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The carbon isotope ratios of nonessential amino acids identify sugar-sweetened beverage (SSB) consumers in a 12-wk inpatient feeding study of 32 men with varying SSB and meat exposures

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ABSTRACT

Background: The carbon isotope ratios (CIRs) of individual amino acids (AAs) may provide more sensitive and specific biomarkers of sugar-sweetened beverages (SSBs) than total tissue CIR. Because CIRs turn over slowly, long-term controlled-feeding studies are needed in their evaluation.

Objective: We assessed the responses of plasma and RBC CIR_{AA}'s to SSB and meat intake in a 12-wk inpatient feeding study.

Methods: Thirty-two men (aged 46.2 ± 10.5 y) completed the feeding study at the National Institute of Diabetes and Digestive and Kidney Diseases in Phoenix, Arizona. The effects of SSB, meat, and fish intake on plasma and RBC CIR_{AA}'s were evaluated in a balanced factorial design with each dietary variable either present or absent in a common weight-maintaining, macronutrient-balanced diet. Fasting blood samples were collected biweekly from baseline. Dietary effects on the postfeeding CIR of 5 nonessential AAs (CIR_{NEAA}'s) and 4 essential AAs (CIR_{EAA}'s) were analyzed using multivariable regression.

Results: In plasma, 4 of 5 CIR_{NEAA}'s increased with SSB intake. Of these, the CIR_{Ala} was the most sensitive ($\beta = 2.81$, SE = 0.38) to SSB intake and was not affected by meat or fish intake. In RBCs, all 5 CIR_{NEAA}'s increased with SSBs but had smaller effect sizes than in plasma. All plasma CIR_{EAA}'s increased with meat intake (but not SSB or fish intake), and the CIR_{Leu} was the most sensitive ($\beta = 1.26$, SE = 0.23). CIRs of leucine and valine also increased with meat intake in RBCs. Estimates of turnover suggest that CIR_{AA}'s in plasma, but not RBCs, were in equilibrium with the diets by the end of the study.

Conclusions: The results of this study in men support CIR_{NEAA}'s as potential biomarkers of SSB intake and suggest CIR_{EAA}'s as potential biomarkers of meat intake in US diets. This trial was registered at clinicaltrials.gov/ct2/show/NCT01237093 as NCT01237093. *Am J Clin Nutr* 2021;113:1256–1264.

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Supplemental Tables 1–3 and Supplemental Figures 1–6 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AA, amino acid; AS, added sugar; cAUC, optimism-corrected AUC; CIR, carbon isotope ratio; EAA, essential amino acid; GC-C-IRMS, GC–combustion–isotope ratio MS; NEAA, nonessential amino acid; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; NIR, nitrogen isotope ratio; NPAAS-FS, Nutrition and Physical Activity Assessment Study Feeding Study; SSB, sugar-sweetened beverage.

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Introduction

Sugar-sweetened beverages (SSBs) have been associated with the risk of multiple chronic diseases (1–4). Most of these associations are based on self-reported intakes, which are prone to error (5, 6), especially for sugar-related intakes (7, 8). Dietary estimates based on objective, unbiased dietary biomarkers may strengthen disease-risk models (9–11). The carbon isotope ratio (CIR) of total tissues has been used as a biomarker of added sugar (AS) and/or SSB intake in the United States (12–16), because the corn and sugarcane constituting the majority of these sweeteners have a naturally occurring CIR that is higher than in most other diet components (17–19). Stable isotope biomarkers, including CIR, have the added benefit of representing usual intake because of their slow turnover rates in blood components (19). However, in several populations, the CIR has been more strongly associated with intakes of meat or animal protein (20–22) due to the high amount of corn in US production (23, 24). Approaches to improve the specificity of the CIR for SSBs are needed.

The CIR of molecules that are metabolically linked to foods of interest may provide biomarkers with improved sensitivity and specificity. For example, the CIR of the nonessential amino acid (NEAA) alanine should proportionally reflect synthesis from sugars, due to its close metabolic link with glucose (25, 26). In an Alaska Native (Yup'ik) population, the CIR_{Ala} was associated with intakes of both SSB and AS, but importantly, not meat (27). Other NEAAs may also be synthesized from dietary sugar and so may also have a higher CIR in response to SSB consumption. Essential amino acids (EAAs) cannot derive from dietary sugar; however, their CIRs may be elevated in response to meat intake.

The goal of this study was to identify valid $CIR_{amino\ acid}$ (CIR_{AA}) biomarkers of SSB intake that are not influenced by meat intake. We measured the responses of CIR_{AA}'s in plasma and RBCs to SSBs and meat in participants of the Developing Biomarkers of Diet Study, a highly controlled, 12-wk inpatient feeding study that varied SSBs and meat in combination. We expected that the CIR_{Ala} and other CIR_{NEAA}'s would be elevated by SSB consumption but not by meat consumption. Conversely, we expected CIR_{EAA}'s to be unaffected by SSB consumption and instead be elevated with meat intake. We also assessed the change over time in CIR_{AA}'s from blood samples collected biweekly. Because of differing rates of protein turnover, we expected plasma CIR_{AA}'s to approach equilibrium with the experimental diets more quickly than RBC CIR_{AA}'s.

Methods

Subjects and study design

The Developing Biomarkers of Diet Study was designed to evaluate the effects of 3 dietary exposures—SSBs, meat, and fish—on total tissue CIRs and nitrogen isotope ratios (NIRs) in a 12-wk inpatient dietary intervention, as described in detail elsewhere (22). The presence or absence of these 3 foods was varied in all possible combinations, resulting in a full-factorial study with 8 experimental diets that were weight-maintaining and macronutrient balanced as described below. Participants were randomly assigned to diets until $n = 4$ had been completed for each. This sample size was determined based on a power analysis of the total tissue stable isotope ratio measurements, the primary outcomes of the Developing Biomarkers of Diet Study (22). This power at the level of $\alpha = 0.05$ was determined using an expected within-group SD of 0.5 and expected effect size of ≥ 1. All laboratory measurements were analyst-blinded.

