Establishing Urinary Biomarkers as Objective Indicators of Dietary Intake in Adolescents

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ABSTRACT

Obesity is a public health concern and cardiometabolic consequences are severe when obesity develops during youth and continues into adulthood.[1] Treatment prior to adulthood confers health benefits,[1–3] but adolescent obesity rates have continually increased, reaching 20.6% in 2013-2014.[4] Quality and quantity of dietary intake contribute to the development of obesity,[5–7] but limitations of self-reported dietary intake are evident in overweight or obese adolescents,[8–11] who frequently misreport nutrients of concern.[12] Added sugar, sodium, and protein intake may indicate diet quality in this population. The 2015-2020 Dietary Guidelines recommend decreasing consumption of added sugars, sodium, and processed protein due to their known contributions to overweight and obesity.[13] Objective dietary intake assessment measures are necessary for investigating the association between dietary intake and health outcomes. Added sugar, sodium, and protein intake could be assessed objectively with a panel of urinary biomarkers. Prior research indicates the potential of these urinary biomarkers to reflect dietary intake,[14–27] but to date, no controlled feeding study has been conducted in adolescents. Using a controlled feeding design, the current study aims to evaluate the validity of urinary sucrose, fructose, sodium, and nitrogen as objective indicators of dietary intake. It is hypothesized that urinary sucrose and fructose will reflect dietary added sugar intake, while urinary sodium and nitrogen will correspond to dietary sodium and protein intake, respectively, in a healthy adolescent population. These biomarkers, if valid, could be used in clinical and epidemiological research to improve understanding of the associations between dietary intake and health outcomes.

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GENERAL AUDIENCE ABSTRACT

An increasing percentage of adolescents are becoming overweight or obese as a result of lifestyle changes that have decreased physical activity and increased access to foods with more calories and less nutrients. Overweight or obese adolescents are typically less heatlhy than their normal weight peers, and they are more likely to become overweight or obese adults, increasing the liklihood that they will develop diabetes and heart disease. It is important to prevent adolescents from becoming overweight or obese to preserve their health, and to treat adolescents who are overweight or obese to improve their health. Eating certain foods in excess contributes to negative health outcomes, including increased weight gain, so dietary change is an important aspect of overweight and obesity prevention and treatment. Current dietary guidelines recommend eating less added sugars, sodium, and processed protein. Quantifying dietary intake of these food components is essential for fully understanding their impact on health. However, self-reporting food intake is a flawed measure, because individuals may not accurately report all of the foods they consume. An objective method is needed to determine dietary intake of specific food components. Fortunately, urinary biomarkers can objectively assess dietary intake of added sugar, sodium, and protein. Controlling consumption of each food component and measuring urinary excretion of each biomarker is necessary to establish how intake corresponds to excretion, but this type of study has not been conducted in adolescents. The current study aims to use a controlled feeding approach to establish relationships between dietary intake of added sugars, sodium, and protein, and urinary excretion of sucrose/fructose, sodium, and nitrogen, in an adolescent population. These biomarkers could be used in future research to advance understanding of the relationships between food intake and overall health.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 1: Introduction

Childhood Overweight and Obesity Prevalence

Rates of childhood and adolescent obesity have been trending upwards, adversely affecting health and impacting many other facets of life for children and adolescents. Children are classified as overweight or obese based on their sex-specific BMI-for-age percentile. A child with a BMI-for-age between the 85th and 95th percentile is considered overweight, a child with a BMI-for-age at or above the 95th percentile is considered obese,[1] and a child with a BMI-for-age that meets or exceeds 120% of the 95th percentile is considered extremely obese.[2] In 2009-2010, almost 32% of US children and adolescents were overweight or obese by this definition.[3] By 2011-2014, 17% of 2-19 year-olds in the US were obese and nearly 6% of US children and adolescents were extremely obese.[2] Compared to 17% in 2011-2014, 16.9% of children and adolescents were obese in 2009-2010,[3] indicating a recent plateau. In fact, obesity prevalence has begun to plateau or decline in certain age groups, leveling off in the 6-11 year-old age group following 2007-2008 , and decreasing in the 2-5 year-old age group after 2003-2004.[2] However, the obese adolescent population continues to trend upwards, with obesity prevalence nearly doubling from 10.5% to 20.6% of 12-19 year-olds between 1988-1994 and 2013-2014.[2]

Childhood and adolescent obesity does not affect everyone equally with regards to race, gender, and socioeconomic status. Racial discrepancies exist among the obese population. In 2011-2014, children and adolescents who were obese included 8.6% of the Asian population, 14.7% of the non-Hispanic white population, 19.5% of the non-Hispanic black population, and 21.9% of the Hispanic population.[2] This data demonstrates the increased risk of obesity for non-Hispanic black and Hispanic children and adolescents. The trend of childhood and adolescent obesity also reveals gender differences, with obesity becoming more prevalent in both genders, but only showing a significant increase among males from 1999-2000 to 2009-2010.[3]

Socioeconomic status is a likely contributor to the multifaceted obesity epidemic, as children and adolescents are more likely to become obese or extremely obese if their parent received less education than a high school degree.[2] Therefore, obesity is not solely a product of the individual, but a product of his or her environment, indicating that prevention and treatment efforts have the potential to decrease the prevalence of obesity and its negative consequences.

