

Validity, Reliability, and Sensitivity of the  $\delta^{13}\text{C}$  Added Sugar Biomarker in Children and Adolescents

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## ABSTRACT

Currently, 17.1% of 2-19 year olds are obese.<sup>1,2</sup> While obesity is a multifactorial disease, energy imbalance is commonly cited as a primary etiology.<sup>3-6</sup> Excess consumption of added sugar (AS) from corn and cane sweeteners has been implicated as a leading contributor to weight gain in youth and adults.<sup>5,7,8</sup> Children and adolescents are among the highest consumers of AS, which account for 16% of their total daily calories (~318 calories/d), which is above American Heart Association, World Health Organization, and Dietary Guidelines for Americans recommendations.<sup>3,5,9</sup> Although a strong temporal relationship has been established between weight gain and increased consumption of corn and cane sweeteners, a causal relationship is difficult to determine due to the inherent limitations of self-report dietary assessments (i.e., measurement errors such as underreporting).<sup>10-12</sup> Further, obtaining accurate dietary intake data from children and adolescents is challenging due to the high dietary variability observed in this population.<sup>11</sup> To overcome the limitations of self-report dietary assessments, the Institute of Medicine has recognized the need to develop and validate objective biomarkers of dietary intake.<sup>13</sup> One such biomarker is the delta ( $\delta$ )  $^{13}\text{C}$  biomarker; preliminary studies suggest that the  $\delta^{13}\text{C}$  biomarker is a valid, objective indicator of AS intake in adults<sup>14</sup> and holds promise for children and adolescents. Establishing  $\delta^{13}\text{C}$  as a valid, reliable and sensitive means for assessing habitual AS intake in children and adolescents provides valuable objective dietary information with the potential to address a pressing public health concern, which is the relationship between AS intake and health.

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## GENERAL AUDIENCE ABSTRACT

Obesity is a major public health concern and worldwide epidemic. Approximately one-third of United States (U.S.) adults are classified as obese.<sup>1</sup> The epidemic is also starting to affect U.S. children and adolescents with an estimated 17% considered obese.<sup>2,3</sup> While several factors contribute to the development of obesity, the combination of reduced physical activity and poor dietary habits including excess calorie intake have been commonly implicated.<sup>4-7</sup> Excess consumption of added sugars (AS), defined as sugars and syrups put in foods during preparation or processing or added at the table,<sup>8</sup> has been cited as a leading contributor to weight gain in youth and adults;<sup>6,9,10</sup> corn and cane sweeteners are the most widely used sweeteners in the U.S., particularly in the form of sugary drinks (SSBs).<sup>11</sup> AS intake accounts for ~16% of children and adolescents' total calories, which is far above the recommended daily intake.<sup>4,6,12</sup> Despite observational evidence of a relationship between excess AS intake and chronic diseases, such as obesity, hypertension, and diabetes, a cause-and-effect relationship is difficult to assess because of the limitations of self-report dietary information (i.e., measurement errors such as inaccurate dietary reporting).<sup>13-15</sup> Because of these limitations, it is important to develop and validate objective measures of intake, better known as biomarkers. The proposed biomarker, delta <sup>13</sup>C ( $\delta^{13}\text{C}$ ), is a ratio of the <sup>13</sup>carbon isotope (<sup>13</sup>C) to the <sup>12</sup>carbon isotope (<sup>12</sup>C); plant tissues can be enriched with <sup>13</sup>C relative to <sup>12</sup>C, if they employ the phosphoenol pyruvate carboxylase enzyme during photosynthesis.<sup>11,16</sup> Corn and cane sugars tend to use this enzyme and therefore demonstrate a  $\delta^{13}\text{C}$  value that is closer to zero (a "high"  $\delta^{13}\text{C}$  value). Therefore, the purpose of this investigation is to assess the reliability, validity and sensitivity of the  $\delta^{13}\text{C}$  biomarker in assessing AS intake in children and adolescents. Doing so would provide valuable and objective dietary information and allow for causal relationships to be assessed to address a pressing public health concern.

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### CHAPTER 2: Validity, Reliability, and Sensitivity of the $\delta^{13}\text{C}$ Added Sugar Biomarker in Children and Adolescents

**Figure 1.** Boxplots depicting difference in  $\delta^{13}\text{C}$  values between quartiles of reported AS intake (% total AS intake) and SSB intake (kcal).

**Figure 2.** Receiver operating characteristic (ROC) curves to assess the validity and sensitivity of the  $\delta^{13}\text{C}$  biomarker to detect high and low AS and SSB consumers.

## CHAPTER 1: Introduction

Obesity is a major public health concern and worldwide epidemic. Since the 1960s, the prevalence of obesity has increased threefold so that 35.5% and 36.5% of United States (U.S.) adult men and women, respectively, are currently classified as obese.<sup>1</sup> More troubling is that some experts are predicting that this generation will have a shortened lifespan compared to their parents;<sup>2</sup> this could be related to the early manifestation of chronic diseases, such as obesity in US children and adolescents. It is estimated that 17% of children and adolescents, aged 2-19 years, are considered obese.<sup>2,3</sup> While obesity has a multifactorial etiology, poor dietary patterns that lead to excess energy intake accompanied with a reduction in physical activity are commonly cited.<sup>4-7</sup>

