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Stable Isotope Ratios as Biomarkers of Diet for Health Research

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Abstract

Diet is a leading modifiable risk factor for chronic disease, but it remains difficult to measure accurately due to the error and bias inherent in self-reported methods of diet assessment. Consequently there is a pressing need for more objective biomarkers of diet for use in health research. The stable isotope ratios of light elements are a promising set of candidate biomarkers because they vary naturally and reproducibly among foods, and those variations are captured in molecules and tissues with high fidelity. Recent studies have identified valid isotopic measures of short and long-term sugar intake, meat intake, and fish intake in specific populations. These studies provide a strong foundation for validating stable isotopic biomarkers in the general United States population. Approaches to improve specificity for specific foods are needed, for example, by modeling intake using multiple stable isotope ratios, or by isolating and measuring specific molecules linked to foods of interest.

Keywords

Carbon-13; Nitrogen-15; Sulfur-34; Nutritional epidemiology; isotope ratio mass spectrometry

INTRODUCTION

There is a pressing need for more objective biomarkers of diet for use in research (11, 77). Because chronic diseases are complex and dietary associations may be modest, even diet-disease associations with strong empirical laboratory support are difficult to establish at the population level (12, 130). Most instruments for dietary assessment rely on self-report, and are prone to random errors and systematic biases that can obscure relationships between diet and disease (12, 70, 142). The need for precise dietary measures is particularly great in studies for which individual exposure matters; for example, nutrigenomics, which evaluates nutrient interactions with specific gene variants (149). Objective biomarkers of diet have reduced error and bias (11, 69, 77), and can clarify diet-disease relationships (125, 126); however, relatively few validated biomarkers are available, and even fewer are feasible to apply in large population studies due to analytical cost and/or participant burden.

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DISCLOSURE STATEMENT

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Stable isotope ratios (SIR) are well-established tools for assessing diet in animal ecology (34) and archaeology (79), and are attracting interest as candidate dietary biomarkers for nutritional epidemiology (27, 42, 66, 102, 112). The SIR of carbon, nitrogen and sulfur vary naturally in many of the foods we eat, are captured in body proteins and other molecules, and can be measured in a variety of tissues. SIR are similar to concentration biomarkers in that they are measured *in situ* rather than recovered (11, 69); however, unlike many concentration biomarkers, SIR are relatively little affected by endogenous processes (see “Stable Isotope Ratios in Tissues”), and thus dietary associations can be quite strong (101). Depending on the growth and turnover rate of the molecule or tissue measured, they can indicate diet over short-term (e.g. blood glucose) or long-term (e.g. red blood cells (RBC)) timescales (120). Once appropriately validated these measures could be used to test dietary hypotheses retrospectively using archived specimens, because SIR are not affected by processes that disrupt molecular structure, such as freeze-thaw cycles or long-term storage. Specimens are available for many epidemiologic studies for which retrospective dietary data would be valuable; for example, in providing dietary information where none may have been collected, or in helping to characterize the structure of measurement error for existing self-report data (127, 128). Thus, there is tremendous potential for SIR to become useful tools in nutritional epidemiology.

In this article I review the concepts and analytical methodologies underlying the measurement of SIR at natural abundance, I explain the ecological factors which cause SIR to vary among foods, I describe the applications of SIR to date as biomarkers of diet in nutritional studies, and I discuss the next steps required for the further use of SIR in nutritional epidemiology. Natural abundance SIR measurements were invented by geochemists, enthusiastically adopted by plant and ecosystem ecologists, and developed as dietary measures by animal ecologists and archaeologists; therefore, relevant literature spans several fields, creating the need for a review. Most researchers in public health are unfamiliar with natural abundance SIR; consequently, nomenclature, methodology and interpretation can be a barrier to their understanding and use of these biomarkers. My hope with this review is to reduce those barriers.

WHAT ARE STABLE ISOTOPE RATIOS?

Biological molecules are built from atoms of different elements, each of which has a distinct chemical character and place in the periodic table based on the number of protons in its nucleus (the atomic number). Isotopes are atoms of the same element that differ in the number of neutrons in the nucleus, and thus in their atomic mass. The term “isotope” refers to the fact that different isotopes of the same element occupy the same (“iso”) place (“topos”) in the periodic table (88). Most elements have multiple naturally-occurring isotopes, and as the name implies, stable isotopes are those that do not undergo radioactive decay. Although it is common for one isotope to be more abundant, in nature each element always occurs as a mixture of its isotopic forms. For example, carbon commonly has 6 protons and 6 neutrons, giving it an atomic mass of 12; however, just over 1% of carbon on earth has an extra neutron, with a mass of 13 (Figure 1). Although ^{13}C is much less abundant than ^{12}C , it isn't exactly rare: a 50-kg person contains about 137 grams of ^{13}C ,

which is over 6×10^{24} atoms! (46). By convention, isotopes with lower atomic mass are termed “light”, and isotopes with higher atomic mass are termed “heavy”.

Although stable isotopes of the same element form the same types of bonds, they may have slightly different reaction rates due to their mass differences. These rate differences can cause isotopic partitioning or “fractionation” in the environment as elements move through various physical, chemical and biological reactions, and are manifested by differences in the ratio of heavy to light isotopes (135). The section of this paper titled “How and Why Stable Isotope Ratios Vary in Food” describes how biological fractionation causes SIRs to differ naturally in the foods we eat, which is the basis for using them as dietary biomarkers. First, however, I describe how SIRs are measured and expressed.

The “delta value”

The stable isotopes of the common elements in biological molecules are given in Figure 1 (omitting phosphorus because it has only one stable isotope). For all of these elements, the lightest isotope is by far the most abundant, and SIRs are presented by convention as heavy/light. Natural variation in the SIRs of these elements typically takes place at the 4th, 5th or even 6th decimal place (135). Given how subtle these variations are, accuracy in measurement and reporting is paramount. Relative differences can be measured more precisely than absolute values (16); therefore, measurements of sample SIRs are always made in tandem with measurements of reference gas, and SIRs are reported in units of relative abundance rather than as absolute isotope ratios (86, 140). These “delta” (δ) values are expressed in units of permil (‰) rather than percent (%) relative abundance, to make the scale more tractable (Figure 2). In order that these measures be comparable across studies, delta values are presented relative to universal international standards, to which lab reference materials and gases are calibrated (Figure 2). Because analyses of SIRs at natural abundance were pioneered by geochemists (62, 140), standards are commonly geological materials; for example, the reference standard for carbon is a limestone (V-PDB), and the reference standard for sulfur is a meteorite (V-CDT). Because tissue from living organisms typically has a lower abundance of ^{13}C than limestone, $\delta^{13}\text{C}$ values from biological studies are rather unfortunately almost always negative.