Childhood Overweight and Obesity Consequence

The prevalence of childhood and adolescent overweight and obesity has numerous consequences, both short-term for the child and long-term as they become adults. The short-term consequences of childhood and adolescent obesity affect both the physical and mental health of the child. Low-self esteem is one of the immediate consequences of childhood obesity, and it puts children at risk for a myriad of psychological or psychiatric problems.[4] Obese adolescent girls have an increased risk for gynecological and obstetric complications, which often have a psychological impact as well. Gynecological consequences include an earlier onset of puberty, reduced fertility, irregular menstruation, decreased use of contraceptives, increased risk of polycystic ovary syndrome, and increased risk of developing breast cancer and endometrial cancer later in life. Obstetric consequences of adolescent obesity include increased risk for preeclampsia, cesarean delivery, neonatal complications, and infants that are large for gestational age.[5] Male and female obese children are more likely to develop symptoms of asthma, and low grade systemic inflammation has been proposed as another consequence of childhood obesity.[4]

Childhood obesity is likely to continue into adulthood, and there is an even greater likelihood for the persistence of adolescent obesity. It is estimated that about 40-70% of obese children will continue to be obese as adults, although studies have found up to 77% persistence of childhood obesity into adulthood.[4] These levels of persistence combined with continually increasing rates of adolescent obesity will lead to the manifestation of long-term consequences in

a larger percentage of the population. Research has consistently shown that childhood and adolescent overweight and obesity are associated with long-term health consequences, such as increased risk of premature mortality and adult morbidity.[6,4] Mortality and morbidity risks are elevated largely due to the associations between childhood overweight or obesity, increased cancer risk, and cardiometabolic risk factors.[7,8] A positive dose-response relationship exists between BMI and cancer risk, whereas an absence of excess body fat lowers the risk for eight different cancer types.[7] BMI measures for individuals 25 years old or less are associated with several of these cancer types, and cancer risk continues to increase into adulthood when body fat remains in excess.[7] Furthermore, overweight and obese children who maintain a high adiposity status into adulthood are more likely to present with type 2 diabetes, hypertension, high LDL cholesterol, low HDL cholesterol, high triglyceride levels, and carotid-artery atherosclerosis than consistently normal-weight individuals.[8] However, overweight and obese children who become non-obese adults have similar risk outcomes as individuals who were never obese.[8] The metabolic status of the individual likely plays a role in this outcome. A study comparing metabolically unhealthy and metabolically healthy obese adolescent boys determined that cardiometabolic risk may be associated with the timing of onset and the duration of obesity.[9] Ultimately, early intervention can protect overweight and obese children from certain cardiovascular and metabolic risk factors and lower their risk for several cancer types.

In fact, a BMI standard deviation score reduction as low as 0.25 can improve insulin sensitivity, blood pressure, and the total cholesterol/HDL ratio in obese adolescents, with more significant improvements in body composition and cardiometabolic risk resulting from greater reductions in BMI standard deviation score.[10] Although the risk for hypertension decreases with weight-loss, obese adults who were obese as children are more likely to be hypertensive than

obese adults who were not obese as children, indicating that the adverse impact of childhood obesity on blood pressure cannot be erased, but it can be diminished.[8] These findings have clinical implications for both the prevention and treatment of childhood obesity. Preventing childhood obesity altogether can protect children from all of the adverse short and long-term impacts of obesity. When prevention is not possible, it is important to treat obese children and adolescents in a timely manner, because treatment has proven most beneficial for individuals with greater insulin sensitivity,[10] and the duration of obesity likely impacts cardiometabolic health.

Dietary Contributions to Childhood Overweight and Obesity

The causes of childhood and adolescent obesity must be understood in order to optimize prevention and treatment efforts. Consistently positive energy balance, or energy intake in excess of energy expenditure for a long period of time, results in weight gain and an increase in body fat percentage that leads to obesity.[11] This trajectory is modified throughout the course of the lifespan by various genetic and environmental determinants.[11] Nutrition is an environmental factor that can modulate gene expression during pre- and post-natal development, subsequently impacting long-term health and disease risk.[12] Of course, diet affects the development of childhood and adolescent obesity well beyond early programming.

As mentioned, overall energy intake plays a role in the development of obesity, with an imbalance as low as 100-200 kilocalories per day resulting in significant weight increases in children.[11] In addition to energy imbalance, there is a relationship between overall diet quality and childhood obesity. Significant associations have been found between overall dietary quality and BMI z-score during mid-childhood, with BMI decreasing as diet quality increases.[13] Consumption of butter and margarine spreads, breaded poultry and fish, potatoes cooked in oil,

processed meats, desserts, sweets, milk, and sugar-sweetened beverages were all positively associated with three-year excess weight gain in UK children ages 7-13.[14] As evidenced by some of the specific foods associated with weight gain in children, the added sugar content, sodium content, and protein content of a child's diet may impact overall diet quality and energy intake, contributing to childhood and adolescent overweight and obesity.

Added Sugar Intake and Childhood Obesity

The desserts, sweets, and sugar-sweetened beverages mentioned above in relation to three-year excess weight gain in children likely all contain added sugars, which are added to foods during preparation, processing, or at the table, as opposed to the sugars occurring naturally in fruit and milk.[15] In fact, sugar-sweetened beverages contribute the most added sugar to the diets of children and adolescents and as such, they have been identified as a target for dietary interventions in that population.[16] The majority of research implicates sugar-sweetened beverage consumption as a contributor to childhood and adolescent overweight and obesity, however, conflicting data exists.[16] A study conducted in the UK found that a high fat, high sugar diet was associated with greater adiposity in children and adolescents than a lower fat, high sugar diet, which was not associated with adiposity.[17] This data indicates that high amounts of sugar were not solely responsible for the increase in adiposity,[17] although the inclusion of sugar in dietary patterns needs to be monitored as it relates to other adverse metabolic effects.