Energy intake has increased concomitantly as US meal composition has been altered; changes to meals include increased consumption of meals away from home,<sup>6,8</sup> increased portion sizes,<sup>6,8</sup> and simultaneous increased intake of sugar-sweetened beverages (SSBs) and decreased intake of milk.<sup>9-11</sup> Despite recommendations by the World Health Organization (WHO),<sup>7</sup> American Heart Association (AHA),<sup>6</sup> and Dietary Guidelines for Americans<sup>12</sup> to limit dietary sugar, AS intake accounts for 16% (~355 kcal per day) of children and adolescents' total energy intake,<sup>5,12</sup> which far exceeds the allowance for discretionary calories (5-15% of total calories) across age and gender.<sup>5,12</sup> The primary source of AS in children and adolescent diets are SSBs,<sup>6,9,10</sup> which account for 41% of total AS intake.<sup>11</sup> Excessive AS intake, particularly in the form of SSBs, has a strong association with obesity that can be largely explained by the weak satiety signals provided by SSBs and failure to compensate for excess calories consumed in the liquid form.<sup>13,14</sup> It has been estimated that with every 100g (less than half a can of soda) increase in SSB intake, BMI z-score and sum of 4 skinfolds significantly increased 0.05 units and 0.86

mm over 6 years, which is mediated by an increase in energy intake and non-energy effects of SSBs, such as the metabolic effects of fructose.<sup>14,15</sup>

The ramifications of high AS consumption are far-reaching and poor dietary intake can negatively impact growth and development while simultaneously predisposing children and adolescents to an early onset of chronic diseases.<sup>5,12-19</sup> Growth and development in children and adolescents may be impaired with increasing consumption of AS due to its tendency to reduce consumption of milk and other dairy products and displace other key nutrients.<sup>5,12,20</sup> Inadequate intakes of calcium, vitamin A, iron and zinc are commonly cited with AS intake exceeding 25% of total energy.<sup>21,22</sup> Furthermore, excessive AS consumption has been associated with other chronic diseases such as diabetes, hypertension, ischemic heart disease, and stroke.<sup>7,16,19</sup> Therefore, the AHA suggests that a prudent upper limit for AS intake is half of discretionary calories allotted.<sup>5</sup>

While a strong temporal relationship exists between an increased consumption of corn- and cane-based sweeteners and obesity, a causal relationship is difficult to determine due to reliance on self-report methods.<sup>27,28</sup> As all self-reported methods have measurement error, there is not a single measure that provides the truth about dietary intake, which results in a compromise between data accuracy and subject burden when considering diet analysis methodology.<sup>23</sup>

Under- and over-reporting are commonly cited limitations of self-report dietary data, but misreporting is not uniform across all populations or food groups.<sup>23,24</sup> Children and adolescents commonly misreport, specifically underreport, energy intake by 20% according to doubly labeled water studies.<sup>28,30</sup> This is attributed to the high dietary variability observed in adolescents<sup>14,28</sup>, the increased contribution of snacks to total energy intake<sup>14,28</sup>, and

developmental milestones;<sup>25,26</sup> therefore, it may be difficult for children and adolescents to estimate portion sizes, conceptualize the foods, understand food preparation, and recall all foods and beverages consumed.<sup>23,25,26</sup> In order to capture usual intake in younger populations, it is critical to collect multiple days of dietary information to account for the high variance ratio (i.e. the proportion of within- to between-person variance).<sup>23</sup>

Using multiple recalls in children and adolescents can provide more accurate data compared to other diet assessments;<sup>27,28</sup> however, it is still impacted by misreporting errors and subject burden making it challenging to assess usual dietary intake and to establish consistent, causal relationships between dietary intake and health.<sup>27-30,38-40</sup> Therefore, there is significant interest in developing and validating dietary biomarkers that can objectively assess dietary intake without bias of self-report.<sup>30,31</sup> Although dietary biomarkers have been identified for protein,<sup>32-35</sup> essential fatty acids,<sup>36-38</sup> corn intake, fruits and vegetables,<sup>39-41</sup> and total sugar<sup>31,42</sup> and added sugar intake,<sup>43-46</sup> the Institute of Medicine (IOM) still recognizes the lack of nutritional biomarkers as a knowledge gap requiring future research to more accurately assess dietary intake in children and adolescents.<sup>47</sup> Dietary biomarkers should be valid, reliable, and sensitive to changes in dietary intake.<sup>30,48</sup> While there are currently no known predictive dietary biomarkers to assess AS intake in children and adolescents, the proposed biomarker, delta <sup>13</sup>C ( $\delta^{13}\text{C}$ ) is a promising biomarker of AS intake demonstrating preliminary validity and reliability in adults.<sup>30,43-46,48</sup>

This biomarker has been used extensively in archaeological and ecological studies,<sup>33,49,50</sup> but current evidence suggests that naturally occurring variations in stable isotope ratios, such as  $\delta^{13}\text{C}$ , can be used as objective measures of diet.<sup>43-46,48,51</sup> When compared to <sup>12</sup>C, <sup>13</sup>C sugars are found more prominently in plants that employ the C4 photosynthetic pathway.<sup>48,51</sup> This pathway

uses phosphoenolpyruvate carboxylase during photosynthesis, which enriches plant tissues with  $^{13}\text{C}$  relative to  $^{12}\text{C}$ .<sup>48</sup> Therefore, when corn and cane sugars and their derivatives (i.e., high fructose corn syrup, brown sugar cane, plain sugar cane, molasses, and corn starch) are consumed in high quantities, it can be detected by a high  $^{13}\text{C}:^{12}\text{C}$  ( $\delta^{13}\text{C}$ ) ratio within human tissues using a minimally invasive fingerstick blood sample;<sup>43,48</sup> high concentrations indicate high AS consumption.<sup>43,45,48,52</sup> If proven valid, reliable, and sensitive, the  $\delta^{13}\text{C}$  AS biomarker can provide objective measures of AS consumption and can overcome the research limitations of relying upon self-report dietary data among children and adolescents.<sup>23,25,26</sup>

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## **CHAPTER 2: Validity, Reliability, and Sensitivity of the $\delta^{13}\text{C}$ Added Sugar Biomarker in Children and Adolescents**