Subjects were recruited between 2011 and 2018 at the Obesity and Diabetes Clinical Research Section of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) in Phoenix, Arizona (clinicaltrials.gov identifier: NCT01237093). Recruitment was restricted to males, because the fish-containing diets exceeded mercury-exposure recommendations for females of reproductive age. Volunteers were screened for overall health prior to admittance, and participants were excluded during an initial 1-wk period of residence in the facility if they presented with type 2 diabetes, but not impaired glucose tolerance, as described below (**Supplemental Figure 1**). The study protocol was approved by the NIDDK Institutional Review Board (#11-DK-N018).

Participants were placed on a standard weight-maintaining diet for 1 wk prior to the dietary intervention. On day 4 of the standard diet, we administered a 75-g, 3-h oral-glucose-tolerance test (glucose samples run on an Analox GM9 glucose analyzer; Analox Technologies). Daily fasting weight was measured, and percentage body fat was determined at the start and end of the study using DXA (DPX-L; Lunar Corp and Prodigy, GE) (28, 29). Fasting blood samples were collected biweekly, from the first day of the experimental diet (baseline) to the end of the final week (week 12, postintervention). Blood was centrifuged to isolate plasma and RBC samples, which were frozen at −20°C prior to analysis.

The experimental diets were designed using Food Processor (version 11.0.2; ESHA Research) to maintain body weight with a fixed macronutrient profile of 50% carbohydrate, 30% fat, and 20% protein. Diets were designed to vary as little as possible apart from the presence or absence of SSBs, meat, and fish at 14%, 19%, and 6% of daily energetic requirement (kilocalories), respectively. The SSB treatment comprised cola and lemon lime soda. The meat exposure included servings of hamburgers, hot dogs, chicken, turkey, ham, roast beef, meatloaf, bacon, and sausage. The fish included salmon, tuna, and pollock.

Outcome measures: CIR_{AA}'s in plasma and RBCs

CIR_{AA}'s are measured and reported as $\delta^{13}C$ values with units of per mil (‰), as follows: $\delta^{13}C = (^{13}C/^{12}C_{sample}/^{13}C/^{12}C_{reference} - 1) \times 1000‰$, where the reference is Vienna Pee Dee Belemnite ($^{13}C/^{12}C = 0.0112372$), the established international reference material for $\delta^{13}C$ measurements. For continuity, we retained CIR_{AA}'s as the variable name when referring to AA $\delta^{13}C$ values. We measured CIR_{AA}'s in plasma and RBC samples using GC–combustion–isotope ratio MS (GC-C-IRMS). We prepared and analyzed baseline and postintervention samples for all participants ($n = 32$) in batches of 8 by tissue type (plasma or RBCs). These batches were designed to ensure even representation of experimental diets. To evaluate change in CIR_{AA}'s over time, we measured CIR_{AA}'s at all time points in a subset of 18 participants whose total plasma CIR changed by

≥0.5‰ over the study duration (hereafter, the "turnover subset"). We used this criterion to exclude participants whose CIR did not change appreciably over the study, presumably due to similarity between the randomly assigned study diet and the participant's usual intake. For analyses of the turnover subset, we batched samples by individual and sample type, and biweekly samples ($n = 7$) were analyzed in random order.

AAs must be hydrolyzed and derivatized prior to measurement of CIR via GC-C-IRMS. We hydrolyzed aliquots of plasma (12–15 μL) or RBCs (2–5 μL) using 1 mL of HCl (6 mol/L) at 110°C for 20 h. We lipid-extracted the hydrolysates using *n*-hexane and dichloromethane (6:5 vol:vol) and dried them down under nitrogen. Hydrolyzed AAs were derivatized to *N*-acetyl methyl esters as follows (30, 31). First, we methylated the AAs with acidified methanol during a 75°C incubation for 1 h. Next, methylated AAs were dried under nitrogen and acetylated by incubation with acetic anhydride, triethylamine, and acetone (1:2:5 vol:vol:vol) at 60°C for 10 min. The derivatized AAs were dried under nitrogen, purified with a phosphate buffer wash (1 mol/L potassium phosphate + 1 mol/L sodium phosphate, pH 7), and extracted using chloroform. After the chloroform was evaporated under nitrogen, derivatized AAs were dissolved in ethyl acetate and were analyzed via GC-C-IRMS within 24 h or stored at −18°C.

The GC-C-IRMS analyses were performed at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks using a GC IsoLink II System (ThermoFisher Scientific). The derivatized AAs were injected onto a VF-35ms column (Agilent) in a TRACE 1310 GC (ThermoFisher Scientific) (32) for peak separation, and the GC effluent was routed through the IsoLink II interface for combustion of each individual peak into carbon dioxide gas and introduction into a Delta V Plus isotope ratio mass spectrometer (ThermoFisher Scientific) for determination of the CIR. CIR_{AA}'s are reported in δ notation ($\delta^{13}C$ values) with units of per mil (‰), as described above, using calibrated carbon dioxide gas as the proximal reference material for Vienna Pee Dee Belemnite. The ^{13}C:^{12}C ratio is calculated by peak integration in the program Isodat (version 3.0; Thermo Scientific). Correct peak identification and integration (width and background assignment) were visually confirmed, as was adequate separation between peaks.

With each batch we also prepared an external standard, containing a mix of commercial AAs (**Supplemental Table 1**) for which the nonderivatized CIR has been measured relative to certified reference materials, and a laboratory check sample of the same tissue type as the experimental samples. To all samples and external standards, we added 3 internal standards, which do not co-elute with AA peaks: 1 requiring derivatization (norleucine) and 2 that are volatile (nonadecane and caffeine). Internal standards were used to monitor instrument performance but were not used to adjust measured CIR_{AA}'s. In a typical analytical sequence, each sample was analyzed in triplicate injections. An injection of the external standard was made between triplicates (n = 8–9/sequence), and an injection of the check sample was made between every other triplicate (n = 4–5/sequence).