Continuous-flow isotope ratio mass spectrometry

Naturally occurring differences in SIR of the light elements are measured with isotope ratio mass spectrometry (IRMS) (93), and there are many academic and commercial IRMS laboratories available. The analysis requires that samples be converted to gases, which occurs via one of several instruments (peripherals) used in tandem with IRMS in a continuous flow system (CF-IRMS). One of the most commonly used peripherals for dietary analyses is an elemental analyzer (EA), which combusts solid organic samples into CO_2 , N_2 , H_2O and SO_2 (Figure 3). Following sample combustion and desiccation to remove H_2O , gases are carried in a stream of helium through a separation column, generating distinct peaks for each gas. A subsample of this gas stream enters the isotope ratio mass spectrometer via an open split interface, which also allows the introduction of reference gases before or after the sample peaks. Gases enter the isotope ratio mass spectrometer at the ion source, where they are bombarded by high-energy electrons. These collisions dislodge

electrons from gas molecules, converting the molecules into positively charged ions. Ions are focused into a beam and aimed down a curved flight tube, where a magnetic field bends their flight path to a degree that depends on mass. For a given charge (+1), molecules with a greater mass bend slightly less, molecules with a smaller mass bend slightly more, so that they strike different ion detectors in an array at the end of the flight tube. For example, $^{15}\text{N}/^{14}\text{N}$ is calculated from the amplified currents from detectors at mass 29 ($^{15}\text{N}^{14}\text{N}^{+1}$) and 28 ($^{14}\text{N}_2^{+1}$), whereas the carbon isotope ratio ($^{13}\text{C}/^{12}\text{C}$) is detected at mass 45 ($^{13}\text{CO}_2^{+1}$) and 44 ($^{12}\text{CO}_2^{+1}$). The magnetics of the isotope ratio mass spectrometer re-tune between the arrival of the N_2 and the CO_2 sample peaks, such that both isotope ratios can be measured in a single analysis using the same detector array.

Sample types and handling

As biomarkers of diet, SIRs at natural abundance are unusual in that the measurement of interest is at the atomic rather than at the molecular level. Consequently, SIRs are not affected by even dramatic alterations to molecular structure, such as autoclaving (74, 158). Short term (2–4 months) cryo-storage had no effect on blood $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values (74), and although the effect of longer-term storage has not been formally tested, the SIR of a sample cannot change unless atoms of the element of interest have been added (i.e., through contamination) or lost (i.e., via microbial respiration). A recent re-analysis of $\delta^{13}\text{C}$ values in insect eggs found values within analytical precision of those originally reported, after samples had been stored dry at room temperature for more than a decade (F.J. Wessels and D.M. O'Brien, unpublished findings). This property makes retrospective analysis of SIRs possible for stored specimens, especially given the very low volumes required for analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Blood additives have the potential to alter SIRs, as the carbon and nitrogen atoms contributed by the additive are likely to have different SIRs from those of the sample. The effect is likely to be small, given the preponderance of sample atoms relative to additive, but should be tested. Recent papers have shown no effect of EDTA, polymerized acrylamide resin, or sodium fluoride additives on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of blood samples (74, 158), although the effect of sodium fluoride on plasma $\delta^{13}\text{C}$ was only marginally non-significant (74).

SIRs have been measured in a variety of human sample types, including hair (100, 116, 118, 150), fingernails (18, 95), urine and feces (76), and blood fractions including whole blood (33), RBC (97, 101), clot (74), plasma (100), and serum (42, 74, 112, 161). In most of those analyses, what is measured is the SIR of the total sample carbon or nitrogen, which is often colloquially referred to as “bulk” analysis. These days, bulk analyses are relatively straightforward, automated, and inexpensive, as they primarily involve weighing a small amount of dried sample into tin capsules for combustion and EA-IRMS. However, blood-borne pathogen risk needs to be considered, as dried samples are enclosed in a crushed tin capsule but not sealed, and the possible transmission risks from dried, powdered material are not well understood. To remove this risk, our lab aliquots liquid samples into tin capsules and autoclaves them prior to drying and analysis (158), using purpose-designed and machined aluminum 96-well plates. SIRs have also been measured in specific molecules; for example, blood glucose (31) and specific amino acids (27, 115, 119). These analyses are usually more involved, as they require isolating specific molecules prior to IRMS, either

using gas chromatography or other separation techniques. Finally, there is a long history of analyzing the $\delta^{13}\text{C}$ values of breath CO_2 at natural abundance, originally as a way to understand baseline variation in breath $\delta^{13}\text{C}$ for metabolic labeling studies (136, 137, 139). Recent analytical advances in the measurement of $\delta^{13}\text{C}$ values in breath CO_2 have sparked a resurgence of interest in this measure for understanding diet and metabolism (19, 157).

HOW AND WHY STABLE ISOTOPE RATIOS VARY IN FOOD

This section of the review will examine where our food comes from and the isotopic imprints that the ecological processes involved in food production leave on those foods. The atoms of each of the elements that make up our food move through an elemental cycle from inorganic to organic forms, and back again. The chemical and biological reactions inherent in each elemental cycle have the potential for isotopic fractionation, and cause a natural partitioning of isotopes through the environment. Although elemental cycling is far outside of the purview of most nutritionists, it is always instructive to consider where our food comes from, and understanding how and why isotopes vary in our foods is important to understanding the strengths and the limitations of SIRs as biomarkers of diet.

Carbon

The carbon in food derives from atmospheric CO_2 , which is captured (fixed) in organic molecules by the process of photosynthesis in plants. Variation in plant photosynthetic physiology generates almost all of the carbon isotope variation in our food. The enzyme responsible for binding and fixing CO_2 in photosynthesis, Rubisco, has a strong preference for CO_2 bearing the lighter isotope of carbon, ^{12}C (43). This causes plants to have proportionally less ^{13}C relative to atmospheric CO_2 and thus a lower delta value. However, plants differ in photosynthetic physiology, specifically in the gas exchange properties of the leaf and whether a preliminary fixation step precedes Rubisco (43). Many plants native to dry environments evolved a CO_2 -concentrating mechanism that reduces water loss from the leaf (38), and also reduces the extent to which Rubisco can discriminate against ^{13}C (43). Consequently, plants that use this strategy (C4 plants) have $\delta^{13}\text{C}$ values that are approximately 12–13‰ higher than plants that do not (C3 plants) (Figure 4)(8, 44, 107). Most plants in the United States food supply are C3, including wheat, rice, soy, potatoes, canola, and the vast majority of fruits and vegetables. However, two important C4 exceptions are widely consumed in the United States diet: corn and sugar cane, which have distinctly higher $\delta^{13}\text{C}$ values than C3 food plants. There are other minor exceptions; for example, millet and sorghum are also C4 (8), and pineapple and agave belong to a 3rd type of photosynthetic physiology, CAM (Crassulacean acid metabolism), which typically has $\delta^{13}\text{C}$ values similar to C4 plants (43). However, corn and sugar cane provide the overwhelming majority of carbon with elevated $\delta^{13}\text{C}$ values in the United States diet.

Plants in the marine environment also have elevated $\delta^{13}\text{C}$ values relative to C3 plants, although to a different extent than C4 plants and for a different reason. Most of the CO_2 dissolved in the ocean is converted to bicarbonate, an equilibrium process that favors ^{13}C . Many phytoplankton take up bicarbonate for photosynthesis, which is elevated in $\delta^{13}\text{C}$ over atmospheric CO_2 by ~9‰ (92). This isotopic difference in the source of carbon for photosynthesis produces characteristic “marine” $\delta^{13}\text{C}$ values of ~ -17‰ to -20‰, which

are passed on to commercially important pelagic fish species such as salmon, cod, tuna, and pollock. On average, people in the United States consume 1–2 servings of fish per week (40), which is likely a small source of carbon compared to the major C3 and C4 sources such as wheat and corn. However, if people consume a very large proportion of calories from fish and other marine foods, their $\delta^{13}\text{C}$ values will be influenced by those sources (97).