### **ABSTRACT**

Reliance on self-reported dietary intake information is a commonly cited limitation in research; misreporting errors may be magnified in children and adolescent populations. To overcome this limitation, it is critical to develop reliable, valid, and sensitive dietary biomarkers. The proposed biomarker,  $\delta^{13}\text{C}$ , has demonstrated preliminary reliability and validity in adults. The purpose of this investigation was to determine the comparative validity, test-retest reliability, and sensitivity of the  $\delta^{13}\text{C}$  biomarker of added sugar (AS), particularly in the form of sugar-sweetened beverages (SSB), using fingerstick blood samples in children and adolescents aged 6-18 years. Three-hundred twenty-six children and adolescents aged 6-18 completed all study visits. Participants' height, weight, and health history were assessed at the first visit; twenty-four hour recalls were conducted at each visit and fingerstick blood samples were collected at two of the four visits. Samples were analyzed for  $\delta^{13}\text{C}$  value using natural abundance stable isotope mass spectrometry.  $\delta^{13}\text{C}$  value was compared to dietary outcomes in all participants and between children and adolescent subgroups. Reported mean AS consumption was  $82.2 \pm 35.8$  g/d and  $329 \pm 143$  kcal/d, and SSB consumption was  $7.5 \pm 8.2$  fl oz/d and  $98 \pm 103$  kcal/d. Mean fingerstick  $\delta^{13}\text{C}$  value was  $-19.65 \pm 0.69\%$ , which differed between age groups.  $\delta^{13}\text{C}$  values were similar across visits and were associated ( $P < 0.001$ ) with intake of total AS (g and kcal,  $r = 0.228$ ) and SSB (fl oz,  $r = 0.350$ ; kcal,  $r = 0.351$ ). The  $\delta^{13}\text{C}$  biomarker was able to better discriminate between high and low SSB consumers than AS consumers, as demonstrated by the area under the receiver-operator characteristic curve (0.697 and 0.618, respectively). Despite the limitations of

the current investigation, the  $\delta^{13}\text{C}$  biomarker holds promise as a minimally invasive, objective biomarker of AS and SSB intake in adults and in children and adolescents.

Key words: dietary assessment, added sugars, obesity, biomarker, validity, sugar-sweetened beverages

## INTRODUCTION

As the global obesity epidemic continues to mount, childhood obesity has also escalated with 30.4% of children and adolescents are now considered overweight or obese with 18.0% and 18.1% of children (6-11 years) and adolescents (12-19 years) classified as obese, respectively.<sup>1-3</sup> While obesity has a multifactorial etiology, excess energy intake and physical inactivity are commonly cited contributors. Energy intake has increased concomitantly as US meal composition has been altered; changes to meals include increased consumption of meals away from home,<sup>4,5</sup> increased portion sizes,<sup>4,5</sup> and simultaneous increased intake of sugar-sweetened beverages (SSBs) and decreased intake of milk.<sup>6-8</sup>

Despite recommendations made by major health organizations,<sup>9-11</sup> to limit added sugar (AS) intake, children and adolescents consume 16% of their total calories (355 kcals) from AS,<sup>10</sup> which exceeds the 10% of total calorie intake recently recommended<sup>12</sup> Sugar-sweetened beverages (SSBs) are the primary source of AS, accounting for 41% of total AS intake,<sup>7,8,11,12</sup> or 8% of total caloric intake.<sup>13</sup> Excessive AS intake, particularly in the form of SSBs, has a strong association with obesity that can be largely explained by the weak satiety signals provided by SSBs and failure to compensate for excess calories consumed in the liquid form.<sup>14,15</sup> High AS consumption is also associated with dental caries<sup>16-19</sup> as well as increased risk for chronic diseases such as diabetes, hypertension, ischemic heart disease, and stroke.<sup>9,20,21</sup> Potentially more concerning are the negative impacts excessive AS intake has on growth and development in children and adolescents; high AS consumption tends to reduce dairy consumption and displace foods containing key nutrients including: calcium, vitamin A, iron and zinc.<sup>11,12,22,23</sup>

Therefore, it is critical to accurately assess children and adolescents' AS and SSB intake. Although several studies indicate a relationship between AS and SSB intake with several

chronic diseases,<sup>9,20,21,24-28</sup> a causal relationship is difficult to assess due to the limitations of self-reported dietary assessment methodologies.<sup>29</sup> Under- and over-reporting are commonly cited limitations, which is more pronounced in younger populations who may have difficulty estimating portion sizes, conceptualizing foods, understanding food preparation and recalling all foods and beverages consumed.<sup>29-31</sup> Furthermore, it is common to underreport foods that are considered unhealthy and are consumed as snacks, which can skew dietary data<sup>31</sup> and make the relationship between dietary intake and disease prevention and progression more challenging to assess. For this reason, the Institute of Medicine, among others, have expressed significant interest in developing and validating dietary biomarkers that can objectively assess dietary intake without the bias of self-report.<sup>32-34</sup> Currently, there are no validated predictive dietary biomarkers of AS intake in children and adolescents. However, the delta  $^{13}\text{C}$  ( $\delta^{13}\text{C}$ ) added sugar biomarker is a promising biomarker of AS intake with preliminary evidence of validity and reliability in adults.<sup>32,35-39</sup>

While this biomarker has been used extensively in archaeological and ecological studies,<sup>40-42</sup> recent evidence indicates that naturally occurring variations in stable isotope ratios, such as  $\delta^{13}\text{C}$ , can be used as objective measures of diet.<sup>35,37-39,43,44</sup> The  $\delta^{13}\text{C}$  biomarker is capable of distinguishing plant tissues enriched by  $^{12}\text{C}$  sugars versus  $^{13}\text{C}$  sugars; when compared to  $^{12}\text{C}$  sugars,  $^{13}\text{C}$  sugars are found more prominently in corn and cane plants due to their use of the C4 photosynthetic pathway, which employs phosphoenolpyruvate carboxylase during photosynthesis.<sup>36,44</sup> Therefore, when corn and cane sugars and their derivatives are consumed in high quantities, it can be detected by a high  $^{13}\text{C}:$  $^{12}\text{C}$  ( $\delta^{13}\text{C}$ ) ratio within human tissues using a minimally invasive fingerstick blood sample.<sup>35,36</sup>

Therefore, the purpose of this investigation was to determine the comparative validity, test-retest reliability, and sensitivity of the  $\delta^{13}\text{C}$  biomarker of AS, particularly in the form of SSBs, using fingerstick blood samples in children and adolescents aged 6-18 years.