We obtained reliable chromatography for 5 NEAAs: alanine (Ala), serine (Ser), aspartic acid (Asp), proline (Pro), and glutamic acid (Glu). Asparagine and glutamine are deamidated during acid hydrolysis; thus, they are indistinguishable from aspartic acid and glutamic acid, respectively. We also measured the CIR of 5 EAAs: valine (Val), leucine (Leu), isoleucine (Ile), threonine (Thr), and phenylalanine (Phe). The CIR_{Ile} was measured only in plasma due to its low concentration in RBCs. The *N*-acetyl methyl ester derivatization allows the CIR measurement of additional AAs, including glycine, methionine, lysine, tyrosine, and histidine. However, here these AAs were excluded based on unreliable chromatography (insufficient peak height or significant co-elution) in human plasma and RBCs. **Supplemental Figure 2** shows typical GC-C-IRMS chromatographs of the external standard and a sample.

Derivatization adds carbon atoms to AAs and causes potential kinetic isotope effects, both of which influence the CIR of derivatized AAs. These influences are accounted for by adjusting the measured CIR of derivatized AAs in samples using the measured CIR of derivatized AAs in the external standard (33). The measured CIR_{AA} of derivatized AAs (termed $CIR_{AA,d}$) were adjusted using the known CIR_{AA} in the external standard, as follows:

$$CIR_{AA(smp)} = \frac{CIR_{AA,d(smp)} - CIR_{AA,d(std)}}{p} + CIR_{AA(std)} \quad (1)$$

In this equation, $CIR_{AA,d(smp)}$ and $CIR_{AA,d(std)}$ are the measured CIRs of the derivatized AAs in the sample and external standard, respectively; $CIR_{AA(std)}$ is the known value of the CIR_{AA} in the external standard; p is the proportion of carbon in the derivatized AAs from the un-derivatized AAs; and $CIR_{AA(smp)}$ is the corrected sample CIR_{AA}.

The propagated analytical error (SE) of CIR_{AA} measurements was estimated for replicate injections of the check sample as described elsewhere (34). In plasma, this ranged from 0.08‰ for CIR_{Phe} to 0.34‰ for CIR_{Thr}, with an average of 0.16‰. In RBCs, measurement error ranged from 0.09‰ for CIR_{Phe} to 0.29‰ for CIR_{Ser}, with an average of 0.12‰. We used measurements of the check sample across batches to evaluate reproducibility. In plasma, this across-batch reproducibility ranged from 0.53‰ for CIR_{Phe} to 1.44‰ for CIR_{Ala}, with an average of 1.04‰. In RBCs, the reproducibility across batches ranged from 0.13‰ for CIR_{Ala} to 1.21‰ for CIR_{Ser}, with an average of 0.76‰. AAs with across-batch reproducibility of SD ≥2.0‰ were considered insufficiently reproducible for inclusion in the paper. In plasma, this included CIR_{Ile} (SD = 2.4‰), and in RBCs this included CIR_{Thr} (SD = 2.8‰). Thus, for plasma, we present the CIRs of Ala, Asp, Glu, Leu, Pro, Ser, Thr, and Val and, for RBCs, we present the CIRs of Ala, Asp, Glu, Leu, Pro, Ser, and Val. Analytical error and reproducibility of the check sample for all CIR_{AA}'s are reported in **Supplemental Table 2**.

Statistical analyses

We modeled the CIR of each AA in plasma and RBCs separately as a function of 3 dietary factors (SSBs, meat, and fish) with 2 levels each (presence/absence) using multiple linear regressions, with baseline CIR_{AA} as a covariate. Baseline CIR_{AA} values were included to account for potentially incomplete isotopic turnover of CIR_{AA} to equilibrium with the study diets. To account for multiple hypothesis testing across covariates, models, and sample types, we adjusted the false discovery rate of $\alpha = 0.05$ with a Benjamini-Hochberg procedure to determine the significance of parameter estimates. Data analyses were conducted in R version 4.0.1 (R Core Team, 2020).

TABLE 1 Baseline characteristics of study participants, both for study cohort and stratified by SSB intake[1]

Characteristic	Total	No SSBs	SSBs
Male sex, *n* (%)	32 (100)	16 (100)	16 (100)
Race/ethnicity, *n* (%)			
White	19 (59.4)	10 (62.5)	9 (56.2)
Native American	10 (31.3)	4 (25.0)	6 (37.5)
Hispanic	2 (6.3)	1 (6.2)	1 (6.2)
African American	1 (3.1)	1 (6.2)	0 (0.0)
Age, y	46.3 ± 10.5	48.8 ± 10.0	43.8 ± 10.7
BMI, kg/m^2	27.2 ± 4.0	28.5 ± 4.2	25.9 ± 3.5
Weight, kg	83.9 ± 13.6	88.0 ± 14.2	79.8 ± 12.1
Body fat, %	27.9 ± 7.6	28.4 ± 8.5	27.5 ± 6.9
Glucose, g/dL	92.7 ± 8.1	92.4 ± 7.7	92.9 ± 8.8

[1]Age and subsequent variables are reported as means ± SDs. SSB, sugar-sweetened beverage.

We performed both univariable and multivariable logistic regressions to predict the exposures of SSBs and meat separately using CIR_{AA}'s as predictors. We assessed the predictive accuracy of the CIR_{AA} biomarkers using the AUC of the receiver operating characteristic curve, as an estimate of the association between the CIR_{AA} and dietary factors, using Harrell's bootstrap estimate of the optimism-corrected AUC (cAUC) to account for potential overfitting by using the same data to fit and test the models, as well as bootstrap 95% CIs for the cAUC using the percentile method (35).