When animals consume plants, they incorporate carbon from those plants into their own tissues. Studies spanning several decades have demonstrated that animal $\delta^{13}\text{C}$ values typically reflect those of their diet (36), with a modest average increase of approximately 1‰ (85). Because the difference in $\delta^{13}\text{C}$ values between animals and their diets is small relative to the average difference in $\delta^{13}\text{C}$ values between C3 and C4 plants, diet is the primary determinant of variation in animal $\delta^{13}\text{C}$ values. In the United States, livestock are heavily fed on corn, which gives store-purchased meats an elevated $\delta^{13}\text{C}$ value relative to C3 plants (~ -18 to -15 ‰) (98, 138).

In summary, varying $\delta^{13}\text{C}$ values in United States food are primarily driven by the balance of C3 vs. C4 sources. In the United States, sweeteners are heavily C4 based: 75% of sugar sold in the United States, and 99% of the sugar in sugar-sweetened beverages (SSBs), derives from corn or sugar cane (52). Much of the current research on stable isotope biomarkers in the United States is exploring and refining measures of sugar intake using $\delta^{13}\text{C}$, a topic reviewed later in this article. Animal products are another important source of elevated $\delta^{13}\text{C}$ in the diet; therefore, biomarkers that can differentiate carbon contributed by sugars and carbon contributed by protein (e.g., meat, eggs) are of particular interest and relevance. A final point is that corn can also contribute to the diet (as whole corn, corn chips, breakfast cereals, corn tortillas, and other products) and thus it will be important to test biomarkers of sugar intake based on $\delta^{13}\text{C}$ values in populations where corn intake is high and in populations where it is low.

Nitrogen

The nitrogen isotope ratio is a potential biomarker of protein source because tissue nitrogen derives almost entirely from dietary protein. Plants take up inorganic nitrogen from the soil and assimilate it into amino acids; therefore, their $\delta^{15}\text{N}$ values are determined by the $\delta^{15}\text{N}$ values of soil nitrogen sources. Because nitrogen assimilation discriminates against ^{15}N , plants may have lower $\delta^{15}\text{N}$ values than soil nitrogen when nitrogen availability is high (and plants can be isotopically “choosy”) (41). The synthetic, inorganic fertilizers used in conventional agriculture have $\delta^{15}\text{N}$ values close to that of atmospheric N_2 (0‰) (6, 152), and conventionally grown crops typically have similar $\delta^{15}\text{N}$ values, approximately -5 ‰ to $+5$ ‰ (7). In contrast, organic fertilizers have higher and more variable $\delta^{15}\text{N}$ values (6), a difference that can aid in determining the authenticity of organically-labeled produce (7).

The $\delta^{15}\text{N}$ value is of particular interest as a biomarker of animal protein intake, because animal $\delta^{15}\text{N}$ values are typically elevated over those of plants (37). When animals consume plants, plant nitrogen is incorporated into animal proteins; therefore, animal $\delta^{15}\text{N}$ values tend to reflect dietary sources (37). However, animals are constantly generating and excreting waste nitrogen, and this process shows a strong preference for the lighter isotope, ^{14}N (72, 91, 141). The disproportionate excretion of ^{14}N and retention of ^{15}N

causes animals to have $\delta^{15}\text{N}$ values that are 3‰ to 4‰ higher than diet (37, 85). This increase in $\delta^{15}\text{N}$ values occurs at each step up a food chain (Figure 5), such that top predators, such as polar bears, have very high $\delta^{15}\text{N}$ values (~20‰)(9). Ecologists have long used the $\delta^{15}\text{N}$ value as a measure of an animal's position in the food web, or "trophic level" (61, 78). Generally the animals consumed in the United States for food are herbivores (cows, chickens, turkeys, pigs), with $\delta^{15}\text{N}$ values higher than those of most conventionally grown crops.

The $\delta^{15}\text{N}$ value is a particularly good marker for seafood consumption. Seafood is an exception to the rule that animals consumed for food in the United States are herbivores. Commonly consumed fish species such as tuna, salmon, cod, pollock, halibut, and swordfish are highly predatory; furthermore, food chains tend to be much longer in the marine environment than in the terrestrial environment. Consequently, the $\delta^{15}\text{N}$ values of fish (~10‰ to 20‰) are markedly elevated relative to other foods (0‰ – 5‰), including other meats (90, 98, 138). Thus, $\delta^{15}\text{N}$ values have the potential to indicate both meat intake and fish intake, with meat having a smaller effect but being more widely consumed, and fish having a bigger effect but being consumed less frequently. Approaches that allow the meat component of variation in $\delta^{15}\text{N}$ values to be disentangled from the fish component will be of particular interest in the development of this biomarker.

Sulfur

The $\delta^{34}\text{S}$ value has the potential to be a useful biomarker of seafood intake but has been comparatively little explored in nutritional studies. There are two major reservoirs of sulfur in the earth: igneous sulfur, with a $\delta^{34}\text{S}$ value of 0‰, and oceanic sulfate with a $\delta^{34}\text{S}$ value of 21‰ (75). Plants assimilate sulfate into biological compounds with relatively little isotope fraction, and their $\delta^{34}\text{S}$ values are typically within 0‰ to –2‰ of the $\delta^{34}\text{S}$ value of their sulfur source (148). Furthermore, unlike nitrogen, $\delta^{34}\text{S}$ values do not exhibit a systematic shift between plants and their animal consumers (85) so that the $\delta^{34}\text{S}$ values of both animals and plants tend to reflect the $\delta^{34}\text{S}$ value of sulfate at the base of the foodweb. Marine plants and animals exhibit $\delta^{34}\text{S}$ values ranging from ~17‰ to 21‰ (50, 75). Values of $\delta^{34}\text{S}$ in terrestrial plants and animals are lower, but are also more variable, as sulfate $\delta^{34}\text{S}$ values in the terrestrial environment vary with the nature of the bedrock (e.g. marine sedimentary vs. igneous), soil bacterial activity, and other sulfate inputs (e.g., pollution or sea spray) (28). Where terrestrial plants are not affected by sea spray or other marine inputs, $\delta^{34}\text{S}$ values typically fall within the range of 0 to 10‰ (114). Thus, $\delta^{34}\text{S}$ values can be a powerful tool for detecting intake of marine-based foods (18), that is independent of trophic level. When measured in combination with $\delta^{15}\text{N}$ values, $\delta^{34}\text{S}$ values could help differentiate individuals with high meat intakes from those with high fish intakes.