High fructose corn syrup is implicated in the development of metabolic syndrome because its metabolism is unregulated.[18,19] The various pathologies of metabolic syndrome include abdominal obesity, insulin resistance, hyerinsulinemia, dyslipidemia, and hypertension, which contribute to an increased risk for developing cardiovascular disease, renal disease, and type 2 diabetes.[18,19] High fructose corn syrup can trigger metabolic damage without resulting in

weight gain, but consumption appears to progressively contribute to obesity.[18] Taste receptors may also play a role in the development of childhood overweight and obesity. In children ages 7-14, sucrose detection thresholds were lower in children with larger waist-to-height ratios, indicating that sweet taste sensitivity may contribute to obesity risk.[20] Variations in the bitter taste receptor gene likely contribute to lower sucrose thresholds, and the diets of children with a bitter-sensitive genotype contained a higher percentage of added sugars.[20]

There is sufficient evidence indicating that added sugar intake contributes to the development of childhood and adolescent overweight and obesity, cardiovascular disease, hypertension, obesity-related cancers, and dental caries.[21] However, research in this area is complicated by flawed dietary intake assessment,[16] the inclusion of fat in the diet relative to sugar, metabolic complexities that may or may not reflect weight gain, and the wide range of sucrose detection thresholds present in children. In children and adolescents aged 4-18, sugar-sweetened soft drink consumption was significantly associated with salt intake,[22] and in an adult population reductions in sugar-sweetened beverage consumption have been associated with subsequent decreases in blood pressure.[23] Therefore, a decrease in sodium consumption could result in subsequent decreases in sugar-sweetened beverage consumption, in addition to alleviating the adverse health effects associated with excess sodium intake.[22]

Sodium Intake and Childhood Obesity

High sodium intake in US children and adolescents increases their risk for hypertension, cardiovascular disease, and death as adults.[24] Weight status mediates the relationship between salt consumption and high blood pressure risk,[24,25] with a 1,000 milligram per day increase in sodium corresponding to a 74% increase in hypertension risk in 8-18 year-olds who were overweight or obese, while normal weight children and adolescents experienced only a 6%

increase in risk for the same increase in sodium.[24] Processed foods are a larger contributor to overall sodium intake than salt added at the table, with food manufacturers responsible for up to 75% of US sodium intake.[26] Therefore, efforts to decrease overall US sodium intake will require a gradual reduction in the amount of salt added by the food industry during processing.[26,27] Achieving this reduction in children's sodium intake is worthwhile, because even small reductions in sodium consumption decrease blood pressure in children, which can decrease the risk for developing hypertension and cardiovascular disease in all children,[27] and minimize the adverse impact of childhood obesity on blood pressure into adulthood.[8]

Protein Intake and Childhood Obesity

The relationship between protein intake and childhood obesity is less understood. It is known that adiposity rebound occurs sooner for children who consume a high protein diet early in life, which results in faster increases in BMI and puts children at risk for obesity.[28,29] Specifically, consumption of a diet containing ≥ 16% protein before age 2 is associated with a higher BMI later in life.[28] Manipulation of protein intake in formula-fed infants revealed that higher protein intake is related to more pre-peritoneal fat accumulation by age 5.[30] This visceral fat distribution is associated with increased risk for metabolic syndrome and cardiovascular disease.[30] In addition to impacting fat distribution, early protein intake may impact hormonal pathways and displace fat in the diet, which is essential for energy production, rapid growth, and nervous system development. [28] For children two years and younger, a diet higher in protein and lower in fat is likely due to the higher protein content of infant formula compared to breast milk,[29] or a premature transition to low-fat milk.[28]

Research concerning the macronutrient composition of children and adolescent diets in relation to adiposity are inconclusive, likely as a result of their dependence on self-reported

dietary intake data.[31] However, one study in 8-12-year-olds identified increased protein intake as a risk factor for overweight and obesity.[31] Overweight and obese children and adolescents obtained more of their total energy from protein than their normal weight counterparts, the ratio of animal protein to vegetable protein consumed was higher, and the percentage of energy from protein sources was positively correlated with BMI and waist-to-height-ratio.[31] These results indicate that protein may be replacing other food groups in the diets of overweight and obese children and adolescents. On the other hand, weight management strategies often target protein intake because of its effect on appetite. Data from healthy adults indicates that higher protein preloads cause more feelings of fullness than lower protein preloads.[32] In obese adults, consumption of a calorie-restricted diet with protein intake (30% of total energy intake) evenly distributed across about six meals per day (protein-paced) was associated with improvements in weight, body composition, and obesity-related biomarkers when compared to a heart-healthy diet of 15% protein, consumed over three meals each day.[33] Protein-pacing combined with calorie restriction also showed improved maintenance of weight-loss,[33] which has implications for obesity treatment.

The relationship between protein consumption and childhood and adolescent overweight and obesity is complex. Protein consumption in children could have a filling effect, especially if it is consumed over the course of several small meals throughout the day. Feelings of fullness attributed to protein intake may lead to less energy intake overall, but they could also limit intake of other food groups such as fruits and vegetables. Ultimately, it is unknown how protein consumption after age two contributes to childhood obesity. However, it appears evident that overall diet quality contributes to the development of childhood and adolescent overweight and obesity, and diet quality can be affected by added sugar, sodium, and protein intake. Therefore,

monitoring dietary intake in children and adolescents is important since diet is indicative of health status and disease risk.