We characterized turnover of CIR_{AA} within individuals by fitting exponential models using nonlinear least squares (36):

$$CIR_{AA,t} = CIR_{AA,\infty} - (CIR_{AA,\infty} - CIR_{AA,0}) \times e^{\lambda t} \quad (2)$$

Here, $CIR_{AA,t}$ is the CIR_{AA} measured at time (wk) t, $CIR_{AA,\infty}$ represents the estimated CIR_{AA} in equilibrium with the diet, $CIR_{AA,0}$ represents the estimated baseline CIR_{AA}, and λ represents the estimated fractional incorporation rate. The half-life and time to 90% turnover can be calculated as $-\ln\left(\frac{100-\%\text{turnover}}{100}\right) \times \lambda^{-1}$. We used Wilcoxon signed-rank tests to compare estimates of λ between plasma and RBC CIR_{AA} and the number of participant models that converged.

Results

Study participants

We screened 55 male volunteers for the 12-wk controlled-feeding study. Forty-one were admitted to the inpatient study and, of these, 37 were randomly assigned and 32 completed the experimental diet through at least week 8 and provided samples for stable isotope analysis (Supplemental Figure 1), as described elsewhere in detail (22). One participant withdrew after week 8 of the feeding study, another participant withdrew after week 10, and the remaining 30 participants completed the full 12 wk. For the participants who withdrew early, the blood samples collected at the week of withdrawal were used for postintervention CIR_{AA} measurements. The baseline characteristics of the participants are shown in **Table 1**. The distributions of age, body weight, BMI, percentage body fat, and plasma glucose were similar among participants assigned to each of the 8 diets (22) and between SSB consumers and nonconsumers (Table 1). The largest differences were in age, BMI, and body weight; however, these variables are not expected to affect CIR_{AA}. Participants maintained constant body weights (day of discharge–first full day of study) within 0.5 ± 2.6 kg (0.7% ± 3.2% body weight). Baseline CIR_{AA} measurements were similar across participants when stratified by SSB and meat intake (**Supplemental Table 3**).

Effects of SSB and meat intake on postintervention CIR_{AA}

Most CIR_{NEAA}'s were elevated in SSB consumers (**Table 2**, **Supplemental Figure 3**), including CIR_{Ala}, CIR_{Ser}, CIR_{Asp}, and CIR_{Glu} in both plasma and RBCs. RBC CIR_{Pro} increased with SSB intake, whereas plasma CIR_{Pro} did not. CIR_{Ala} showed the largest effect of SSB intake in both plasma ($\beta_{SSB} = 2.81$, SE = 0.38) and in RBCs ($\beta_{SSB} = 1.66$, SE = 0.30). No CIR_{EAA}'s, in plasma or RBCs, were influenced by SSB consumption.

All CIR_{EAA}'s measured in plasma, and 2 out of 3 measured in RBCs, were elevated in meat consumers (Table 2, **Supplemental Figure 4**): CIR_{Leu} and CIR_{Val} in both plasma and RBCs and CIR_{Thr} and CIR_{Phe} in plasma only. Effect sizes were similar across plasma EAAs, with the largest effect in CIR_{Leu} ($\beta_{meat} = 1.26$, SE = 0.23). The CIR of a single NEAA, plasma CIR_{Pro}, showed an increase with meat intake, and this increase was similar in magnitude to the increase in CIR_{EAA}'s.

No CIR_{AA} showed an increase in fish consumers, but there was a small but significant decrease in plasma CIR_{Glu}.

Baseline CIR_{AA} was included as a covariate in these multivariable regression models to account for the potential influence of preintervention diets due to incomplete equilibration of postintervention CIR_{AA} with the study diets. In plasma, only CIR_{Thr} was significantly associated with baseline, whereas in RBCs 4 CIR_{AA}'s (CIR_{Ala}, CIR_{Asp}, CIR_{Val}, CIR_{Phe}) were associated with baseline. Furthermore, the effect sizes measured in RBCs tended to be smaller than in plasma (Table 2).

Plasma CIR_{NEAA}'s were highly predictive of SSB intake based on their ability to discriminate between consumers and nonconsumers, measured as the AUC of the receiver operating characteristic curves for the logistic regressions (**Figure 1**). The CIR_{Glu} had the highest predictive accuracy (cAUC = 1; 95% CI: 1, 1), closely followed by the CIR_{Ala} (cAUC = 0.97; 95% CI: 0.93, 1). Using all plasma CIR_{NEAA}'s as predictors was highly discriminatory for SSB intake (cAUC = 1; 95% CI: 0.99, 1). In RBCs, CIR_{NEAA}'s were also highly predictive of SSB intake, achieving, in combination, a cAUC of 0.97 (95% CI: 0.94, 1) (**Supplemental Figure 5**). Individual CIR_{NEAA}'s in RBCs were slightly less discriminatory than in plasma (e.g., RBC CIR_{Ala} cAUC = 0.92; 95% CI: 0.81, 0.98). However, even the least discriminatory CIR_{NEAA}'s had a moderately high cAUC (RBC CIR_{Pro} cAUC = 0.79; 95% CI: 0.57, 0.93).

Plasma CIR_{EAA}'s and CIR_{Pro} were highly predictive of meat intake (**Figure 2**). The CIR_{Leu} achieved the highest predictive accuracy (cAUC = 0.92; 95% CI: 0.83, 0.99) and was as discriminatory as the combination of all plasma predictors of meat intake (cAUC = 0.93; 95% CI: 0.82, 1). All other plasma CIR_{EAA}'s had cAUC scores >0.8 for meat intake. In contrast, RBC CIR_{Leu} was only moderately predictive of meat intake (cAUC = 0.76; 95% CI: 0.53, 0.93), while RBC CIR_{Val} was less so (cAUC = 0.64; 95% CI: 0.30, 0.85). Using all RBC CIR_{EAA}'s as predictors did not improve their cAUC (0.73; 95% CI: 0.37, 0.92) (**Supplemental Figure 6**).