## **MATERIALS AND METHODS**

### **Subjects and Design**

Three-hundred sixty-four children and adolescents aged 6-18 years were screened online from a local university community between January 2014-September 2015; three-hundred twenty-six children and adolescents completed all study sessions (90% completion rate). The Virginia Tech Institutional Review Board approved the study protocol. Interested participants were enrolled in the study following parental consent and if they met eligibility criteria, as follows: aged 6-18 years old, can read, write, and speak English, and willing to comply with all study procedures. Participants completed the four laboratory sessions within a three-week period. This investigation is registered at clinical trials.gov (NCT02455388).

### **Procedures**

Following parental consent and participant assent, a healthy history questionnaire was administered to gather information regarding participants' age, race, ethnicity, medical conditions, and medication and supplement use. Height was measured in meters without shoes using a wall-mounted stadiometer and weight measured in light street clothing without shoes to the nearest 0.1 kg (Scale Tronix 5002; Carol Stream, IL); values were used to calculate body mass index (BMI) and BMI percentile<sup>45</sup> to categorize the weight status of participants. Resting energy expenditure (REE)<sup>46</sup> was calculated to identify potential under-reporters. Participants were classified as under-reporters if their reported total caloric intake was less than 80% of their estimated REE.

At each lab session, a record-assisted 24-hour diet recall (24-HR) was administered by a trained research assistant using the automated multiple pass method (AMPM); visual aids, including food diagrams and food models, were used. The four 24-HR were obtained on non-consecutive days, including one weekend day. Consistent with National Health and Nutrition Examination Survey (NHANES) methodology, participants under 11 years of age had a proxy present to assist them with diet recalls while participants over 12 years of age were considered capable of providing an accurate diet recall;<sup>30,31,47</sup> however, all participants were encouraged to have an adult present to aid in completing the 24-HR. The 24-HRs were analyzed using NDS-R (Nutrition Data System for Research [NDS-R] 2013; University of Minnesota, Minneapolis, MN) to quantify dietary intake variables including usual AS and SSB intake.

Non-fasting fingerstick blood samples were obtained at two of the four visits via routine fingersticks (One Touch Fine Point Lancet, Johnson and & Johnson Company; New Brunswick, NJ). Blood samples were blotted onto sterilized, binder-free glass microfiber filters (Whatman, type GF/D, 2.5cm diameter; Whatman, Inc, Piscataway, NJ) and air-dried. Blood samples were analyzed in triplicate for  $\delta^{13}\text{C}$  value via natural abundance stable isotope mass spectrometry (Isoprime; Micromass UK Ltd, Manchester, UK), as described previously.<sup>35</sup>  $\delta^{15}\text{N}$  values were used as covariates to control for the potential confounders of meat (i.e., livestock consuming corn) consumption on  $\delta^{13}\text{C}$  values. Total variability across the three measurements never exceeded 0.2‰, with an intra-assay coefficient of variation of 0.04‰.

## **Data Analysis**

Statistical analyses were performed using statistical analysis software (SPSS version 22.0 for Windows, SPSS Inc, Chicago, IL). Descriptive statistics (mean  $\pm$  standard deviation) are reported for participant demographic information and dietary intake variables (total kcal, total

dietary AS (g, kcal), and total SSB (fl oz, kcal)). Simple and bivariate correlations, paired sample t tests, independent sample t tests, Chi-squared test and one-way analysis of variance (ANOVA) were used to assess associations among variables, group differences, and differences within and between assessment methods. Difference in quartiles of AS and SSB intake and  $\delta^{13}\text{C}$  were represented graphically using box-and-whisker plots. T-tests between the high and low AS consumers were conducted to assess group differences in  $\delta^{13}\text{C}$  values; participants were characterized as high AS consumers if their AS consumption (kcal) constituted greater than 20% of total calories (i.e., twice the recommended 10% of total calories) and as high SSB consumers if their SSB intake (kcal) was twice the recommended intake suggested by the American Heart Association.<sup>12,48</sup> A Pearson correlation coefficient between  $\delta^{13}\text{C}$  values obtained at the two laboratory visits assessed test-retest reliability while a Pearson correlation coefficient between mean  $\delta^{13}\text{C}$  values and mean reported dietary AS intake evaluated concurrent validity. Finally, 2-sided, parametric receiver operating characteristics (ROC) curves were generated to evaluate the relationship between the sensitivity and false-positive rate for the fingerstick blood and 24-HR results. ROC curves for children and adolescents were calculated separately and the variability in ROC curves was assessed to ensure that the difference in the area under the curve (AUC) was not significantly different with reference to age.

## RESULTS

Participant demographic characteristics and dietary intake are presented in **Table 1**. The sample (n=326) was balanced with regard to sex (49% males, 51% females) while 93% of participants considered themselves white (2% were Asian, 1% were African American, and 1% were other) and non-Hispanic (93%). Age ranged from 6 to 18 years (mean age  $12\pm 3$  years). Mean BMI was in the normal range with most participants being categorized as 5<sup>th</sup> to 85<sup>th</sup>



percentile. Age group differences were noted between the percentage of children and adolescents classified as underweight and obese ( $p < 0.05$ ). Mean resting energy expenditure was  $1893 \pm 470$  kcals and as expected adolescents had greater energy expenditure than children. Resting energy expenditure for the sample was lower than reported total caloric intake from 24HR (mean difference =  $-169.8 \pm 659.0$  kcals,  $p < 0.001$ ). Approximately 12% of our sample were classified as under-reporters.

