that EPA and DHA intake attenuated associations between obesity and CRP in a subset of this study population. Therefore, the lack of association may be due to the unique dietary patterns of this study population and may not be relevant to other US populations.

Finally, our finding that high sugar intake was associated with higher levels of circulating leptin and lower levels of adiponectin are also in contrast to those from other observational studies, which have demonstrated an inverse (8) or no (10, 63) association

of sugar intake with leptin and no association with adiponectin (8, 63). This pattern may lead to increased risk of cardiovascular disease, renal disease (64-66), and type 2 diabetes (67). These results remained statistically significant when adjusted for either BMI or WC, measures of adiposity that are known to affect these adipokines. These results may be unique to this population, and require further exploration using a larger, prospective study.

The primary strength of this study was that it used biomarker-based estimates of sugar intake to examine associations with chronic disease risk in a large sample of Yup'ik people. These estimates were likely more reliable than those from self-report, and were available on a much large number of study participants than would have been available had we assessed diet using self-report. This study is also one of very few which examine the association of non-traditional food intake and chronic disease risk in the Yup'ik population (68, 69). The primary limitation of this study was that it was based on a cross-sectional study sample. Therefore, we cannot exclude the possibility of reverse causality, or residual confounding from intake of other dietary components, physical activity, or other lifestyle factors. Finally, we note that some of the associations presented here, particularly those with blood lipids, are the inverse of those found with biomarkers of EPA and DHA intake in a subset of this study population (29). Thus, these relationships could partially reflect the negative association between intakes of sugar and traditional foods, which are high in EPA and DHA. However, many of the risk factors shown here to be associated with sugar intake were not associated with EPA and DHA, including HOMA-IR, insulin, SBP, DBP and adiponectin. Furthermore, the associations between sugar intake and blood lipids were consistent with our *a priori* hypotheses based on findings from other study populations. Therefore, we are confident that our findings were valid and reflect real associations of sugar intake with chronic disease risk factors.

This study was the first examination of the effects of sugar intake on Yup'ik health, and used an objective biomarker of sugar intake that was developed specifically for use with Yup'ik people. We found that sugar intake was not associated with BMI or WC in this Yup'ik study population, but that it was positively associated with blood

pressure, TG, insulin, insulin resistance, and leptin, and inversely associated with HDL, LDL, total cholesterol and adiponectin. These findings suggest that although sugar intake is not associated with obesity in the Yup'ik population, high intakes of sugar have adverse effects on chronic disease risk factors often related to obesity. Longitudinal studies are warranted to confirm the findings of this study and better understand associations of sugar intake with disease risk in the Yup'ik population.

4.6. ACKNOWLEDGEMENTS

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Table 4.1. Associations of demographic and health related characteristics with sugar intake.

	n (%)	Sugar intake[1] *g/d*	*P*
Total study population	1076 (100)	95.3 ± 37.0	
Sex			0.0017
Male	499 (46)	99.1 ± 37.9	
Female	577 (54)	92.0 ± 35.9	
Age			<0.0001
19 - <40 y	549 (51)	112.5 ± 32.4	
40 - <60 y	375 (35)	85.3 ± 32.8	
> 60 y	152 (14)	57.9 ± 23.0	
Smokers			<0.0001
Current	333 (31)	108.9 ± 36.0	
Non smoker	722 (67)	88.6 ± 35.5	
Body Mass Index			0.0004
<25 kg/m^2	384 (36)	100.9 ± 35.3	
25 – <30 kg/m^2	354 (33)	94.0 ± 37.4	
> 30 kg/m^2	338 (31)	90.3 ± 37.6	

[1]Mean ± SD

Table 4.2. Associations of sugar intake with BMI and waist circumference, stratified by age class and sex[1,2].

		BMI, kg/m^2				Waist circumference, *cm*		
	n	Mean ± SE	BMI > 30, %	β[3] (95% CI)	*P*	Mean ± SE	β (95% CI)	*P*
Total	1076	28.1 ± 0.2	31	-0.09 (-0.37, 0.18)	0.50	92.0 ± 0.4	0.07 (-0.63, 0.77)	0.85
Age								
19 - <40 y	549	27.7 ± 0.3	28	-0.30 (-0.67, 0.07)	0.12	89.5 ± 0.6	-0.67 (-1.60, 0.26)	0.16
40 - <60 y	375	28.3 ± 0.3	34	0.19 (-0.23, 0.60)	0.37	93.2 ± 0.7	0.44 (-0.64, 1.51)	0.43
> 60 y	152	29.0 ± 0.5	38	-1.38 (-2.44, -0.32)	0.011	98.0 ± 1.3	-2.42 (-5.34, 0.49)	0.10
Sex								
Male	499	26.5 ± 0.2	19	-0.00 (-0.30, 0.31)	0.96	91.5 ± 0.6	0.24 (-0.71, 1.10)	0.67
Female	577	29.5 ± 0.3	42	-0.20 (-0.65, 0.27)	0.39	92.5 ± 0.6	0.23 (-1.14, 1.00)	0.90