Dietary Intake in Children

Current dietary intake of US children and adolescents reveals a discrepancy between the amount of energy consumed and the amount of nutrients obtained from that energy. Average dietary intake for males and females in the 9-18 year-old age group falls short of recommendations for vegetables, fruits, dairy, legumes, whole grains, seafood, nuts, oils, while daily consumption exceeds saturated fat, added sugar, and sodium recommendations.[34] Consuming too many calories from energy-dense fats and sugars and not enough calories from nutrient-dense sources results in over-consumption of calories and under-consumption of key nutrients for this population.[35]

Added Sugar Intake in Children

To begin to address this discrepancy, the 2015-2020 Dietary Guidelines for Americans recommend limiting intake of added sugars, saturated fats, and sodium as one of five overall guidelines to support a healthy eating pattern.[34] The American Heart Association, The American Academy of Pediatrics, The Institute of Medicine, and the World Health Organization all support the effort to decrease added sugar intake.[36] The American Heart Association recently released a statement with instructions for children less than two years old to avoid added sugars entirely, and for children older than two years of age to limit their added sugar intake to less than six teaspoons (about 100 calories) per day.[21] This level of added sugar intake is less than the maximum amount suggested by the Dietary Guidelines for Americans, which recommends obtaining less than 10% of one's daily calories from added sugars.[34] Roughly 70% of Americans ages one and older exceed the Dietary Guidelines recommendation for added sugar intake,

indicating that an even greater percentage of individuals exceed the American Heart Association's new recommendation.[34] Males ages 9-13 and females ages 14-18 consume the most added sugar, with average consumption for both males and females ages 9-18 exceeding 16% of calories.[34] NHANES data suggests that adolescents aged 14-18 were also the highest added sugar consumers in 2001-2004, exceeding thirty-four teaspoons per day compared to the national average, which was about twenty-two teaspoons per day at that time.[37] Thirty-four teaspoons of sugar adds nearly 550 calories to one's diet daily, surpassing the discretionary calorie allotment for nearly all calorie levels.[37] These continually high levels of adolescent added sugar consumption may begin to explain why obesity rates are still trending upwards for this population.

Added sugars are available in many solid and liquid sources, both of which contribute to excess consumption by the US population. Beverages account for 47% of added sugar intake in the US, with sugar-sweetened beverages such as soda, fruit drinks, and sports drinks comprising 39% and coffee, tea, and alcoholic beverages contributing the remaining 8%.[34] Solid food categories include snacks and sweets (31%), grains (8%), mixed dishes (6%), dairy (4%), fruits and vegetables (2%), and condiments (2%).[34] Solid and liquid sources of added sugars are consumed and metabolized differently. As a result, consumption of liquid calories is associated with weaker satiety signals and increased overall energy intake, and weight loss strategies that decrease intake of caloric beverages have shown improved outcomes over diets that reduce calories from solid foods.[37] This explains why childhood obesity interventions targeting sugar-sweetened beverage consumption have proven effective.[38] These interventions typically reduce sugar-sweetened beverage intake by encouraging participants to drink plain water instead. Another acceptable alternative may be non-nutritive sweeteners, which are currently considered

safe for use, but their efficacy for long-term weight management has not yet been established.[34] Regardless of the intervention, obesity prevention and treatment efforts could be even more successful if added sugar information was readily available on food and beverage items.

Nutrition Facts Labels do not currently list added sugars separately from total sugars, but they are in the process of being updated to reflect the new recommendations, and changes will be enforced starting in July 2018.[39] Added sugars will be listed in grams and as a percent daily value, with fifty grams of sugar being considered 100% DV for a 2000-calorie diet.[39] Changing the Nutrition Facts Label is intended in part to help Americans limit their added sugar consumption to an amount that allows them to meet other food group requirements without exceeding their calorie needs.[34] The Dietary Guidelines encourage Americans to use their daily added sugar allotment to make nutrient-dense foods taste better.[34] For example, added sugars can sweeten yogurt, milk, whole-grain cereal, and tart fruits to increase average consumption, especially since child and adolescent dietary intake is low in these categories.[34] By using some added sugars to increase the nutritional density of childhood and adolescent diets while decreasing overall added sugar consumption, energy intake and nutrient intake should begin to reflect one another.

Sodium Intake in Children

Decreases in added sugar consumption and sodium intake likely complement each other due to their presence in processed foods. NHANES data from 2003-2008 revealed that 57% of sodium intake and 75% of added sugar intake can be attributed to processed foods,[40] so a dietary shift towards consuming less processed foods or altering the composition of the food supply could lower intake in both of these categories, which aligns with current nutritional goals for the American population. The 2015-2020 Dietary Guidelines recommend reducing sodium intake to

less than 2,200 milligrams daily for 9-13 year-olds and less than 2,300 milligrams per day for adolescents 14 years and older.[34] These recommendations are based on the Upper Limit set by the Institute of Medicine, and they differ because older children and adolescents likely have higher calorie needs, and additional caloric intake is associated with more sodium consumption.[34] Similarly, males generally consume more sodium than females.[34] Currently, over 80% of Americans are consuming more sodium than the recommended amount,[34] and intake continues to rise in children and adolescents aged 2-18 according to Healthy Eating Index-2010 scores measured between 1999 and 2012.[41] On average, male 9-13 year-olds are consuming 3,500 milligrams of sodium, and male 14-18 year-olds consuming upwards of 4,000 milligrams.[34] Females ages 9-18 currently consume about 3000mg of sodium per day, which still exceeds recommendations.[34] Excess sodium consumption can be attributed to processed foods, which are responsible for over half of US sodium intake as described above. As of the 2011-2012 school year, the ten highest food categories contributing to sodium intake in US school children had sodium added to them during processing or preparation, with the exception of milk, which contains sodium prior to processing.[42] The combination of foods naturally containing sodium and foods with sodium added during processing, preparation, or at the table, results in a wide variety of food sources that are high in sodium. Mixed dishes represent 44% of American's sodium intake,[34] including pizza, burgers, sandwiches, and pasta dishes, with protein foods contributing an additional 14% to US sodium consumption.[34]