TABLE 2 Multivariable linear regression results for postintervention CIR_{NEAA}'s and CIR_{EAA}'s[1]

	Plasma			RBCs		
	R^2	β (SE)	P	R^2	β (SE)	P
NEAAs						
CIR_{Ala}						
SSBs	0.65	2.81 (0.38)	<0.001	0.66	1.66 (0.30)	<0.001
Meat		0.01 (0.37)	0.980		0.20 (0.31)	0.518
Fish		−0.32 (0.37)	0.390		−0.25 (0.29)	0.410
Baseline		0.01 (0.11)	0.967		0.41 (0.10)	<0.001
CIR_{Asp}						
SSBs	0.32	1.25 (0.36)	0.002	0.59	0.73 (0.24)	0.006
Meat		0.50 (0.35)	0.168		0.53 (0.25)	0.042
Fish		−0.40 (0.35)	0.257		−0.11 (0.23)	0.650
Baseline		0.14 (0.15)	0.350		0.58 (0.13)	<0.001
CIR_{Glu}						
SSBs	0.78	1.47 (0.16)	<0.001	0.45	1.39 (0.33)	<0.001
Meat		0.37 (0.16)	0.027		0.31 (0.34)	0.363
Fish		−0.43 (0.15)	0.010		−0.38 (0.33)	0.245
Baseline		0.10 (0.09)	0.249		0.30 (0.14)	0.044
CIR_{Pro}						
SSBs	0.47	0.62 (0.26)	0.025	0.38	0.74 (0.22)	0.003
Meat		1.23 (0.27)	<0.001		0.52 (0.24)	0.037
Fish		0.06 (0.26)	0.827		0.22 (0.23)	0.332
Baseline		−0.17 (0.15)	0.268		0.30 (0.14)	0.038
CIR_{Ser}						
SSBs	0.39	1.61 (0.35)	<0.001	0.40	0.95 (0.26)	0.001
Meat		0.40 (0.35)	0.265		0.29 (0.26)	0.282
Fish		−0.30 (0.35)	0.401		0.35 (0.26)	0.185
Baseline		0.01 (0.12)	0.938		0.24 (0.10)	0.023
EAAs						
CIR_{Leu}						
SSBs	0.46	0.21 (0.24)	0.379	0.23	0.22 (0.20)	0.262
Meat		1.26 (0.23)	<0.001		0.58 (0.20)	0.006
Fish		0.05 (0.23)	0.195		0.02 (0.20)	0.921
Baseline		0.05 (0.16)	0.777		0.27 (0.14)	0.054
CIR_{Phe}						
SSBs	0.43	0.08 (0.17)	0.656	0.45	0.38 (0.19)	0.050
Meat		0.86 (0.17)	<0.001		0.29 (0.19)	0.137
Fish		−0.20 (0.17)	0.244		0.05 (0.19)	0.800
Baseline		0.10 (0.15)	0.499		0.81 (0.17)	<0.001
CIR_{Thr}[2]						
SSBs	0.60	0.21 (0.26)	0.429			
Meat		1.23 (0.25)	<0.001			
Fish		0.57 (0.26)	0.035			
Baseline		0.38 (0.10)	<0.001			
CIR_{Val}						
SSBs	0.30	0.31 (0.31)	0.319	0.53	0.34 (0.24)	0.163
Meat		1.08 (0.31)	0.001		0.70 (0.24)	0.007
Fish		0.40 (0.31)	0.203		0.10 (0.24)	0.677
Baseline		0.10 (0.15)	0.480		0.58 (0.10)	<0.001

[1] $n = 32$. Results are presented as regression coefficients and SEs. Coefficients for dietary intakes refer to presence/absence. R^2 presented is the adjusted R^2 for the full model. Significant P values ($\leq$0.010) were significant based on the Benjamini-Hochberg correction for the total number of comparisons across both sample types and a false discovery rate of $\alpha = 0.05$. CIR, carbon isotope ratio; EAA, essential amino acid; NEAA, nonessential amino acid; SSB, sugar-sweetened beverage.

[2] CIR_{Thr} reported in plasma only due to high measurement error in RBCs.

CIR_{AA} turnover

We characterized the change in CIR_{AA}'s over time in individuals by fitting nonlinear models. These models did not converge for all individuals, likely due to the small number of data points and the magnitude of isotopic change, which varied randomly among individuals based on their baseline CIR_{AA} and the treatment assigned. For models that converged, fractional incorporation rates (λ) and weeks to 50% and 90% turnover are presented by AA and specimen type in **Table 3**. Models of plasma CIR_{AA} tended to converge for a greater number of study participants than did models of RBC CIR_{AA} (a median of 11 compared with 7.5 participants; Wilcoxon signed-rank = 64.5, $P = 0.007$), and median (25th, 75th percentile) fractional incorporation rates were higher in plasma compared