[1]Associations in the complete study sample are age and sex adjusted. Associations within age and sex strata are sex and age adjusted, respectively.

[2]Sugar intake was estimated using the formula: ln(total sugar) = 13.07 + 0.33 ($\delta^{13}C$) – 0.23 ($\delta^{15}N$) (26)

[3]Slopes are interpreted as change in obesity measure (BMI or WC) for each 25g increase in total sugar intake

Table 4.3. Linear associations of sugar intake with chronic disease risk factors, and means of those risk factors stratified by quartile of sugar intake (n = 360-1039)[1,2].

		Total sugar intake[3] (range g/d)					
	n	Quartile 1 (23 - <66)	Quartile 2 (66- <93)	Quartile 3 (93 - <121)	Quartile 4 (121 - <217)	β[4] (95% CI)	*P*[5]
SBP, *mm Hg*	880	116.6 ± 1.0	118.1 ± 0.8	117.8 ± 0.9	120.2 ± 0.9	0.80 (0.15, 1.46)	0.0161
DBP, *mm Hg*	882	68.6 ± 0.8	70.8 ± 0.6	71.2 ± 0.6	72.1 ± 0.7	0.90 (0.41, 1.40)	**0.0003**
Triglycerides, *mg/dL*[6]	912	65.5 (61.9, 69.3)	74.9 (71.2, 78.8)	80.1 (76.0, 84.4)	81.9 (77.3, 86.0)	18.7 (9.35, 29.0)	**<0.0001**
Quadratic						-1.29 (-2.21, -0.37)	**0.0066**
Cholesterol, *mg/dL*	922	228.8 ± 3.1	232.9 ± 2.7	217.8 ± 2.8	211.5 ± 3.1	-5.12 (-7.23, -3.01)	**<0.0001**
HDL, *mg/dL*	920	65.0 ± 1.2	64.3 ± 1.0	60.0 ± 1.1	58.8 ± 1.2	-1.64 (-2.45, -0.84)	**<0.0001**
LDL, *mg/dL*	922	150.5 ± 2.7	151.1 ± 2.4	140.1 ± 2.5	133.6 ± 2.7	-4.66 (-6.50, -2.82)	**<0.0001**
Leptin, *ng/mL*[6]	811	6.5 (6.0, 7.0)	6.7 (6.2, 7.2)	6.9 (6.3, 7.4)	7.4 (6.8, 8.1)	5.12 (1.70, 7.00)	**0.0007**
Adiponectin, *μg/mL*	960	10.4 ± 0.4	9.6 ± 0.3	9.3 ± 0.3	9.2 ± 0.4	-0.34 (-0.60, -0.08)	0.0103
Ghrelin, *pg/mL*	809	404.4 ± 11.5	417.9 ± 10.9	408.5 ± 11.7	412.8 ± 12.8	-0.63 (-9.05, 7.80)	0.88
HbA1c, *%*	961	5.5 ± 0.2	5.5 ± 0.2	5.4 ± 0.2	5.4 ± 0.2	-0.01 (-0.03, 0.00)	0.13

Table 4.3 continued

Glucose, *mg/dL*	1039	94.3 ± 0.7	93.1 ± 0.7	92.7 ± 0.6	93.5 ± 0.7	-0.04 (-0.51, 0.44)	0.88
Insulin, *μU/dL*[6]	788	11.8 (11.0, 12.6)	11.7 (11.0, 12.5)	12.5 (11.7, 13.2)	12.6 (11.7, 13.5)	2.93 (0.45, 5.47)	0.0203
HOMA-IR[6]	783	2.7 (2.5, 2.9)	2.6 (2.5, 2.8)	2.9 (2.7, 3.1)	2.9 (2.7, 3.1)	3.11 (0.47, 5.89)	0.0210
IGF-I, *ng/mL*	387	267.6 ± 9.8	248.1 ± 9.3	256.5 ± 9.0	260.7 ± 9.5	0.56 (-0.61, 7.23)	0.87
CRP, *mg/dL*[6]	783	0.08 (0.07, 0.10)	0.11 (0.09, 0.13)	0.10 (0.08, 0.12)	0.13 (0.10, 0.16)	6.79 (-0.15, 14.2)	0.055
IL-*6*, *μg/L*[6]	380	0.09 (0.07, 0.13)	0.09 (0.07, 0.13)	0.08 (0.06, 0.11)	0.06 (0.05, 0.09)	-8.19 (-17.5, 2.12)	0.12