Protein Intake in Children

Of the nutrients discussed, children and adolescents are closest to meeting the recommendations for protein intake, which should account for 10-30% of total calories for males and females ages 9-18.[34] Consumption of 34 grams of protein per day is the recommendation for

males and females ages 9-13, while 14-18 year-old females should consume 46 grams per day, and 14-18 year-old males should consume 52 grams daily.[34] Males ages 9-13 and 14-18 are within these recommended ranges for protein intake, but average protein intake for females ages 9-13 is barely meeting the recommendation, and females ages 14-18 are not meeting the recommendations.[34] Average protein consumption for children and adolescents appears to be concentrated within the meat, poultry, and egg categories, resulting in under-consumption of other protein sub-groups such as seafood, nuts, seeds, soy, and legumes.[34] However, consumption of plant proteins and total protein has increased in children ages 2-18 according to Healthy Eating Index-2010 Scores measured between 1999 and 2012.[41] Since protein consumption is increasing and the majority of US children and adolescents are meeting but not exceeding overall protein requirements, this macronutrient is less of a concern as it relates to childhood and adolescent overweight and obesity. However, as researchers explore the relationship between protein consumption and satiety, the quality and quantity of protein intake should continue to be monitored, with protein foods currently accounting for 14% of US sodium intake and 15% of US saturated fat intake.[34] Choosing lean, unprocessed protein sources could maintain adequate protein intake while decreasing consumption of sodium and saturated fat, which may improve childhood and adolescent obesity outcomes.

Current dietary intake in US children and adolescents demonstrates a general overconsumption of added sugars, sodium, and saturated fats, with certain protein sources contributing to excess intake of sodium and saturated fats. The 2015-2020 Dietary Guidelines for Americans recommends decreasing consumption of added sugars, sodium, saturated fats, and processed protein sources, recognizing the contribution of these dietary components to childhood and adolescent overweight and obesity.

Dietary Intake Assessment in Children

Prevention and treatment of obesity requires assessing the diets of children and adolescents by comparing their current dietary intake of key nutrients to levels that are known to support healthy growth and development. Obtaining accurate dietary intake information from children and adolescents has implications across the translational spectrum from research to practice. Dietary intake assessment is essential for exploring the relationship between dietary intake and health outcomes, developing appropriate interventions to improve nutritional status and diet quality, and analyzing the impact of those interventions. Methods for obtaining dietary information include diet histories, dietary recalls, food records, and food frequency questionnaires, and there are variations regarding how each of these is conducted. Specific to 24-hour multiple-pass dietary recalls, which are used most frequently,[43] variables that could impact the accuracy of a child's dietary intake assessment may include: presence of a parent, use of food models or serving size diagrams, use of technology, conducting the interview in person or over the phone, and the length of time between consumption and the recall.

Furthermore, research indicates that a child's age, BMI, social desirability, food preferences, and cognitive ability unsystematically impact the accuracy of dietary reporting.[43] As a result of these variables, objective methods of dietary assessment are needed to evaluate the validity of current dietary assessment measures. For example, doubly-labeled water estimates total energy expenditure, and in free-living, weight-stable individuals, this corresponds to energy intake.[44] Therefore, doubly-labeled water is considered the gold standard for determining the validity of various methods of energy intake assessment.[44] A systematic review of studies comparing doubly-labeled water to methods of dietary intake assessment indicates that overweight and obese children and adolescents are more likely to under-report dietary intake data.[44]

Doubly-labeled water data also indicates that under-reporting may increase with age.[45] Although children less than eight-years-old may not be able to recall foods independently, by 12 years of age children are likely capable of self-reporting dietary intake data with accuracy.[44] However, even though adolescents may be able to remember and report this information, their reports may be biased due to age or any combination of the factors mentioned above. For example, age and weight-related reporting biases combine to explain the unreported energy intake levels of up to 40% in obese adolescents, compared to 25% in obese 10-year-olds.[46] Low energy reporters, defined as those who report less energy intake than physiologically plausible, characteristically report less sweet and savory snacks, and significantly less soft drinks.[47] Therefore, it is likely that foods high in sugar and sodium are frequently under-reported in the target population. Characteristics of low energy reporters and doubly-labeled water studies reveal the limitations associated with self-reported measures of dietary intake assessment in children and adolescents, demonstrating the need to establish valid methods of dietary intake assessment in this population.