Among children and adolescents in this sample, reported total daily AS intake was similar to the mean intake of AS by persons older than 2 years (83.9 g, 336 kcals/d) in the United States.<sup>7,11</sup> AS and SSB intakes were significantly different between children and adolescents ( $p < 0.001$ ), with children reporting a lower consumption than adolescents.

Mean  $\delta^{13}\text{C}$  values at time one and time two, respectively, were  $-19.66 \pm 0.68\text{‰}$  (range:  $22.26\text{‰}$  to  $-17.79\text{‰}$ ) and  $-19.64 \pm 0.68\text{‰}$  (range:  $-22.39\text{‰}$  to  $-17.84\text{‰}$ ). Biomarker measurements were strongly correlated across the two visits ( $r = 0.992$ ,  $p < 0.001$ ) and for subsequent analyses the  $\delta^{13}\text{C}$  value at time one was used. The  $\delta^{13}\text{C}$  values differed between children and adolescents with lower values noted in children, which is consistent with the self-reported AS and SSB intake data. The lowest and the 50<sup>th</sup> percentile of AS intake demonstrated significant differences in  $\delta^{13}\text{C}$  values when compared to the highest quartile of AS intake (% AS contribution to total intake). Similarly, significant differences in  $\delta^{13}\text{C}$  values were found between the lowest percentile and the 50<sup>th</sup> percentile when compared to the 75<sup>th</sup> percentile and highest percentile of SSB intake (kcals). These results are depicted graphically in **Figure 1**.  $\delta^{13}\text{C}$  values also differed between normal weight and obese participants (mean difference =  $-0.551$ ,  $p < 0.001$ ), but not between normal weight and overweight participants (mean difference =  $-0.196$ ,  $p = 0.111$ ) or by gender (mean difference =  $0.112$ ,  $p = 0.140$ ).

Significant correlations were noted between  $\delta^{13}\text{C}$  and several variables from 24-HR including: total AS in grams and kcals ( $r=0.228$ ,  $p<0.001$ ), SSB in fl oz ( $r=0.350$ ,  $p<0.001$ ) and kcal ( $r=0.351$ ,  $p<0.001$ ). BMI was also correlated with  $\delta^{13}\text{C}$  values ( $r=0.276$ ,  $p<0.001$ ). After controlling for BMI and  $\delta^{15}\text{N}$ , associations remained significant (AS g:  $r=0.180$ ,  $p=0.001$ ; AS kcal:  $r=0.180$ ,  $p=0.001$ ; SSB fl oz:  $r=0.281$ ,  $p<0.001$ ; SSB kcal:  $r=0.276$ ,  $p<0.001$ ).

Using ROC curve analysis, twice the recommended intake for AS and SSBs were used as reference standards and compared with  $\delta^{13}\text{C}$  biomarker values. As indicated by the area under the curve, the  $\delta^{13}\text{C}$  biomarker was able to better discriminate between high and low SSB consumers (AUC = 0.697) than between high and low AS consumers (AUC = 0.618) presented in **Figure 2**. The AUC for the  $\delta^{13}\text{C}$  for AS and SSB intakes did not differ after controlling for BMI and  $\delta^{15}\text{N}$ . Furthermore, variances for AUC were not different when analysis of children and adolescent curves were conducted separately (data not shown). The adolescent subgroup was further separated into gender subgroups to assess the potential differences in  $\delta^{13}\text{C}$  turnover rate in varied stages of puberty; AUC values were similar between adolescent boys (AUC for AS = 0.553, SSB = 0.667) and adolescent girls (AUC for AS = 0.590, SSB = 0.602).

## **DISCUSSION**

Our findings represent the first investigation to evaluate the validity, reliability and sensitivity of the  $\delta^{13}\text{C}$  AS biomarker assessed in fingerstick blood samples in children and adolescents. The biomarker demonstrated strong test-retest reliability, similar to findings from previous studies.<sup>35,38</sup> The  $\delta^{13}\text{C}$  biomarker demonstrated concurrent validity as it was moderately correlated with AS intake, expressed in g and kcal, and SSB intake, expressed in fl oz and kcal. The association between  $\delta^{13}\text{C}$  and SSB intake variables was stronger when compared to AS intake, consistent with the literature.<sup>35,38,39,49</sup> This is likely due to the fact that the sugars present

in SSBs are mostly derived from corn and cane sugar while AS could be derived from C4 or C3 plant sources.<sup>36</sup>

As further evidence of the  $\delta^{13}\text{C}$  biomarker's validity, the ROC analyses revealed that this biomarker is able to discriminate high and low SSB consumers (AUC=0.697) more accurately than high and low AS consumers (AUC=0.618). While the AUC for each of these variables was slightly below the acceptable range (0.7-0.9),<sup>50</sup> the use of self-report dietary data may underestimate biomarker validity.<sup>51</sup> Therefore, this technique merits further investigation using controlled feeding study designs in children and adolescents, given its potential to contribute to the investigations of the health consequences of AS and SSB intake.<sup>9,11,12,16-22</sup>

The current investigation reported mean intakes of SSB intake for children (79 kcals) and adolescents (110 kcals) which were below the current estimates of 129 kcals and 350 kcals from SSB alone for children and adolescents in the United States, respectively.<sup>6</sup> Johnson et al. found that the mean AS intake for children aged 4-8 was approximately 336 kcals while adolescent males and females reported much higher intakes (467 kcals and 371 kcals, respectively);<sup>11</sup> the results presented are consistent with the current literature indicating our sample is generalizable with respect to AS and SSB intake. These estimates of AS and SSB intake far exceed recommendations made by the Dietary Guidelines for Americans,<sup>12</sup> American Heart Association,<sup>11,48</sup> and World Health Organization.<sup>9</sup>

The current investigation recruited a large sample size (n=326) with a wide age range (6-18 years). This yielded variety in dietary patterns and a wide range of reported AS and SSB intakes. Our approach also allowed us to evaluate differences in dietary intake and biomarker values between children and adolescents. The current investigation assessed the age group differences and controlled for age when analyzing relationships between variables to account for

any mediating effects. Utilizing fingerstick blood samples minimized the degree of invasiveness, which is a commonly cited limitation of biochemical data collection, and no adverse effects were reported.