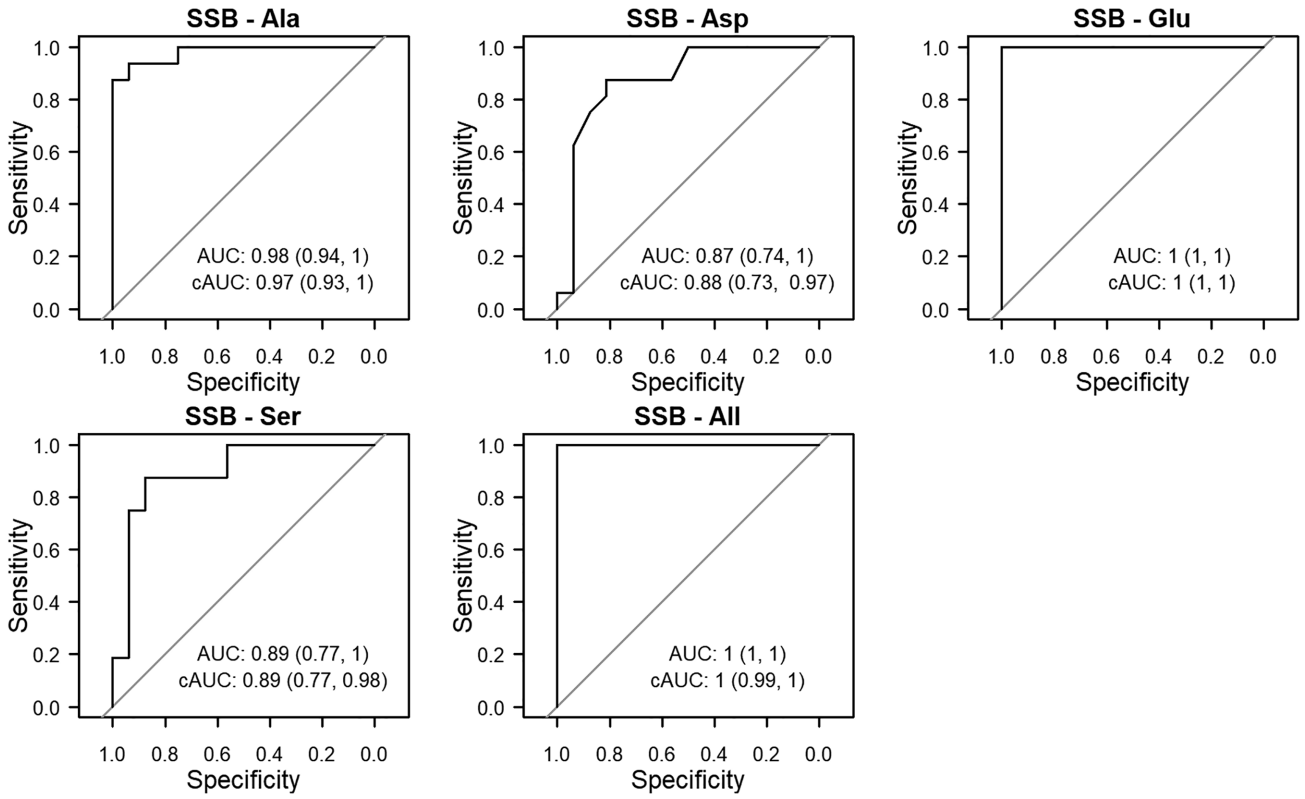

FIGURE 1 Receiver operating characteristic curves for logistic regression models ($n = 32$) predicting SSB consumption using plasma CIR_{NEAA}'s as predictors. The cAUC (95% CI) was calculated using Harrell's bootstrap to adjust for optimism. cAUC, corrected AUC; CIR, carbon isotope ratio; NEAA, nonessential amino acid; SSB, sugar-sweetened beverage.

with RBCs [0.38 wk^{-1} (0.30, 0.39) compared with 0.15 wk^{-1} (0.12, 0.19); Wilcoxon signed-rank = 69.5, $P = 0.0015$]. We estimated times to 90% turnover ranging from 5 to 9 wk for plasma AAs and 8–23 wk for RBC AAs. For 75% of RBC CIR_{AA}'s the time to 90% turnover exceeded the 12-wk study feeding period.

Discussion

We measured the responses of plasma and RBC CIR_{AA}'s to SSB and meat intakes in a 12-wk fully inpatient controlled-feeding study. Most plasma CIR_{NEAA}'s and all RBC CIR_{NEAA}'s increased significantly with SSB intake, with the largest effect in plasma CIR_{Ala}, and no CIR_{NEAA}'s were significantly associated with meat intake. CIR_{Ala} and CIR_{Glu} were the most promising individual estimators of SSB intake due to their high predictive accuracy (cAUC $\geq$0.97). In combination, CIR_{NEAA}'s of both plasma and RBCs were highly predictive of SSB intake (cAUC $\geq$0.98). Meanwhile, most plasma and RBC CIR_{EAA}'s increased significantly with meat intake. Plasma CIR_{Leu} was the most promising individual predictor of meat intake (cAUC = 0.92) and combining multiple CIR_{EAA}'s did not improve predictive accuracy. These results further the validation of CIR_{AA}'s as biomarkers of SSB and meat intakes.

A key goal of this study was to determine whether CIR_{AA}'s were more predictive of SSB intake than total tissue CIR. The effects of SSB intake on CIR_{NEAA}'s were 2–5 times larger than on total plasma and RBC CIRs (22), reflecting their greater sensitivity. CIR_{NEAA}'s that increased with SSB intake were also more specific, because, unlike total tissue CIR, they were not affected by meat intake. Several prior studies have found that total tissue CIR was more strongly associated with intakes of meat and/or animal protein than AS/SSBs in US populations (13, 20, 21). Although combined total plasma CIR and NIR had moderate success at identifying SSB consumers in the Developing Biomarkers of Diet Study (cAUC = 0.78), the predictive accuracy of CIR_{NEAA}'s for SSB intake was higher (22). These findings suggest strong potential for CIR_{NEAA}'s as biomarkers of SSB intake.