Biomarkers of Dietary Intake in Children

Biomarkers of dietary intake are "components of body fluids or tissues that have a direct relationship with the dietary intake component of interest or that reflect intake in a predictable manner".[48] For example, doubly-labeled water serves as a biomarker for energy intake.[48] This biomarker is unspecific because assessment of energy intake alone does not lend insight to overall diet quality. Doubly-labeled water is well suited to assess the validity of dietary intake assessment methods as described above,[45] but there is a demonstrated need for the development of more cost-effective biomarkers that can indicate specific consumption of food categories emphasized by the U.S. Dietary Guideline recommendations, such as added sugars.[49] Additional biomarkers of dietary intake are being investigated because they can serve as objective indicators

of dietary intake, free from the biases associated with self-reported data.[48] Table 1 summarizes the biomarker research that has been done, with a focus on child and adolescent dietary intake of the following key nutrients: whole-grains, sugars (added sugars, total sugars, sucrose, and fructose), protein, sodium, potassium, and fruits and vegetables (hippuric acid, carotenoids, flavonoids). These biomarkers are categorized by the body fluid or tissue from which they were isolated, including blood, urine, skin, and hair.

Urinary Biomarkers

There are a variety of factors to consider during the development of biomarkers. In addition to being cost-effective and representing a specific, relevant component of dietary intake, the degree of invasiveness, amount of subject burden, and tissue turnover time should all be considered.[49] Urinary biomarkers are considered minimally-invasive[50], relative to more invasive biomarkers that require the collection of blood or adipose tissue.[49] Urine collections typically only require the addition of a preservative (thymol) to the collection container to preserve excreted components, and sample processing is minimal.[50] Para-aminobenzoic acid (PABA) can also be used to determine urine collection compliance; for example, recovery of 85% or more PABA could classify a urine sample as complete.[50] Participant burden can be high due to multiple 24-hour urine collections being required, but the progression of research using spot urine samples may lessen participant burden in the future.[51,52] Another limitation of urinary biomarkers is their rapid turnover time of less than or equal to approximately 24 hours, which corresponds to the length of time they are capable of reflecting dietary intake.[50] Gaining insight into the prior day's dietary intake, which could be manipulated, is less valuable than understanding someone's habitual intake. However, a panel of urinary biomarkers including sucrose, fructose, sodium, and nitrogen can provide information about a wide range of dietary

factors such as sugar, sodium, and protein intake. In addition, these biomarkers can serve as measures of compliance when levels of expected intake and excretion are known.

Urinary Sugars

The body does not produce sucrose or fructose, so any excretion of these sugars can be directly linked to dietary intake.[53] Therefore, urinary sugar excretion can represent both added sugars and those naturally occurring in foods,[50] but sucrose and fructose excretion have shown stronger correlations with extrinsic sugars because they are more readily available for metabolism than intrinsic sugars, which are naturally incorporated into foods.[54] Urinary sucrose and fructose are only excreted in small amounts. The majority of dietary sucrose is hydrolyzed into glucose and fructose in the duodenum, but some un-hydrolyzed sucrose can pass through gastrointestinal mucosa, entering circulation prior to excretion in the urine.[52] Dietary fructose is absorbed in the small intestine, comprising a fraction of the urinary fructose excreted. The remaining urinary fructose is derived from dietary sucrose that is broken down but not taken up by other tissues.[52] Urinary sugars have potential as a biomarker for dietary sugar intake in normal weight, overweight, and obese children and adolescents because they are sensitive to changes in dietary intake, and BMI did not appear to affect validity in a sample of 19 adults.[55] However, controlled feeding studies are needed to validate urinary sugar biomarkers in adolescent populations.

Urinary Sodium

A urinary sodium biomarker can serve as a measure of dietary intake and dietary compliance, because sodium is excreted at a known value of approximately 90% of intake.[51] Predictive equations have been developed to assess population-wide sodium intake in adults, but individual measures currently lack validity.[56] Furthermore, 24-hour recall data has shown poor

correlations with urinary sodium excretion in adults.[57] Aside from the limitations of self-reported dietary intake and the burdensome nature of urine collections addressed previously, sodium can be lost through sweat. Sweat losses vary in accordance with physical activity, climate, acclimation to climate, and sodium intake.[51,57] In an adolescent population, sweat losses may also vary based on the timing of puberty and sweat gland development. Although there are challenges associated with using urinary sodium as a biomarker, there are benefits to establishing an objective indicator of dietary sodium intake in a child and adolescent population. Lower urinary sodium excretion has been associated with better diet quality in 6-year-old children, but these findings are based on a cross-sectional study where only one 24-hour urine sample was collected.[58] In addition, higher urinary sodium excretion has been associated with increased body weight and body fat percentage in 3-18 year olds based on data from three-day weighed food records.[59] Ultimately, urinary sodium excretion could indicate the amount of sodium consumed by free-living individuals or provide a measure of dietary compliance in controlled settings.

Urinary Nitrogen

Urinary nitrogen excretion reflects 80% of dietary nitrogen intake, which accounts for losses in the feces and skin.[60,61] This excretion rate relies on the assumption that participants are in nitrogen balance, but since individuals may not be in balance on any given day, urinary nitrogen excretion is more reliable over a period of a few days.[61] Dietary protein intake of 1.5 grams per kilogram of body weight appears to be sufficient for achieving positive nitrogen balance in adolescent athletes, regardless of growth rate, although dietary protein intake was self-reported.[62] A study in children and adolescents indicated that weighed food records estimated protein intake with reasonable validity, but validity was lower for adolescents and average

protein intake was significantly underestimated.[63] When dietary protein intake and urinary nitrogen excretion are known, this biomarker can also be used a measure of dietary compliance.