[1] Sample size varies because of outliers and missing data. SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model of insulin resistance; IGF-I, Insulin-like growth factor I; CRP, C-reactive protein; IL-6, interleukin-6. Multiple linear regression models were adjusted for age (continuous), sex, BMI (continuous), and smoking status (yes or no).

[2] Means of chronic disease risk biomarkers are least squares means (±SE), adjusted for age (continuous), sex, BMI (continuous), and smoking status (yes or no). Geometric means (95% CI) are given for log-transformed variables.

[3]Total sugar intake was estimated using the formula: $\ln(TS) = 13.07 + 0.33\ (\delta^{13}C) - 0.23\ (\delta^{15}N)$ (26)

[4]Slopes are interpreted as change in chronic disease risk factor for each 25g increase in total sugar intake

[5]Associations which remained statistically significant after Bonferroni-Holm correction are highlighted in bold

[6]Log-transformed values were used for regression analyses; slopes have been back transformed for ease of interpretation and are interpreted as percentage change in the chronic disease risk factor for each 25g increase in total sugar intake

CONCLUSION

In recent decades, the Yup'ik people have experienced a shift in dietary intake patterns known as the nutrition transition; however, the extent to which this dietary change has affected Yup'ik health is unknown. Demonstrating causal associations between dietary intake and disease risk requires valid estimates of intake that are easily measured in large numbers of study participants. Unfortunately, self-reported measures of intake that are suitable for use in large, population-based studies (e.g., food frequency questionnaire) suffer error and biases that may obscure associations of intake with disease risk (1, 2). Conversely, more reliable methods (e.g., repeated 24 h recall) can be prohibitively expensive, labor intensive and burdensome on both the study participant and the researcher (3, 4). Objectively measured biomarkers of dietary intake provide a promising alternative to self-reported methods of dietary assessment because they can provide unbiased, reliable estimates of intake (5-7). Validated biomarkers of commonly consumed Yup'ik foods would likely be invaluable in studies of the association between dietary change and disease risk in the Yukon-Kuskokwim Delta.

The stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) were candidate biomarkers of Yup'ik dietary intake because several commonly consumed traditional and market foods are distinct in their carbon and nitrogen isotope ratios (8). The nitrogen isotope ratio is high in fish and marine mammals (9), foods which comprise a large proportion of the Yup'ik traditional diet (10). The carbon isotope ratio is uniquely elevated in market-purchased corn and sugar cane-based foods (C_4; 11), such as sweeteners, corn-products and corn fed meats. These foods comprise a large proportion of the Yup'ik market diet. Therefore, the carbon and nitrogen isotope ratios could potentially provide estimates of both Yup'ik traditional and market food intake that would be informative in studies of how the nutrition transition had affected, or is affecting, the health of Yup'ik people.

This dissertation presented evidence that moves these isotope ratios from "candidate" to "validated" markers of Yup'ik dietary intake. The first chapter addressed

the validation of the $\delta^{15}N$ and $\delta^{13}C$ markers in three ways. First, a comprehensive analysis of the $\delta^{15}N$ and $\delta^{13}C$ values of a suite of traditional and market foods important to Yup'ik people allowed me to determine expected relationships between tissue isotope ratios and dietary intake specific to Yup'ik people. Second, an evaluation of associations between isotope ratios and self-reported dietary intake measures demonstrated strong associations of red blood cell (RBC) $\delta^{15}N$ with traditional food intake, and of RBC $\delta^{13}C$ with C_4 and total market food intake. Third, the ability of these markers to indicate dietary patterns by age, sex, community location and cultural identification demonstrated the potential of stable isotope ratios to provide useful and novel information about dietary intake in this Yup'ik study population.