These findings are broadly consistent with published studies of CIR_{AA}'s from a cohort of Alaska Native (Yup'ik) males and females (27) and a cohort of postmenopausal women from the Women's Health Initiative Nutrition and Physical Activity Assessment Study Feeding Study (NPAAS-FS) (37). In the Yup'ik cohort, median intakes of AS and SSBs were high (80 g/d and 1.5 servings/d, respectively) and RBC CIR_{Ala} was associated with both AS and SSBs ($r = 0.6$ and 0.7, respectively) (27). In that study, however, other CIR_{NEAA}'s were either not or only weakly associated with SSB intake, including CIR_{Glu}. In the NPAAS-FS, the median intake of AS was low (48 g/d), the median intake of SSBs was very low (<0.5 servings/d), and serum CIR_{Ala} was associated with AS only ($r = 0.3$). Estimation of AS was improved by using multiple CIR_{AA}'s: CIR_{Ala}; CIR_{Gly}, which was inversely associated with AS; and

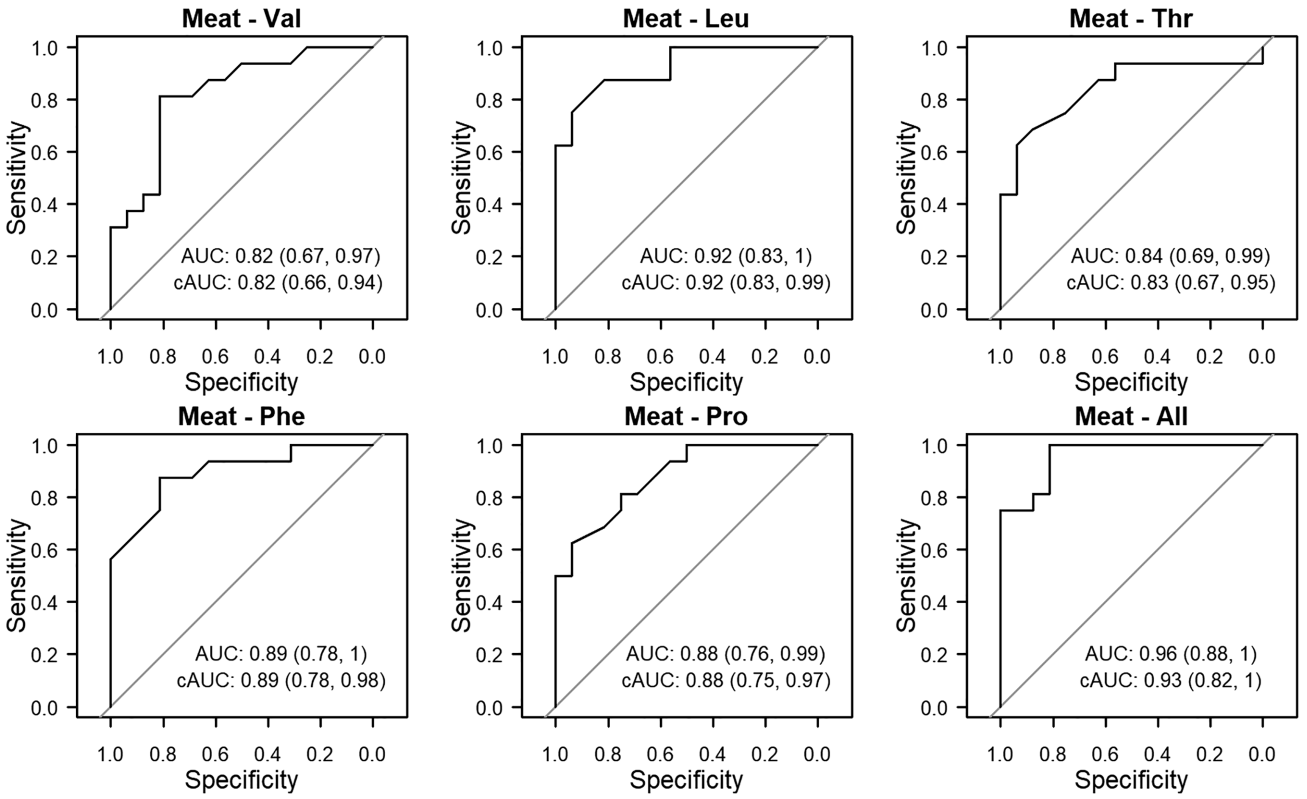

FIGURE 2 Receiver operating characteristic curves for logistic regression models ($n = 32$) predicting meat consumption using plasma CIR_{EAA}'s and 1 CIR_{NEAA} (Pro) as predictors. The cAUC (95% CI) was calculated using Harrell's bootstrap to adjust for optimism. cAUC, corrected AUC; CIR, carbon isotope ratio; EAA, essential amino acid; NEAA, nonessential amino acid.

CIR_{Ile}, which was associated with animal protein intake (37). Thus, the studies differed in whether single or multiple CIR_{AA}'s were required to estimate AS/SSB intake. Comparing all CIR_{AA} responses with diet across these studies is difficult because they used different blood fractions and AA derivatization methods, resulting in different suites of AAs that were reliably measured. Despite these differences, these studies indicate that CIR_{Ala} is a robust biomarker of SSB and/or AS intake across diverse US study populations (38) and suggest that using multiple CIR_{AA}'s may improve estimation of SSB intake in certain contexts.

We also evaluated whether CIR_{EAA}'s were more sensitive and specific measures of meat intake in relation to total tissue CIR and NIR. The effects of meat intake on individual CIR_{EAA}'s were comparable to those on total tissue CIR in both plasma and RBCs (22). Furthermore, the predictive accuracy of CIR_{Leu}, the best CIR_{AA} for detecting meat intake, was identical to that of the total plasma CIR and NIR combined (cAUC = 0.92) (22). Although CIR_{Leu} did not improve upon total tissue CIR and NIR as a measure of meat intake, it would be a useful covariate representing meat intake in models of SSBs using CIR_{AA}'s because it is measured in the same analysis. A similar approach was used in the NPAAS-FS, in which a model of AS intake was improved by the inclusion of CIR_{Ile}, presumably due to its association with animal protein (37). Similar to the present findings, associations of CIR_{AA}'s with animal protein intake in the NPAAS-FS study were not stronger than those with total serum CIR and NIR (21, 37), nor were associations of hair CIR_{AA}'s with animal protein stronger than those of total hair CIR and NIR in a German cross-sectional study (39).