Hydrogen/Oxygen

The SIRs of hydrogen ($^2\text{H}/^1\text{H}$, usually expressed as D/H for "deuterium/hydrogen") and oxygen ($^{18}\text{O}/^{16}\text{O}$) vary in broad geographical patterns, due to isotope fractionation in the water cycle (62). The H and O in both drinking water and foods from the land derive from precipitation, which supplies water to lakes, rivers, and groundwater. Most of the water in precipitation derives from evaporation over tropical and subtropical oceans, and evaporation

shows a strong preference for the lighter isotopes of both H and O (15, 62). As the water vapor in air moves northward and across continents, the heavy isotopes condense into precipitation more readily than the light isotopes, causing progressively lower δD and $\delta^{18}O$ values in the remaining vapor and in successive precipitation events (15). This process creates striking continental-scale variation in the δD and $\delta^{18}O$ values of water (14). This geographic pattern of SIRs in water is captured by local plants, and subsequently, animals (58, 60). A major effort in wildlife research has been devoted to understanding the use and limits of δD and $\delta^{18}O$ values to identify the geographic origins of migratory animals (59). In human studies, δD and $\delta^{18}O$ values have largely been explored as forensic tools to constrain regions of origin, and differentiate local from non-local residents (39, 122). There is a strong geographic association between the δD and $\delta^{18}O$ values of water and human hair in the United States (39).

Naturally-occurring isotope variations in H and O have been little explored as potential biomarkers of diet. Both δD and $\delta^{18}O$ values should be elevated in food deriving from the marine relative to the terrestrial environment, and limited available data supports this (26). It is also possible that δD or $\delta^{18}O$ values could differentiate foods with specific regions of production (e.g. sugar cane) (103); however, there has not yet been a systematic investigation of multiple food types and their variation in δD and $\delta^{18}O$ values. One of the issues in dietary applications is that H and O can derive from both water and from diet (56, 159, 160); however, human population studies suggest that the contribution of diet to both H and O is high, based on the low slopes of the relationships between δD and $\delta^{18}O$ values in hair and local drinking water (39, 103). Thus, although there is potential for variations in natural abundance SIRs of H and O in human tissues to contribute information as dietary biomarkers, such an application is still undeveloped. Because of the very early state of knowledge about O and H as human dietary biomarkers, the focus for the remainder of this review is on C, N and S.

STABLE ISOTOPE RATIOS IN TISSUES

In this section of the review we turn our attention from the SIRs in foods to how they are captured in human tissues as the molecules from food are broken down and reassembled into new molecules and cells. This point may be obvious, but is worth stating: all of the carbon, nitrogen and sulfur in our bodies derives from the food we eat; there are no other sources of those elements and there is no way for the body to synthesize them *de novo*. This characteristic distinguishes SIRs from many molecules that can serve as dietary biomarkers, which may be of either dietary or endogenous origin or which may undergo modification through metabolism. However, the SIR of a given tissue does not always perfectly match current diet. Here we consider some of the factors that can affect the tissue SIR.

Elemental turnover

Our bodies are in a constant process of elemental turnover as C, N and S are lost from the body (as respiratory CO_2 , urine, sloughed cells, and incrementally growing structures like hair and nails) and replaced by C, N and S from our diet. Tissues vary in their rates of elemental turnover, and these differences govern the time frame over which dietary information is captured and integrated by that tissue. Tissue elemental turnover should be

Another issue to consider in the use of tissue SIRs as dietary biomarkers is whether elements from foods of interest are incorporated into (or routed to) the tissues measured. This is a straightforward question for nitrogen and sulfur, as they are primarily supplied by dietary proteins and incorporated into tissue proteins, which means they show almost perfect protein-to-protein routing. However, the situation is more complicated for carbon, which is supplied by all major macronutrients in the diet: protein, lipids, and carbohydrates. In the

ecological and archeological literature, researchers often assume that the carbon in tissue protein reflects only the carbon from dietary protein sources; in other words, that there is protein to protein routing for carbon, as some studies have shown (1). However, other animal studies have demonstrated that there can be a high contribution of non-protein dietary carbon to protein-rich tissues such as RBCs (123), which indicates that there has been significant synthesis of non-essential amino acids from non-protein carbon sources.

The question of whether carbon is or is not routed from dietary protein to tissue protein is particularly relevant for biomarkers of sugar intake based on $\delta^{13}\text{C}$ values. If the carbon in tissue protein came exclusively from dietary protein, it would be impossible to detect intake of corn and cane-based sugars by measuring the $\delta^{13}\text{C}$ values of hair or RBCs. In that scenario, tissue $\delta^{13}\text{C}$ values would reflect only dietary protein sources. However, recent studies show that dietary intake of both sweeteners and corn-fed animals affect blood $\delta^{13}\text{C}$ values (97, 161), and thus, that carbon from both dietary sugars and dietary proteins is routed into blood proteins. Approaches that maximize the routing of elements from the foods of interest to the tissue or molecules measured will be particularly valuable in the further development of stable isotope biomarkers, and current approaches are discussed in greater detail in the section titled "Validation Studies".

Physiological effects

The primary determinant of carbon, nitrogen and sulfur SIRs in tissue is diet; however, physiological state has the potential to affect the SIR if it differentially alters the rate at which heavy vs. light isotopes are incorporated into tissues or excreted. In this section we examine some of the physiological factors that differentially impact heavy and light isotopes and discuss why tissues vary systematically in SIR.

Tissue $\delta^{15}\text{N}$ values have the potential to be influenced by nitrogen balance. In the section on nitrogen isotopes we discussed the fact that $\delta^{15}\text{N}$ values of animal tissues are elevated by 3–4‰ over those of the diet, due to discrimination against ^{15}N during the formation of nitrogenous waste. This difference in $\delta^{15}\text{N}$ values between animal tissues and diet is termed "trophic fractionation". Factors that alter the rate of nitrogen excretion relative to the rate of nitrogen intake, for example, growth (49), starvation (80), and major differences in dietary protein quality (133), have the potential to alter the magnitude of nitrogen trophic fractionation. According to a simple box model of this process (72), N trophic fractionation is predicted to be high when the proportion of dietary N lost to excretion is high (insufficient dietary protein, low protein quality), and low when the proportion of dietary N lost to excretion is low (growth, high protein quality). In a memorable pair of studies, Fuller and colleagues demonstrated that the $\delta^{15}\text{N}$ values of hair from pregnant women decreased during periods of weight gain and increased during periods of weight loss due to morning sickness (47, 48). Similarly, hair $\delta^{15}\text{N}$ values increased with weight loss in anorexia nervosa patients (55, 89). Finally, hair $\delta^{15}\text{N}$ values were markedly lower in liver cirrhotic patients relative to matched healthy participants, an effect that is not well understood but is presumably due to alternations in liver amino acid metabolism (117). Studies using $\delta^{15}\text{N}$ values as a dietary biomarker should exclude participants who are pregnant, who are undergoing rapid weight gain or loss, and who have chronic liver disease.

The primary physiological influence on tissue $\delta^{13}\text{C}$ values is lipid content. Lipids have $\delta^{13}\text{C}$ values that are typically 5–7‰ lower than those of tissue protein or carbohydrate (143), due to an enzymatic preference for ^{12}C at one of the early biochemical steps in lipid synthesis (35). Because of this effect, ecological researchers commonly lipid-extract muscle samples being measured for dietary studies to remove the potentially confounding effect of variation in tissue lipid content on $\delta^{13}\text{C}$ values (124). However, lipid-extraction can also alter tissue $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values (110), which is an undesirable side-effect. An alternative approach is to mathematically correct $\delta^{13}\text{C}$ values for tissue lipid content using the tissue C:N ratio, an accurate proxy for lipid content (124). The lipid content of samples such as hair and RBC is too low to be a confounding factor on $\delta^{13}\text{C}$ values; however, it could be an issue for serum or plasma samples if their lipid content was high. In general, trophic fractionation for carbon is more modest than for nitrogen, averaging approximately +1‰ (36). This effect is likely caused by the fact that respired CO_2 has a low $\delta^{13}\text{C}$ value relative to the body (36), providing an avenue for disproportionate loss of ^{12}C . In one animal study this effect was observed only in fasted individuals (154), supporting its relationship to lipid metabolism.