There are several limitations that are acknowledged. Despite a large sample size there was a lack of racial and ethnic diversity in our sample, which may skew results since minority populations are more likely to consume SSB than white counterparts.<sup>6</sup> Pubertal status was not assessed; however, this was addressed by dichotomizing our sample into children and adolescent subgroups, which did not present any significant differences in AUC values. The  $\delta^{13}\text{C}$  biomarker is limited to assessing intake of C4 plants that demonstrate a conspicuously high  $\delta^{13}\text{C}$  value.<sup>36,43,44</sup> While many high AS foods carry the C4 signature in their carbon isotope composition, there are several other sweeteners with a C3-signature that can be used in products including: beet sugar, honey, and maple syrup. However, these comprise the minority of AS used in the United States.<sup>36</sup> Furthermore, the  $\delta^{13}\text{C}$  value in whole blood is not able to distinguish between corn consumption and corn-derivative consumption.<sup>36,39,52</sup> This limitation is compounded by livestock consumption of corn products as feed; when their meat is ingested, the carbons from the corn feed can be integrated into the individual's  $^{13}\text{C}$  pool. To address this potential limitation,  $\delta^{15}\text{N}$  was included as a covariate to control for meat intake,<sup>37,38,53</sup> although our findings are consistent with recent evidence indicating that this does not enhance the  $\delta^{13}\text{C}$  biomarker's ability to predict AS and SSB intake in populations of mostly white US residents.<sup>54</sup>

## **CONCLUSIONS**

Despite the limitations of the current investigation, the  $\delta^{13}\text{C}$  biomarker holds promise as a minimally invasive, objective indicator of AS and SSB intake in adults and in children and adolescents. This biomarker has the potential to overcome limitations posed by self-report

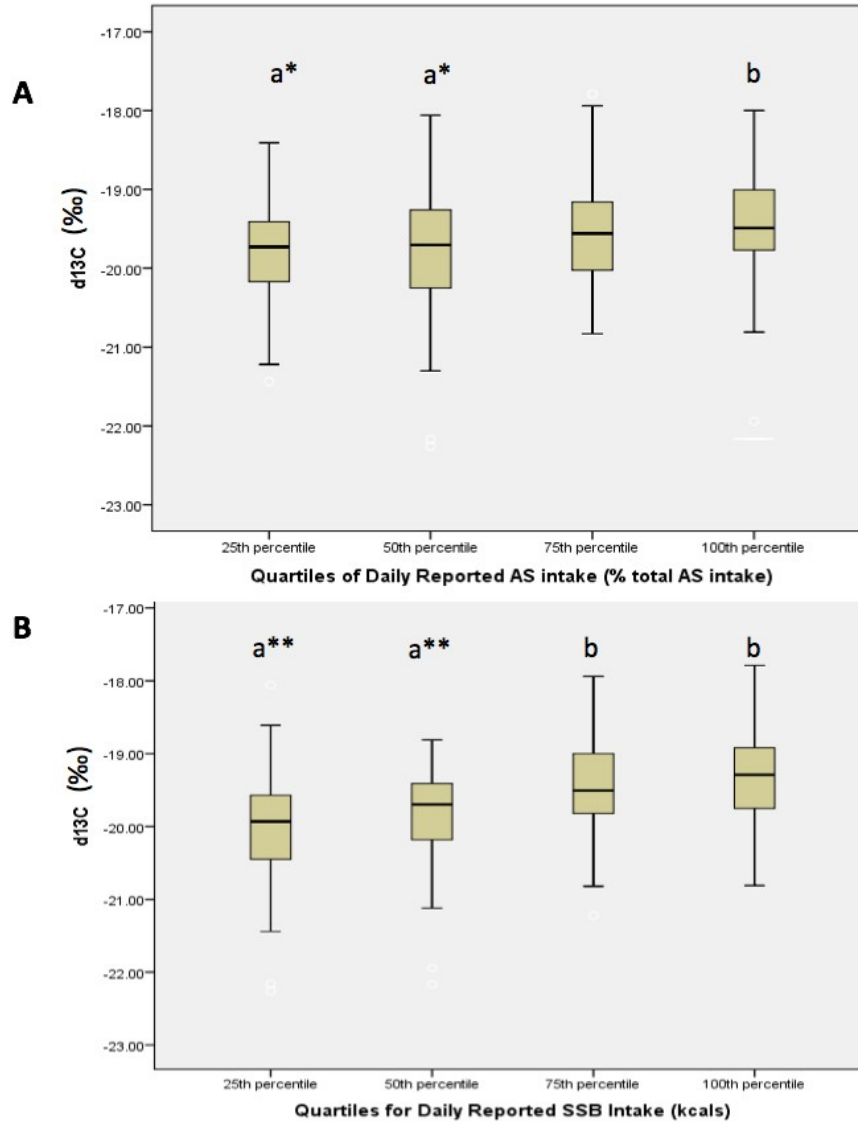
dietary data that may aid in discovering consistent and causal relationships between AS and SSB intake and health.<sup>32,35,36,55</sup> Further research is warranted to characterize  $\delta^{13}\text{C}$  values; a controlled feeding study where AS intake is manipulated would serve as the next step in validating the  $\delta^{13}\text{C}$  AS biomarker. This type of study would clarify the dose-response relationships between  $\delta^{13}\text{C}$  values and AS and SSB intake,<sup>56</sup> which could be translated into clinically-relevant thresholds to objectively characterize children and adolescents' AS and SSB consumption.