Urinary Creatinine

Urinary creatinine is derived from creatine and phosphocreatine through a process that occurs in muscle tissue.[64] As a result, urinary creatinine measures are reflective of muscle mass and body composition.[64] Since reference values have been established in healthy, white children and adolescents, measures of urinary creatinine provide an indication of urine collection compliance.[64] Based on the established reference values, it is recommended for samples with creatinine values of less than 0.1 millimoles per kilogram of body weight per day to be excluded.[64,65] However, it is still recommended to establish additional urine collection completeness criteria.[64]

Establishing Urinary Biomarkers in Children

Table 1 includes examples of objective biomarkers of dietary intake that have been studied in children, including biomarkers from a variety of bodily fluids and tissues, but with an emphasis on urinary biomarkers. Therefore, adult urinary biomarker studies are also included. Of the studies reviewed, only two had a controlled-feeding design, and both of these were conducted in an adult population.[55,66] Therefore, controlled-feeding studies conducted in younger populations are lacking, but they are necessary to circumvent the limitations of self-report data and establish urinary biomarkers of dietary intake in children. Controlling dietary intake and evaluating urine samples for completeness would contribute to the validity of the study, while collecting two 24-hour urine samples at each assessment would enable evaluation of reliability. Finally, altering dietary intake would establish if urinary sugar biomarkers are responsive to dietary changes.

Objectives

The current study aims to use a randomized controlled crossover feeding study design to establish urinary sucrose, fructose, sodium, and nitrogen as objective indicators of dietary intake. It is hypothesized that urinary sucrose, fructose, and urinary total sugars will reflect dietary added sugar intake, while urinary sodium and nitrogen will correspond to dietary sodium and protein intake, respectively, in a healthy adolescent population.

TABLE

Table 1. Objective biomarkers of dietary intake, with an emphasis on urinary biomarkers and biomarkers evaluated in childhood and adolescent populations.

Biomarker		Dietary Intake Assessment	Author	Year	Population	Significant Findings	Limitations
Blood	Plasma alkylresorcinol	Whole Grain Intake	Biltoft-Jensen A, Damsgaard CT, Andersen EW, et al[67]	2016	8-11 year olds	Reported whole grain wheat and rye intake moderately correlated with plasma AR	Plasma AR does not reflect consumption of oats, self-administered recording of dietary intake
	Fingerstick blood $\delta^{13}C$	Added Sugar and SSB Intake (Corn & Cane Sugar)	Davy BM, Jahren AH, Hedrick VE, Comber DL[68]	2011	>21 years old	$\delta^{13}C$ associated with AS and SSB intake	Adult population; non-AS dietary items with similar $\delta^{13}C$ values may confound results, self-reported data
Urine	Doubly-Labeled Water	Total Energy Intake	Burrows TL, Martin RJ, Collins CE, et al[44]	2010	Review including children and adolescents 0.5 – 18 years old	Weighed food records best age 0.5-4; 24-hour MPR for at least 3 days(weekdays+ weekend day) with parent reporter best for age 4-11, diet histories best for age 16 and up	Compared to self-report data; collected during a time period that may or may not reflect typical intake
	Urinary Nitrogen	Protein Intake	Bingham, SA[61]	2003	Review	Data continues to support initial estimate (80% of dietary nitrogen intake[60]); several collections verified for completeness are needed for accurate comparison	Assume subjects are in nitrogen balance
	Urinary Nitrogen	Protein Intake	Bokhof B, Gunther ALB, Berg-Beckhoff G, et al[63]	2009	Ages 3-4, 7-8, 11-13, or 18-23	Weighed dietary records underestimated protein consumption by 11% compared to urinary nitrogen excretion predictions	Use of one 24-hour urine collection
	Urinary Sugars	Total Sugars	Tasevska N[52]	2015	Review	Need for controlled feeding studies in different populations to validate urinary sugar biomarkers	Current data limited by sample size, location, and age
	Urinary Sucrose and Fructose	Sucrose, Total Sugars	Tasevska N, Runswick SA, McTaggart A, Bingham S[66]	2005	Males aged 25-77	Sum of sucrose and fructose excretion significantly correlated with sucrose intake and total sugar intake; controlled feeding design	May not represent daily variation of free-living individuals' diets
	Urinary Fructose	Fructose	Johner SA, Libuda L, Shi L, et al[53]	2010	Mean age= 9.3±0.8 (boys), 7.9±0.7 (girls)	Urinary fructose is more associated with dietary fructose than added sugars or total sugars	One 24-hour urine collection; sugar-intake estimated from weighed food records

Biomarker		Dietary Intake Assessment	Author	Year	Population	Significant Findings	Limitations
Urine (cont.)	Urinary Sucrose & Fructose	Sugar Consumption	Joosen AMCP, Kuhnle GGC, Runswick SA, Bingham SA[55]	2008	20-85 years (n=10 normal weight, n=9 obese)	Urinary sucrose and fructose are valid biomarkers of sugar intake regardless of BMI; controlled feeding design	Low sample size; medications not controlled for
	Urinary Sodium & Potassium	Dietary Sodium & Potassium	Kristbjornsdottir OK, Halldorsson TI, Thorsdottir I, Gunnarsdottir I[58]	2012	6 year olds	Better diet quality associated with lower sodium excretion and higher potassium excretion	Cross-sectional study; one 24-hour urine collection
	Hippuric Acid	Fruit & Vegetable Consumption	Krupp D, Doberstein N, Shi L, Remer T[69]	2012	9-10yo; 12-14yo	Hippuric acid biomarker associated with fruit and vegetable (whole and juice) consumption in healthy children and adolescents	One 24-hour urine collection; 3-day weighed dietary records collected
	Hippuric Acid	Flavonoid Intake	Penczynski KJ, Krupp D, Bring A, et al[70]	2015	9-16 years old	24-hour urinary hippuric acid (adjusted for body surface area) validated in healthy adolescents as indicator of flavonoid fruit and vegetable consumption	No manipulation of flavonoid intake; HA precursors other than flavonoids not controlled for
	Urinary Sodium	Intake of salty foods	Libuda L, Kersting M, Alexy U[59]	2011	3-18 years old	Consumption of salty foods associated with body weight and body fat percentage, relationship not completely explained by SSBs or total energy intake	Sodium intake determined from 3-day weighed food records; physical activity not controlled for
Other	Skin	Carotenoid Status	Nguyen LM, Scherr RE, Linnell JD, et al[71]	2015	9-12 year olds	Resonance Raman Spectroscopy can predict plasma carotenoids and dietary intake of carotenoids	Food choices and portion sizes limited by FFQ; skin pigmentation may be confounding variable
	Hair Carbon and Nitrogen Ratios	Added Sugar Intake	Chi DL, Hopkins S, O'Brien D, et al[72]	2015	6-17 year old Yup'ik children Mean = 10.8 ± 3.3	Added sugar intake from hair biomarker was significantly correlated with dental caries	Cross-sectional design