The second chapter of this dissertation focused more specifically on the potential for isotope ratios of hair to indicate dietary intake. Hair samples can be collected easily and non-invasively, which makes them ideal for estimating dietary intake in situations where drawing a blood sample is undesirable, for example in young children. They also have an additional advantage: because hair grows continuously and does not remodel after growth, hair isotopic markers could provide a dietary record over the length of the participant's hair (~1 month/cm of hair). In this chapter, I showed that the $\delta^{15}N$ and $\delta^{13}C$ values of RBC and hair were highly correlated, and provided a metric to compare isotopic measurements in hair and RBC in Yup'ik people. I also showed that hair $\delta^{15}N$ values were equally as associated with % RBC membrane eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as the previously validated RBC $\delta^{15}N$ marker (12). These results demonstrated the potential for hair isotope ratios to indicate dietary intake with similar validity to RBC measurements, and will also enable the comparison of results from studies that utilize these different sample types.

In the third and fourth chapters of this dissertation, I switched my focus to the validation and application of stable isotope biomarkers of sweetener intake. Markers of sweetener intake are of particular interest because sugar intake, in particular high fructose corn syrup, has been linked to several intermediate risk factors for chronic disease,

including excess energy intake, body mass index (BMI), dyslipidemia, insulin resistance and markers of inflammation (13-21). The carbon isotope ratio has been proposed as a marker of corn- and cane sugar-based sweetener in several US populations (22, 23); however, the validity of this marker is low because of confounding by consumption of other ^{13}C enriched foods. Here, I showed that the primary confounders of the association between $\delta^{13}C$ and sweetener intake in the Yup'ik population were traditional marine foods and commercial meats (Chapter 3). Because $\delta^{15}N$ values are a marker for the marine component of the Yup'ik traditional diet, a model based on both the carbon and nitrogen isotope ratios provided a more valid marker of sweetener intake than one based on $\delta^{13}C$ values alone. Unfortunately, because $\delta^{15}N$ was not associated with commercial meat intake in this Yup'ik study population, $\delta^{15}N$ adjustment could not account for the effect of this confounder on RBC $\delta^{13}C$. Nevertheless, the dual isotope marker explained 48% of the variation in reported total sugar intake in this Yup'ik study population, a surprisingly high amount considering the potential inaccuracy of dietary self-report.

I used this dual isotope model of total sugar intake in the final chapter of this dissertation to examine associations with BMI and other chronic disease risk factors in participants from the CANHR I study. I found that while sugar intake was not associated with BMI in this Yup'ik study population, it was associated with blood pressure, blood lipids, and the adipokines leptin and adiponectin. This chapter is important in two ways: first, it demonstrates the ability of isotopic markers to be informative in studies of disease risk in Yup'ik people, and second, it discovers potentially adverse associations of sugar intake on health that were previously unknown for this population. Because this study was conducted in a cross-sectional study sample, it is not possible to infer causality from these results. However, my hope is that this study will provide the basis for longitudinal studies of sugar intake and chronic disease risk in Yup'ik people.

The focus of this dissertation was the validation and application of these novel biomarkers; however, this research frequently touched upon another theme that warrants

further discussion: that of dietary patterns. Nutritional epidemiologists have traditionally examined associations of chronic disease risk with a single nutrient or food (24-26); however, the assessment of dietary patterns has begun to gain popularity in recent decades (24, 27, 28). The strengths and weaknesses of these two approaches are contrasting. Nutrient analysis allows for the identification of key nutrients, such as polyunsaturated fatty acids (26, 29, 30), fiber (31, 32), and saturated and *trans* fats (33-35) that are associated with chronic disease risk, but this method cannot account for complex interactions or associations among nutrients. In contrast, dietary pattern analysis allows the study of how foods or nutrients consumed in combination affect disease risk, but cannot provide details on specific nutrients, or inform about the biological relationships between dietary components and disease risk. Isotope ratios are direct biomarkers of specific foods, and thus can be used as indirect measures of either nutrients or diet patterns, depending on how closely linked they are to those foods. This dissertation validates stable isotope ratios as biomarkers of both dietary pattern (Chapter 1) and specific nutrients (Chapters 2 and 3). For example, fish intake, measured via $\delta^{15}N$, is tightly correlated with both traditional food intake (diet pattern) and the fatty acids EPA/DHA (nutrients). Thus, $\delta^{15}N$ is a biomarker for both a diet pattern and a specific nutrient.