Associations between AS and SSB and  $\delta^{13}\text{C}$  should also be assessed in population-based studies in the United States since this would test for how biomarker values may be affected by various diet composition.<sup>56</sup> While investigations have been carried out in the Yup'ik population,<sup>37,38</sup> the dietary patterns of this population are very different from the standard Western diet. As mass spectrometry technology advances to include portable mass spectrometers,<sup>57</sup> this biomarker technique could have substantial public health impact both for research and clinical practice.

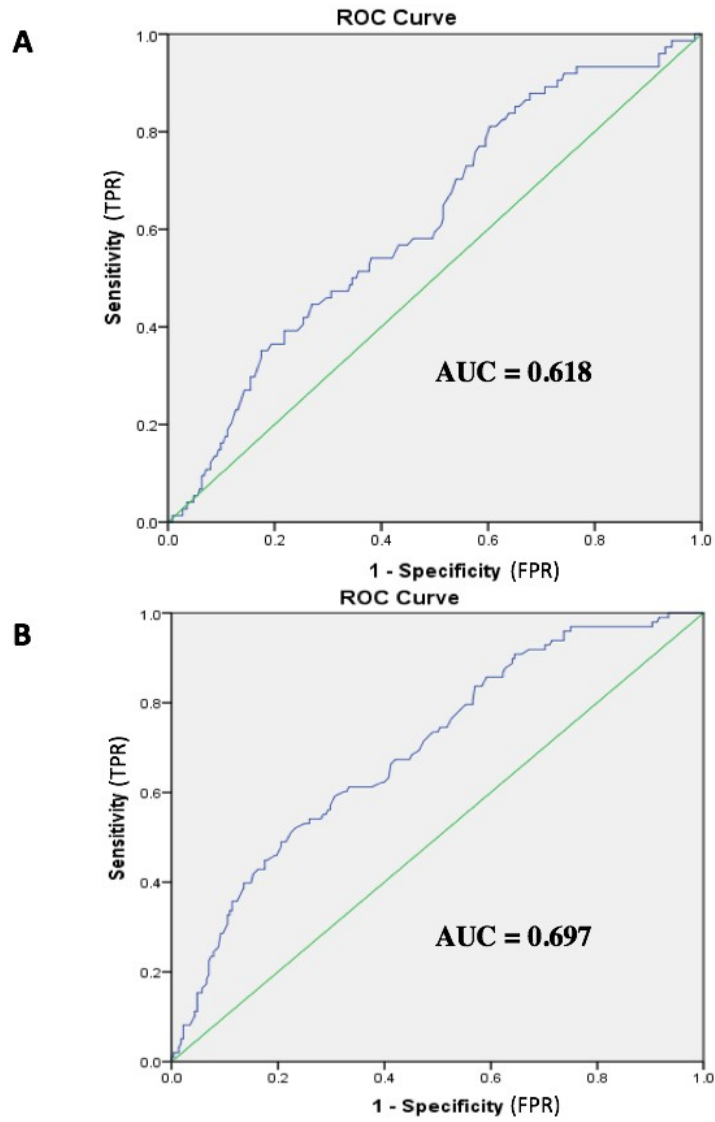
**TABLE**

<b>Table 1.</b> Participant characteristics of the full sample, and among children and adolescent subgroups.				
<b>Participant Characteristics</b>	<b>Total Sample</b>	<b>Children (6-11 years)</b>	<b>Adolescents (12-18 years)</b>	<b>p-value</b>
Total number of participants, <i>n</i>	326	126	200	
Male, n (%)	159 (49)	71 (56)	88 (44)	<sup>a</sup> p=0.030
Female, n (%)	167 (51)	55 (44)	112 (56)	
Age (y), mean±SD <sup>c</sup>	12±3	9±2	15±2	<sup>b</sup> p<0.001
<b>BMI Status, n (%)</b>				
Underweight (<5 <sup>th</sup> percentile)	12 (4)	9 (7)	3 (2)*	<sup>a</sup> p=0.010
Normal weight (>5 <sup>th</sup> – <85 <sup>th</sup> percentile)	254 (78)	101 (80)	153 (77)	
Overweight (>85 <sup>th</sup> – <95 <sup>th</sup> percentile)	35 (11)	11 (9)	24 (12)	
Obese (>95 <sup>th</sup> percentile)	25 (8)	5 (4)	20 (10)*	
BMI (kg/m <sup>2</sup> ), mean±SD	20.0±4.5	17.0±2.8	21.8±4.4	<sup>b</sup> p<0.001
BMI-for-age (percentiles), <sup>c</sup> mean±SD	56.1±27.9	49.6±28.4	60.1±26.8	<sup>b</sup> p=0.001
Resting Energy Expenditure (kcal), <sup>d</sup> mean±SD	1891±472	1736±412	1992±485	<sup>b</sup> p<0.001
Reported Energy Intake (kcal), <sup>e</sup> mean±SD	2063±552	1862±418	2190±588	<sup>b</sup> p<0.001
δ <sup>13</sup> C (‰), mean±SD	-19.65±0.69	-19.80±0.67	-19.56±0.67	<sup>b</sup> p=0.002
<b>Added sugar, mean±SD</b>				
g	82.2±35.8	73.2±28.1	87.8±38.9	<sup>b</sup> p<0.001
kcal	329±143	293±112	351±155	<sup>b</sup> p<0.001
<b>Sugar-sweetened beverage consumption, mean±SD</b>				
fl oz	7.5±8.2	6.0±7.1	8.5±8.6	<sup>b</sup> p=0.005
kcal	98±103	79±88	110±110	<sup>b</sup> p=0.006
<sup>a</sup> χ <sup>2</sup> tests were used to compare proportions across groups				
<sup>b</sup> Independent sample t-tests were used to compare age group means;				
<sup>c</sup> BMI-for-age percentiles were calculated according to CDC standards <sup>45</sup>				
<sup>d</sup> Resting Energy Expenditure was calculated based upon <sup>46</sup>				
<sup>e</sup> Reported Energy Intake was the average energy intake from the four 24HRs				
*Significant differences (α <0.05) noted between children and adolescents				