A strength of stable isotope biomarkers is that they integrate diet over weeks to months (19, 22, 40), providing estimates of usual intake. This contrasts with other proposed biomarkers of sugar (41) and meat (42) intake, which integrate over the scale of hours or days. The time responses of plasma and RBC CIR_{AA}'s have not previously been described. By including baseline CIR_{AA}'s as a covariate in models of postintervention CIR_{AA}'s, we were able to evaluate whether individual CIR_{AA}'s had equilibrated to the study diet within the 12-wk feeding period. With 1 exception, plasma postintervention CIR_{AA}'s were not associated with baseline, suggesting that they were at or close to equilibrium with intervention diets. In contrast, all RBC CIR_{AA}'s had some association with baseline, suggesting that RBC CIR_{AA}'s were not in equilibrium, although several were marginally nonsignificant following correction for multiple testing. These findings are further supported by our estimates of the fractional incorporation rates of CIR_{AA}'s, which suggested that plasma CIR_{AA}'s were at or near equilibrium with study diets within the 12-wk period, whereas most RBC CIR_{AA}'s were not. Thus, dietary effects on RBC CIR_{AA}'s are likely underestimated, which may explain the generally higher dietary effect sizes in plasma CIR_{AA}'s relative to RBCs. Generally, fractional incorporation rates of CIR_{AA}'s in plasma and RBCs were similar to those of total plasma and RBCs (22).

TABLE 3 Fractional incorporation rates [median (25th, 75th percentile)] of CIR_{AA}'s estimated from biweekly measurements[1]

Tissue	CIR_{AA}	Number converged	Median λ[2] (25th, 75th)	Median $t_{0.5}$,[3] wk	Median $t_{0.9}$, wk
Plasma	Ala	13	0.31 (0.15, 0.53)	2.2	7.4
Plasma	Val	9	0.25 (0.19, 0.37)	2.8	9.2
Plasma	Leu	13	0.38 (0.19, 0.49)	1.8	6.1
Plasma	Thr	7	0.39 (0.23, 0.51)	1.8	5.9
Plasma	Ser	10	0.50 (0.22, 0.87)	1.4	4.6
Plasma	Asp	11	0.42 (0.22, 0.64)	1.6	5.5
Plasma	Pro	15	0.26 (0.13, 0.36)	2.7	8.9
Plasma	Glu	8	0.38 (0.27, 0.59)	1.8	6.1
Plasma	Phe	14	0.30 (0.21, 0.40)	2.3	7.7
RBCs	Ala	9	0.15 (0.11, 0.39)	4.6	15.4
RBCs	Val	6	0.14 (0.05, 0.26)	5.0	16.4
RBCs	Leu	7	0.12 (0.10, 0.52)	5.8	19.2
RBCs	Ser	5	0.30 (0.24, 0.55)	2.3	7.7
RBCs	Asp	8	0.18 (0.15, 0.35)	3.8	12.3
RBCs	Pro	8	0.10 (0.07, 0.16)	6.9	23.0
RBCs	Glu	8	0.20 (0.16, 0.27)	3.5	11.5
RBCs	Phe	6	0.12 (0.06, 0.17)	5.8	19.2

[1]Estimated using nonlinear least squares. The number of converged models is out of 18 subjects. CIR_{AA}, carbon isotope ratio of amino acid.

[2]Units of λ, the fractional incorporation rate, are $week^{-1}$.

[3]Median weeks until 50% turnover (half-life) and 90% turnover calculated as $t_x = -\ln(1 - x)/\lambda$.

The primary strengths of this study were the high dietary control, factorial design, and 12-wk duration of the fully inpatient feeding study. The factorial design of the experimental diets allowed us to evaluate the responses of CIR_{AA}'s to SSB intake in the context of varying meat intake at a similar percentage of energy (and vice versa). The inpatient design gave high confidence in subject compliance, and the 12-wk duration allowed CIR_{AA}'s in plasma to achieve equilibrium with experimental diets. This study also had some limitations. One limitation was that we measured responses to dietary variables at only 2 levels (presence or absence). Future work should focus on establishing the dose–response relations between CIR_{AA}'s and dietary variables. The 12-wk duration was insufficient for RBC CIR_{AA}'s to achieve equilibrium with study diets, potentially attenuating dietary associations. Thus, an explicit comparison of dietary effects in plasma and RBCs would require a longer dietary intervention. There was 15% missingness among randomly assigned participants. While we do not expect the missing data to have a strong effect on our results, we cannot rule out the possibility of some bias in our complete-case analysis. Finally, the lack of an extended run-in period prior to the dietary intervention meant that participants had widely varying baseline CIR_{AA}'s, which limited our ability to model the time course of CIR_{AA} turnover to experimental diets. GC-C-IRMS methodology is increasing in availability as it gains popularity in nutrition and other fields (forensics, environmental science) and its costs are intermediate to other biomarker approaches.

In summary, this study in men strongly supports plasma and RBC CIR_{NEAA}'s, particularly CIR_{Ala} and CIR_{Glu}, as sensitive and specific measures of SSB intake in the US diet. CIR_{NEAA}'s will also likely reflect AS intake, based on the high contribution of corn and sugarcane to both AS and SSBs. Furthermore, CIR_{EAA}'s appear to be discriminatory measures of meat intake. These promising candidate biomarkers warrant further validation, including dose-response assessment (38). Ultimately, the use of long-term intake biomarkers such as CIR_{AA}'s may calibrate or replace self-report measures and so reduce error in diet-disease risk models.

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The authors' responsibilities were as follows—DMO, JK, MJW, and SBV: designed the study; JK and SBV: oversaw the inpatient study; MJW and DMO: oversaw the stable isotope analyses; JJJ and SM: conducted the stable isotope analyses; HYY, SM, and TL: provided essential methods development; DMO, EJO, JJJ, and PAS: conducted data analyses; JJJ and DMO: wrote the manuscript; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

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