Physiological effects on tissue $\delta^{34}\text{S}$ values have not been well explored; however, estimates of trophic fractionation for sulfur average around 0‰ (51, 85), suggesting that any such effects are minor. There is some evidence that sulfur diet to tissue fractionation may increase when dietary protein content is inadequate, similar to the effect seen for nitrogen; however, the evidence is based on a very small number of observations (131). More studies are needed to better understand the physiological effects that may impact tissue $\delta^{34}\text{S}$ values.

Finally, it is important to be aware that the SIRs of different tissues, while very highly correlated, differ predictably within an organism. In humans, for example, hair $\delta^{13}\text{C}$ values are 2‰ higher than plasma and RBC $\delta^{13}\text{C}$ values, whereas plasma and hair $\delta^{15}\text{N}$ values are 1.5‰ higher than RBC $\delta^{15}\text{N}$ values (74, 99, 100). These observations are consistent with the findings of a number of animal studies (57, 147). This effect is related to differences in amino acid composition among proteins and the fact that individual amino acids vary consistently in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (53, 105). One example is the amino acid glycine. Glycine has a high $\delta^{13}\text{C}$ value relative to other amino acids (87), and is relatively abundant in keratin, the protein comprising hair (83). These differences do not alter the potential for SIRs to serve as biomarkers of diet, but it is important for researchers to take these differences into account, particularly when comparing the results of studies using measurements from different tissues.

STABLE ISOTOPE RATIOS IN HUMAN DIETARY STUDIES

Archaeology

Stable isotopes have a long and rich history of use as dietary proxies in human archeology (79). It is beyond the scope of this article to review this large literature, so I simply note that the use of SIR as biomarkers of human diet has its precedent in studies of our forebears. SIRs can be measured from the proteins collagen and dentine and from apatite carbonates preserved in bones and teeth (111). These measurements have provided considerable insight into the diets of ancient populations. For example, the appearance of greatly elevated $\delta^{13}\text{C}$ values in human bones from wooded regions of North America, where C4 plants were not

naturally occurring, identified the point in time approximately 1000 years ago when corn cultivation became widespread and corn became a major component of the diet (Figure 7) (151, 153). Modest increases in $\delta^{15}\text{N}$ values are commonly used as indexes of meat consumption, whereas more striking increases in $\delta^{15}\text{N}$ values are interpreted as consumption of marine proteins, especially when accompanied by artifacts that indicate fishing or marine food consumption (45, 132). Increases in plant (and thus, herbivore) $\delta^{15}\text{N}$ values may also be caused by arid, denitrifying environments (54); therefore, archeologists have proposed using independent measures of marine food intake, such as patterns of $\delta^{13}\text{C}$ values from specific amino acids (32), or $\delta^{34}\text{S}$ values (129). Such approaches are likely also to prove useful in nutritional epidemiology, as approaches for improving biomarker specificity, as discussed later in this review.

Proof-of-concept studies

Among the earliest studies to examine isotope variations in the modern human diet were those by Schoeller and colleagues, who noted that intake of sugars caused a marked increase in the baseline $\delta^{13}\text{C}$ value of breath CO_2 (139). They followed up on this finding by publishing an isotopic survey of a wide range of United States foods (137), to better understand dietary effects on baseline breath CO_2 $\delta^{13}\text{C}$ values. This and subsequent studies in the 1980's and early 1990's identified major differences in the $\delta^{13}\text{C}$ values of food plants based on whether they used C3 (wheat, rice, beans, most fruits and vegetables, maple syrup) or C4 (corn, millet, cane sugar) photosynthetic pathways (90, 94, 137, 138). As described previously in this review, foods based on animal products (meat and dairy) have $\delta^{13}\text{C}$ values that are intermediate between C3 and C4 plants, with beef and pork typically having the highest $\delta^{13}\text{C}$ values due to high corn intakes and dairy products having the lowest $\delta^{13}\text{C}$ values. Marine foods have $\delta^{13}\text{C}$ values of approximately -20‰ , reflecting the typical $\delta^{13}\text{C}$ values in marine food webs. A comparison of foods from the United States, Japan and Germany showed that these patterns were robust across geographical region, although meat, eggs, and dairy from Germany had lower $\delta^{13}\text{C}$ values, reflecting less use of corn for animal feed. Furthermore, German food plants had $\delta^{13}\text{C}$ values that were approximately 2‰ lower than those measured in the United States and Japan (94), possibly due to the uptake of industrially-produced CO_2 , which has a low $\delta^{13}\text{C}$ value. Food $\delta^{15}\text{N}$ values were lowest in plants, intermediate in meat, eggs, and dairy, and highest in fish (90, 138), although plant $\delta^{15}\text{N}$ values were quite variable. Measurements of hair $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ from residents of the United States, Germany, and Japan were consistent with isotopic measurements of food, when modeled using population-wide estimates of intake (90, 94, 138). These early studies of the modern human diet are particularly impressive in light of the fact that automated CF-IRMS measurements of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were not yet available at that time; therefore, the "library" of stable isotope values from human foods and tissues they provide represents a major analytical effort.

More recent studies have reported $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of foods that are consistent with early papers, but have provided additional measurements of United States sweeteners (65, 98), freshwater and marine fish (64) and marine mammals (98, 158), as well as other foods. A recent study has provided the most comprehensive dataset of food $\delta^{15}\text{N}$ values to date (64), documenting consistent patterns of $\delta^{15}\text{N}$ among types of food plants and animals,

particularly fish. A survey of SIRs from food and human fingernails in Brazil showed similar patterns to the United States and elsewhere; however, $\delta^{15}\text{N}$ values were higher in Brazil than in the United States throughout the foodweb, suggesting differences in the types of agricultural fertilizers used (95). Differences in SIRs among foods from Brazil, Germany, the United States and Japan highlight the importance of validating isotopic biomarkers of diet in the country (ideally the population) in which they will be used, due to differences in geography, food sourcing, and agriculture that can effect SIRs.

The consistent difference in $\delta^{15}\text{N}$ values between foods deriving from plants and animals suggested that $\delta^{15}\text{N}$ values in human tissues could differentiate individuals on the basis of animal protein intake. This idea was initially tested in exploratory studies measuring the hair or fingernails of volunteers who identified themselves as vegan (no animal products in the diet), ovo-lacto-vegetarian (OLV, diet includes eggs and dairy but no meat), or omnivorous. Although sample sizes were small, vegans had markedly lower $\delta^{15}\text{N}$ values than OLV or omnivores (13, 104), and had lower $\delta^{13}\text{C}$ values in one study (13) but not the other (104). Differences between OLVs and omnivores were not consistent; OLVs had lower $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in two populations (13, 95), but were indistinguishable from omnivores in others (95, 104). In one study, hair $\delta^{15}\text{N}$ values were strongly associated with self-reported frequency of animal protein consumption (on a 4-point scale), for both OLVs and omnivores (104).