## FIGURES



**Figure 1** – Boxplots depicting difference in  $\delta^{13}\text{C}$  values between quartiles of daily reported AS intake (% total AS intake) in **panel A** and SSB intake (kcal) in **panel B**. Differences exist between different letters. \* significant difference,  $p < 0.05$ , \*\* significant difference,  $p < 0.001$



**Figure 2** – Receiver operating characteristic (ROC) curves to assess the validity and sensitivity of the  $\delta^{13}\text{C}$  biomarker to detect high and low AS (**panel A**) and SSB consumers (**panel B**). The true positive rate (TPR) is plotted against the false positive rate (FPR)



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### CHAPTER 3: Conclusions and Future Directions

The findings of this investigation represent the first evaluation of the validity, reliability and sensitivity of the  $\delta^{13}\text{C}$  biomarker of AS and SSB intake in children and adolescents. Similar to findings reported in adults,<sup>1</sup> the  $\delta^{13}\text{C}$  biomarker demonstrated reliability and validity in children and adolescents. Our findings suggest that the  $\delta^{13}\text{C}$  biomarker may be more valid and sensitive when differentiating between low and high SSB consumers than AS consumers. This is likely due to the fact that the sugars present in SSBs are mostly derived from corn and cane sugar while AS could be derived from C4 or C3 plant sources.<sup>1-3</sup> Despite the moderate associations and predictive ability of  $\delta^{13}\text{C}$  expressed in this investigation, this method warrants further research since the use of self report dietary data could underestimate these findings.<sup>4</sup>

Future research should utilize a controlled-feeding design that actively manipulates AS and SSB intake to further characterize the  $\delta^{13}\text{C}$  biomarker.<sup>5</sup> A controlled-feeding study would clarify the dose-response relationship between  $\delta^{13}\text{C}$  values and AS and SSB intake,<sup>5</sup> which could be translated into clinically-relevant thresholds to objectively characterize client AS and SSB consumption. Such a study would also test the responsiveness of the  $\delta^{13}\text{C}$  biomarker to changes in dietary intake.

Associations between AS and SSB intake and  $\delta^{13}\text{C}$  should also be assessed in population-based studies in the United States since this would test the effect of various diet compositions on  $\delta^{13}\text{C}$  values.<sup>5</sup> While investigations have been carried out in the Yup'ik population,<sup>6,7</sup> the dietary patterns of this population are very different from the standard Western diet. Therefore, characterizing  $\delta^{13}\text{C}$  values with various Western diet compositions is warranted. As mass spectrometry technology advances to include portable mass spectrometers,<sup>8</sup> validation of these tools is necessary to make this a novel biomarker for research as well as for clinical practice.

In conclusion, our findings indicate that the  $\delta^{13}\text{C}$  biomarker assessed in fingerstick blood holds promise as a minimally invasive, objective biomarker of AS and SSB intake in adults and in children and adolescents. This biomarker has the potential to overcome limitations posed by self-report dietary data that may aid in discovering consistent and causal relationships between AS and SSB intake and health.<sup>1,9-11</sup>

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## MEMORANDUM

**DATE:** January 13, 2016

**TO:** Brenda Davy, Madlyn Irene Frisard, Valisa Ellen Hedrick, Tina Savla, Elaina Lynn Marinik

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)

**PROTOCOL TITLE:** d13C Added Sugar Intake Biomarker: Determining Validity in Children

**IRB NUMBER:** 15-067

Effective January 13, 2016, the Virginia Tech Institutional Review Board (IRB) Chair, David M Moore, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

### PROTOCOL INFORMATION:

Approved As: **Expedited, under 45 CFR 46.110 category(ies) 3,4,6,7**  
Protocol Approval Date: **February 11, 2016**  
Protocol Expiration Date: **February 10, 2017**  
Continuing Review Due Date\*: **January 27, 2017**

\*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

### FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

*Invent the Future*



Date*	OSP Number	Sponsor	Grant Comparison Conducted?
01/28/2015	13173902	National Institute of Child Health & Human Development	Compared on 02/02/2015

\* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

## MEMORANDUM

**DATE:** August 19, 2015  
**TO:** Brenda Davy, Shaun Karl Riebl  
**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)  
**PROTOCOL TITLE:** Determining the Validity, Reliability, and Sensitivity of the d13C Added Sugar Biomarker and BEVQ-15 in Adolescents and a Qualitative Analysis of Beverage Choices in Adolescents and Their Parents using the Theory of Planned Behavior.  
**IRB NUMBER:** 13-810

Effective August 18, 2015, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

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## PROTOCOL INFORMATION:

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Continuing Review Due Date\*: **September 2, 2016**

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## MEMORANDUM

**DATE:** February 13, 2015

**TO:** Brenda Davy, Madlyn Irene Frisard, Valisa Ellen Hedrick, Tina Savla, Elaina Lynn Marinik

**FROM:** Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)

**PROTOCOL TITLE:** d13C Added Sugar Intake Biomarker: Determining Validity in Children

**IRB NUMBER:** 15-067

Effective February 11, 2015, the Virginia Tech Institutional Review Board (IRB) Chair, David M Moore, approved the New Application request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

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**IRB NUMBER:** 13-810

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