Any type of dietary analysis in the Yup'ik population must take diet pattern into account, because all foods fit into the category of traditional or market, and the inverse association between traditional and market food intake is very strong. Any measure of traditional food intake will be inversely correlated with market food intake, even when the association is entirely indirect. Disentangling the direct from the indirect associations between markers and diet has been a major and central challenge to this work. For example, in chapter 3, I discussed why dietary pattern may, in part, explain the substantially better performance of the dual isotope model ($\delta^{13}C$ and $\delta^{15}N$) to predict sweeteners, compared to $\delta^{13}C$ alone: it could be because ^{15}N adjustment factors out the significant effect of high fish and marine mammal intakes on $\delta^{13}C$, or because of the strong, age-related diet pattern. In chapter 4, I discussed the possibility that intake of

traditional marine foods may have affected associations of sugar intake with several chronic disease risk factors; traditional foods show a strong negative association with sugar intake, and have the potential to positively affect chronic disease risk factors including blood lipids, C-reactive protein and insulin resistance (36-38). Although I discuss this diet pattern effect within the context of this Yup'ik study population, the challenge of how dietary patterns affect associations with dietary biomarkers is more broadly applicable. A consideration of dietary patterns is particularly important for biomarkers such as stable isotope ratios, because their associations are with foods rather than specific nutrients.

The ultimate aim of the research presented in this dissertation was to develop markers which could be used in studies of how diet affects chronic disease risk in Yup'ik people. Currently, the nitrogen isotope marker of traditional marine intake is being used in several such studies being conducted by investigators with the Center for Alaska Native Health Research (CANHR). For example, O'Brien and colleagues (unpublished) have used $\delta^{15}N$ values to show that traditional food intake may have beneficial effects on fasting lipids and adiponectin levels. A recent study by Lemas et al. (39) showed that associations between carnitine palmitoyltransferase 1A gene variants and fasting lipids were modified by n-3 PUFA intake, the latter of which was estimated using $\delta^{15}N$. Values of $\delta^{15}N$ are also being used in pharmacogenetic studies to evaluate the effect of EPA and DHA intake on clotting time and evaluate whether dosage of the blood thinner warfarin should be adjusted or personalized for Yup'ik people. Finally, the Fish to School project, led by Dr Bersamin, is hoping to use the $\delta^{15}N$ values of hair to monitor the efficacy of an intervention to increase Alaskan fish consumption in schools across the state.

In contrast, neither the carbon isotope marker of market intake, nor the dual isotope marker of sweetener intake, is currently being used by other CANHR investigators. However, I anticipate that these markers will be of great interest in the near future. For example, CANHR investigators are working with a dentist from the University of Washington on a proposal in which this marker will be used to study

associations of sugar intake with dental caries. Dental caries are a particular problem in Yup'ik children and adolescents (40, 41); this marker will provide a valuable tool to monitor interventions for sugar reduction towards the improvement of Yup'ik oral health. Furthermore, I think it will be of great importance to continue the work that I started in the final chapter of this dissertation. CANHR is developing a longitudinal cohort of participants for whom we have both isotopic and health data, which will provide the opportunity to determine whether the associations I observed in a cross-sectional study sample are causal, to monitor whether dietary change is currently occurring in the Yup'ik population, and to determine what the impact of this change is on disease risk.

In addition to the potential of these markers to be informative in studies of Yup'ik health, my research also represents a substantial contribution towards the development and application of stable isotope markers for use in nutritional epidemiology more generally. The use of naturally-occurring variations in stable isotope ratios to indicate dietary intake is a relatively new concept in this field, and this body of work represents a substantial proportion of those studies that develop stable isotope markers using an epidemiologic framework. In particular, I think that the dual isotope marker presented in chapter 3 will be of great interest to the many researchers who are interested in the effect of sweetener intake on health (42). Sugar intake is notoriously difficult to measure using self-report, as it is commonly underreported, especially by women and overweight individuals (43, 44). Therefore, a biomarker of sweetener intake such as that presented here will be very useful in strengthening inferences from observational studies and helping to resolve the role of sweetener intake in the development of chronic disease.

In summary, this dissertation provided substantial evidence for the validity of the carbon and nitrogen stable isotope ratios to indicate the diets of Yup'ik people. It also illustrated the utility of one of these markers in a study of the associations between isotopic estimates of sweetener intake and chronic disease risk factors in a cross-sectional sample of Yup'ik people. These markers will be useful in current and future studies of

disease risk in the Yup'ik population, and this work also provides a basis for further validations in other US populations.

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