Isotopic measures of marine food intake were tested in a unique proof-of-concept study involving Inuit from North Greenland. This population has very high intakes of local marine foods, including seals, whales, and fish; in addition, their market foods are imported from Denmark, which includes almost no foods of C4 origin. Thus, diets would be expected to vary along an isotopic gradient, from C3-based market foods (low $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values) to marine foods (higher $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values), depending on dietary pattern. The study measured $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values of fingernails from 82 Greenland Inuit individuals and 32 Danes, and found striking linear relationships between all three SIRs (Figure 8), with Danes forming a group at the low end of all isotope distributions, and the Greenland Inuit varying depending on their extent of marine food intake. The fact that SIR can identify dietary differences so clearly in a population consuming such isotopically distinct foods is perhaps not surprising; however, the strength of the correlations among these three independent isotopic measures (all exceeding 0.9) demonstrates the potential magnitude of the dietary signal relative to other sources of error (individual variation and/or analytical error) in SIR measurement.

Two final proof of concept studies evaluated the relationship between diet and SIRs experimentally. The first verified the isotopic response in hair samples to changing dietary SIRs, with a controlled feeding study of four individuals who were switched to a C4/marine-based diet for 28 days (63). That study nicely demonstrated large isotopic deflections in serial hair sections for both carbon and nitrogen, although the magnitude varied among participants, potentially due to their differential selection of the food items provided. The study also found that incorporation of dietary N into hair was more rapid than incorporation of dietary C; an interesting finding that needs replication because it contradicts several animal studies showing either no difference or more rapid incorporation for dietary C (5, 17,

21, 109). The second study estimated the diet to tissue fractionation for nitrogen in human RBCs using a 30-day controlled feeding study of 11 individuals. This study found that RBC $\delta^{15}\text{N}$ values were 3.5‰ higher than those of diet (106), an estimate that is right in line with animal studies (37, 85).

Validation studies

In this section of the article I will review studies validating tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values as measures of fish, animal protein (meat), and sugar intake, all published within the last 10 years. These studies are grouped by the location (Europe or the United States), because these regions differ enough in food SIRs, particularly those of sugars and meats, that results from one region may not be directly applicable to the other. The studies also varied in how diets were assessed: by controlled feeding studies, by alternative biomarkers, or by self-reported methods. Although a controlled feeding study is the gold standard for measuring intake in a biomarker validation study, studies comparing SIRs to self-report have been valuable, because they show how SIR can be associated with multiple components of the diet. This knowledge is very important when interpreting associations between stable isotope biomarkers and disease risk, as I review in the next section. Although self-reported estimates of intake have high error, their errors are uncorrelated with the errors inherent in stable isotope measurements – something that is not true when validating self-reported measures against each other (108).

European validation studies

Petzke and colleagues were the first to validate stable isotope biomarkers in an epidemiological context in 2005 (115), by reporting associations with diet (seven-day food record) in a German nutrition study {VERA (Verbundstudie Ernährungserhebung und Risikofaktoren-Analytik) (Nutrition Survey and Risk Factor Analysis Study)} (115, 116). Participants ($n = 126$) were randomly selected from the study sample of 1,988 adults, with oversampling of those reporting little to no meat intake. Hair $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were associated with each other ($r = 0.39$), and both were associated with intake of meat and % animal protein intake, with R^2 values for regression models of both isotopes on dietary intake ranging from 0.2 to 0.3 (115). Hair $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values easily differentiated omnivores, OLVs, and vegans (116). Associations with other dietary variables, such as fish intake, were not presented. A recent paper by Patel and colleagues based on the Norfolk population of the EPIC (European Prospective Investigation into Cancer and Nutrition) Study similarly assessed associations of serum $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values with diet [food frequency questionnaire (FFQ)], in 1,178 participants (112). In this United Kingdom population, the serum $\delta^{15}\text{N}$ value was associated with intakes of fish, dairy, and meat and animal protein, whereas the serum $\delta^{13}\text{C}$ value was only associated with fish intake. The discrepancy in the association of $\delta^{13}\text{C}$ values with meat/animal protein intake in Germany and the United Kingdom could be caused by differences in the proportion of corn fed to livestock in those countries. Spearman r ranged from ~ 0.1 to ~ 0.2 in the EPIC study, and the strongest associations were with fish intake. Biomarker – diet associations were weaker in the EPIC study compared to the VERA study, likely due in part to differences between the studies in dietary assessment methodology and error.

The effects of meat and fish intake on isotopic measures in the United Kingdom and Germany have also been investigated in controlled feeding studies. Kuhnle and colleagues investigated meat and fish intake in 14 United Kingdom volunteers in a cross-over design, in which each dietary treatment lasted 8 days, and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were measured in urine, feces, and RBC and compared to a vegetarian control group (76). Dietary effects on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of urine and feces were similar to those found in the EPIC study: $\delta^{15}\text{N}$ values were elevated in the meat treatment and highest in the fish treatment compared to a vegetarian control group, whereas $\delta^{13}\text{C}$ values were elevated only in the fish treatment. There were no differences in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of RBC, given the short duration of the feeding treatments. The German study, by Petzke & Lemke (119), was a semi-controlled, cross-over feeding study in which 14 female volunteers either added a daily serving of pork filet to their habitual diet or omitted meat and meat products from their diets for 4 weeks. In this study, there were no effects on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of hair or plasma, and only a weak effect on SIRs of urine. This result was unexpected, as 4 weeks should be sufficient to see effects in both plasma and hair SIRs (63). Measures of urinary urea and 3-methylhistidine excretion confirmed the differences among treatments in meat intake; however, it is possible that consumption of other forms of animal protein, such as eggs or dairy, increased in a compensatory fashion as meat intake was altered, obscuring dietary effects on tissue $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values.

US validation studies

Stable isotope measures of fish intake were assessed in a Yup'ik population in Southwest Alaska. Yup'ik people have a strong cultural tradition of subsistence hunting and fishing, and traditional foods, predominantly fish and marine mammals, account for an average of 22% of energy intake (10). We validated the $\delta^{15}\text{N}$ value as a biomarker of fish and marine mammal intake against an independent biomarker of marine food intake, RBC ω -3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in a sex-, age-, and community- stratified sample of 497 Yup'ik adults from the Center for Alaska Native Health Research (CANHR) study. $\delta^{15}\text{N}$ values in RBC and hair were strongly associated with RBC EPA and DHA ($r > 0.8$, Figure 9), due to the large variability in marine food intake in the population and the comparatively small errors with which the biomarkers were measured (99, 101). RBC $\delta^{15}\text{N}$ values were also associated with self-reported fish and marine mammal intake (one 24-hour recall + one 3-day food record) in a community-based sample of 230 Yup'ik people ($r = 0.52$) (98). Unlike in the VERA study, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were uncorrelated. In the Yup'ik diet, corn-based United States market foods (primarily sugar-sweetened beverages and commercial meats) have the highest $\delta^{13}\text{C}$ values, and their intake is inversely associated with intake of traditional foods, including fish and marine mammals.

Several studies in the last 5 years have investigated $\delta^{13}\text{C}$ values as biomarkers of sugar intake for United States populations, where approximately 75% of the sugars sold, and 99% of sugars in SSBs, derive from corn or sugar cane (52). United States meat products also have high $\delta^{13}\text{C}$ values (94), as livestock are heavily corn-fed (67). Yeung and colleagues examined associations of serum $\delta^{13}\text{C}$ values with self-reported intake (FFQ) in 186 participants of the Atherosclerosis Risk in Communities (ARIC) magnetic resonance imaging study (161), with oversampling at the high and low ends of SSB consumption.

Serum $\delta^{13}\text{C}$ values were associated with sweetened beverage intake ($r = 0.18$), as well as measures of animal protein ($r = 0.28$) and fat ($r = 0.37$) intake. In a study published the following year, Davy and colleagues examined associations of the whole blood $\delta^{13}\text{C}$ value from a fingerstick with added sugar intake (four-day food record) and sweetened beverage intake (beverage questionnaire) in 60 adults and found associations with added sugar intake ($r = 0.37$) and total SSB ($r = 0.35$) (33). Associations with $\delta^{15}\text{N}$ values and with animal protein intake were not assessed. Biomarker – diet associations may have been stronger in the Davy study than the Yeung study due to the method of diet assessment used (4d food record vs. FFQ).

Two recent studies of the $\delta^{13}\text{C}$ value as a biomarker of sugar intake have used $\delta^{15}\text{N}$ values to control for confounding dietary effects. Nash and colleagues evaluated the RBC $\delta^{13}\text{C}$ value as a measure of sugar intake in 68 Yup'ik (Alaska native) adults for whom dietary intake was assessed by four, weekly 24-hour recalls (97). The RBC $\delta^{13}\text{C}$ value was associated with total sugar, meat and fish intake, and the association with total sugar was the weakest of the three. To reduce confounding dietary effects on RBC $\delta^{13}\text{C}$ values, they included the $\delta^{15}\text{N}$ value as a covariate to control for high and varying fish intake. The resultant two isotope model strongly predicted intake of total sugar ($R^2 = 0.48$) (Figure 10), as did models using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from hair and plasma (100). Adjustment by the $\delta^{15}\text{N}$ value did not remove association with meat intake in the Yup'ik population, as $\delta^{15}\text{N}$ values were related to fish intake only. In another recent study of a subset of participants in the PREMIER dietary intervention trial ($n = 144$), Fakhouri and colleagues (42) found that even modest reductions in SSB intake were associated with reduced serum $\delta^{13}\text{C}$ values. In this study, $\delta^{13}\text{C}$ values were also associated with $\delta^{15}\text{N}$ values, presumably due to co-association with animal protein intake, and adjustment by $\delta^{15}\text{N}$ values improved association between $\delta^{13}\text{C}$ values and SSB intake, although only slightly. To my knowledge this is the first study to report changes in $\delta^{13}\text{C}$ values with decreasing intake of sugars or SSB.

Another way to increase the specificity of $\delta^{13}\text{C}$ values for sugars in United States populations is to measure the $\delta^{13}\text{C}$ values of molecules that favor the incorporation of glucose carbon. Two recent studies have taken this approach. Cook and colleagues conducted a seven-day cross-over feeding study of five adults consuming three diets that differed in the percent of carbohydrate deriving from C4 sugars (corn and cane-based), and measured the $\delta^{13}\text{C}$ value of isolated blood glucose at multiple time-points throughout the day (31). They found a strong relationship between C4 sugar intake and the $\delta^{13}\text{C}$ value of postprandial blood glucose, but no effect of the 7 day dietary treatments on blood glucose measured after overnight fasting. A recent follow-up study by Nash and colleagues confirmed this result, finding that the $\delta^{13}\text{C}$ value of fasted blood glucose in 68 Yup'ik adults was not associated with differences in usual total or added sugar intake (100). Thus, the $\delta^{13}\text{C}$ value of blood glucose, although an excellent short term measure of sugar intake, does not appear to integrate sugar intake over multiple days or weeks. An alternative approach was proposed by Choy and colleagues, who measured the $\delta^{13}\text{C}$ values of RBC amino acids in 68 Yup'ik adults using GC-C-IRMS, and compared them to self-reported intake of sugars, meat and fish (weekly 24-hour recall for four weeks) (27). They found a strong ($r = 0.7$) relationship between the $\delta^{13}\text{C}$ value of RBC alanine and SSB intake (Figure 10), and no

relationship with meat or fish intake. Alanine has a close metabolic connection to glucose via the glucose-alanine cycle (113, 155), and appears to capture a long term record of average blood glucose $\delta^{13}\text{C}$ values in RBC and hair (27).

Nutritional epidemiology

Relatively few studies to date have used stable isotope biomarkers to examine diet – disease associations in human populations, due to the fairly early state of validation for most of these biomarkers. However, as a validated biomarker of marine food (fish and marine mammal) intake for the Yup'ik population of SW Alaska, the RBC $\delta^{15}\text{N}$ value has been used to examine how gene $\times$ diet interactions are associated with risk factors for obesity and chronic disease (2, 71, 81, 82), and it was recently used to demonstrate that high and low consumers of marine foods exhibit very different patterns of gene methylation (3). We found that the RBC $\delta^{15}\text{N}$ value captured most of the same diet - risk factor associations that were previously identified for Yup'ik people using RBC EPA and DHA (84), but that in addition, high RBC $\delta^{15}\text{N}$ values were associated with high adiponectin, which promotes insulin sensitivity, and were inversely associated with blood pressure (102). However, some of these effects could result from inverse associations with market food consumption, including sugars and meats (96), which cannot be disentangled in a cross-sectional study.

A recent case-cohort (112) study from the EPIC study (Norfolk cohort) examined biomarker associations of serum $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values with incident type 2 diabetes; it is the first study of its kind. Patel and colleagues found that $\delta^{15}\text{N}$ values were associated with diabetes incidence [hazard ratio per tertile of $\delta^{15}\text{N}$ (HR) = 1.23, CI 1.09, 1.38], whereas $\delta^{13}\text{C}$ values were inversely associated with diabetes incidence (HR = 0.74, CI 0.65, 0.83) (112). In this population, $\delta^{13}\text{C}$ values were associated with fish intake, whereas $\delta^{15}\text{N}$ values were associated with intakes of meat, dairy, fish, and animal protein (112), as described previously. Because of these differential patterns of association, Patel and colleagues propose that meat/animal protein intake may be associated with increased diabetes risk in this population, whereas fish intake may be inversely associated with diabetes risk. If they are correct, the true HR for meat/animal protein intake may be higher, given that $\delta^{15}\text{N}$ values reflect both meat/animal protein and fish intake, and those intakes have opposing associations with diabetes incidence.

FUTURE DIRECTIONS

SIRs have tremendous potential as biomarkers of diet, due to their variability among foods, the fidelity with which they are captured in tissues and molecules, and their comparative ease of measurement. Because of their stability in stored samples, SIR biomarkers offer the potential for retrospective analyses. However, more validation work remains to be done for these measures to achieve their potential as tools for nutritional epidemiology. This validation work needs to include both controlled feeding studies and population-level studies, because each study type offers different, complementary information. Furthermore, future work needs to address how specificity can be improved for stable isotope biomarkers.

Controlled feeding studies are needed in order to understand the dose-response relationships between isotopic biomarkers and specific food intakes. These studies need to be fairly long-

term, as stable isotope measures integrate dietary signals over long periods, depending on the rates of elemental turnover of the tissue or molecules being studied. Although a fully randomized cross-over design is desirable for biomarker validation (145), such a study risks underestimating effect sizes if tissue or molecular isotopic measures do not equilibrate with study diets within the period of each treatment arm. This is a pervasive problem with controlled studies in animal ecology, and has led to flawed conclusions; for example, the conclusion that trophic fractionation depends on dietary SIR (23), which is impossible. Controlled studies that seek to replicate and maintain usual intake in participants may be a better alternative for validating stable isotope measures (106, 146), because experimental diets should involve less perturbation from baseline. An additional advantage of such studies is that multiple food – isotope associations can be evaluated within a single study.

Diet-biomarker associations also need to be evaluated at the population level in diverse populations to test how they may be affected by differences in diet composition. For example, the $\delta^{13}\text{C}$ value of alanine was associated with intake of SSBs but not corn in an Alaska Native population; however, this population consumes few foods produced from whole corn. Dietary co-variables of alanine $\delta^{13}\text{C}$ values should be assessed in populations with higher corn intakes to verify the measure's specificity for SSBs in those populations. Such studies will necessarily rely on self-reported dietary measures, and should use the best measures available (149), with the understanding that biomarker-diet relationships will be attenuated from their true associations. A recent study assessing measurement error in self-reported sugars intake found the least attenuation for multiple 24-hour recalls, followed by four-day food record, and finally FFQ (144), although all three measures were significantly misreported.

New approaches to increase the specificity of stable isotope measures for specific foods are needed. An approach that has been underexplored is the use of multiple SIRs to achieve better validity for foods of interest (97). For example, $\delta^{34}\text{S}$ values have the potential to resolve whether elevated $\delta^{15}\text{N}$ values reflect fish or meat intakes (112). Such an approach could also combine SIR biomarkers with other biomarkers of dietary exposure. Approaches that measure isotope ratios in specific molecules with more direct metabolic links to foods of interest may also help to refine SIR biomarkers where multiple foods affect bulk tissue SIR measurements (27). Regardless of the approach, it is important that validation studies examine and report associations with multiple components of the diet and not only the specific nutrient or food of interest. This information is needed to correctly interpret SIR biomarkers in an epidemiological context.

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Element	Stable Isotopes	Abundance (%)*
Hydrogen	^1H (H)	99.985
	^2H (D)**	0.015
Carbon	^{12}C	98.892
	^{13}C	1.108
Nitrogen	^{14}N	99.635
	^{15}N	0.365
Oxygen	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Sulfur	^{32}S	95.0
	^{33}S	0.75
	^{34}S	4.21
	^{36}S	0.014

*Abundance does not sum to 100% because radioisotopes are omitted from this table

**D = deuterium, $^2\text{H}:^1\text{H}$ is often given as D:H

Figure 1.
Naturally occurring stable isotopes of light elements common in biological molecules.

δ

$$\delta X = ((R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}) \times 1000\text{‰}$$

X = heavy isotope (e.g., ^{13}C)

R = the ratio of heavy to light isotope (e.g., $^{13}\text{C} / ^{12}\text{C}$)

Delta values are expressed in "permil" relative abundance. Permil (‰, $\times 1000$) is analogous to percent (% , $\times 100$) and is used because differences in stable isotope ratios at natural abundance are so small.

Table 2. Standards used to calculate δ (relative abundance of heavy isotope)

Element	International standard	Isotope ratio of the standard
Hydrogen	Vienna Standard mean ocean water (V-SMOW)	$^2\text{H}/^1\text{H} = 0.00015576$
Carbon	Vienna Pee-Dee Belemnite (V-PDB)	$^{13}\text{C}/^{12}\text{C} = 0.0112372$
Nitrogen	Atmospheric N_2 (air)	$^{15}\text{N}/^{14}\text{N} = 0.0036765$
Oxygen	V-SMOW*	$^{18}\text{O}/^{16}\text{O} = 0.0020052$
Sulfur	Vienna Canon Diablo Triolite (V-CDT)	$^{34}\text{S}/^{32}\text{S} = 0.0450045$

*When $\delta^{18}\text{O}$ is measured in carbonates commonly V-PDB is used as the O standard ($^{18}\text{O}/^{16}\text{O} = 0.0020672$)

Figure 2.
Delta demystified

Figure 3.

Measurement of carbon isotope ratios by continuous flow-isotope ratio mass spectrometry (CF-IRMS). A) Elemental analysis – isotope ratio mass spectrometry (EA-IRMS), in which solid samples are combusted and separated into gases by an elemental analyzer before being introduced into the IRMS. B) Gas chromatography – Combustion – isotope ratio mass spectrometry (GC-C-IRMS), in which samples are injected into a gas chromatograph for separation prior to combustion and introduction into the isotope ratio mass spectrometer. Figure used with permission from Reference 93, copyright Royal Society of Chemistry.

Figure 4.

The distribution of $\delta^{13}\text{C}$ values (‰) in C3 and C4 plants, based on approximately 1000 analyses from five different laboratories. Figure adapted with permission from Reference 107, copyright Oxford University Press.

Figure 5. The stepwise increase in $\delta^{15}\text{N}$ values in with trophic level, or position in a food web. Figure adapted with permission from Reference 20, copyright Pearson-Benjamin Cummings.

Figure 6.

Carbon turnover captured in plasma, whole blood, and claw of house sparrows. Figure used with permission from Reference 22, copyright John Wiley and Sons.

Figure 7.

The shift in $\delta^{13}\text{C}$ values (‰) of bone collagen from skeletons recovered from Archaic, Early Woodland, and Late Woodland sites in North America. The dramatic increase in $\delta^{13}\text{C}$ values following 1000 AD reflect the advent of corn agriculture and significant corn consumption. Figure used with permission from Reference 79, original data from References 151 and 153. Copyright John Wiley and Sons.

Figure 8.

Associations between fingernail $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values (‰) sampled from Inuit residents of Uummannaq District, Greenland ($n = 82$) and residents of Denmark ($n = 32$). Abbreviations: AIR, reference standard for nitrogen, V-CDT, Vienna Cañon Diablo Troilite (reference standard for sulfur), V-PDB, Vienna Pee-Dee Belemnite (reference standard for carbon). Figure used with permission from Reference 18, copyright Elsevier Publishing.

Figure 9.

The association between the ω -3 fatty acid eicosapentaenoic acid [as % red blood cell (RBC) fatty acids] and (a) RBC $\delta^{15}\text{N}$ values and (b) hair $\delta^{15}\text{N}$ values in Yup'ik adults ($n = 497$ and 44 , respectively) living in Southwest Alaska. Both % EPA and $\delta^{15}\text{N}$ values increase with intake of marine foods (fish and marine mammals). Figure adapted with permission from References 101 and 99, copyright American Society for Nutrition.

Figure 10.

(a) Modeled total sugar intake, based on red blood cell (RBC) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ versus actual total sugar intake in 68 Yup'ik adults living in Southwest Alaska. (b) $\delta^{13}\text{C}_{\text{alanine}}$ vs. sugar-sweetened beverage (SSB) intake in 68 Yup'ik adults living in Southwest Alaska. Reproduced with permission from References 27 and 97, copyright American Society for Nutrition.