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CHAPTER 2: Establishing Urinary Biomarkers as Objective Indicators of Dietary Intake in Adolescents

ABSTRACT

Self-reported dietary intake has known limitations in adolescents. The aim of this study was to establish urinary sodium (Na), nitrogen (N), and total sugars (TS) excretion as objective indicators (i.e., biomarkers) of dietary Na, protein, and added sugar (AS) intake in adolescents. Non-obese adolescents (n=32, 12-18y) completed a randomized controlled crossover feeding study. Participants were fed a 5% AS diet (55% carbohydrate, 30% fat, 15% protein) and a macronutrient-matched 25% AS diet in a randomly assigned order (1 week each with ≥4 week washout). Two consecutive 24-hour urine collections were obtained at the end of each feeding period. Urinary Na excretion was not different from expected recovery of 90% of intake (87.9±17.8%, $p=0.502$). Urinary N excretion was strongly correlated with dietary protein intake ($r=0.694$, $p<0.001$), but dietary N recovered through urinary excretion was significantly different from the expected 80% recovery (62.2±6.8%, $p<0.001$). N recovery was significantly lower during the 5% AS feeding period (5% AS: 58±7%, 25% AS: 66±8%, $p<0.001$). Urinary TS were strongly correlated with AS intake during the 25% AS feeding period ($r=0.77$, $p<0.001$). Urinary TS excretion was 0.226±0.09mg/d (0.00014% recovery) during the 5% AS feeding period, and 0.365±0.16mg/d (0.00017% recovery) during the 25% AS feeding period. Urinary Na appears to be a valid biomarker for Na intake in adolescents. Urinary N has the potential to become a valid biomarker for protein intake in adolescents, but factors that may contribute to differences between adolescent and adult N recovery (e.g. growth) and feeding period differences in N recovery (e.g. fiber) should be considered. Urinary TS appears to be a valid biomarker for AS intake in adolescents at 25% AS consumption, and it was responsive to the change in AS intake.

These findings suggest that biomarkers can be used in clinical research to improve understanding of the associations between dietary intake and health outcomes.

Keywords: children and adolescents, urinary biomarkers, dietary intake assessment, added sugars, sodium, protein

INTRODUCTION

Obesity rates in the United States have begun to plateau at 17% for 2-19-year-olds, but the prevalence of adolescent obesity has continued to increase, reaching 20.6% of 12-19-year-olds by 2013-2014.[1] Diet quality likely plays a role in the development of obesity; foods such as processed meats, desserts, and sugar-sweetened beverages have been associated with three-year excess weight gain in United Kingdom children ages 7-13.[2] Over-consumption of sodium, protein, and added sugar may negatively impact overall diet quality, contributing to adolescent overweight and obesity. Increased dietary sodium intake has been associated with an increased risk for hypertension and cardiovascular disease;[3,4] this relationship appears to be mediated by weight status, with a 1000mg/d increase in sodium intake corresponding to a 74% increase in pre-high/high blood pressure risk in 8-18-year-olds, compared to a 6% increase for healthy weight children and adolescents. Sodium intake in 2-18-year-olds increased between 1999-2012,[5] with average adolescent consumption reaching 3000mg/d in female 9-18-year-olds and 3500mg/d in male 9-13-year-olds, and exceeding 4000mg/d in male 14-18-year-olds.[6] As of the 2011-2012 school year, the ten highest food categories contributing sodium to the diets of US schoolchildren had sodium added during processing or preparation, with the exception of milk.[7] Increased dietary protein intake has been identified as a risk factor for overweight and obesity in 8-12-year-olds.[8] The majority of adolescents are meeting their protein needs, but current dietary recommendations encourage male teenagers and adults to decrease overall protein consumption by reducing intake of meat, poultry, and eggs.[6] Furthermore, protein foods account for 14% of sodium intake and 15% of saturated fat intake in the US.[6] Added sugar intake has been positively associated with the development of cardiovascular disease and type 2 diabetes, both directly (by impacting metabolism) and indirectly (by increasing body fat and weight), although its

relationship with health remains controversial.[9] Currently, adolescents are the US population's highest consumers of added sugars, with average consumption exceeding 16% of total energy intake[6] compared to recommendations of <10%[6,10] and <5% of total energy intake.[10]

Valid methods for assessing dietary intake in adolescents are necessary for improving current understanding of the relationship between dietary components and the development of adverse health outcomes, including overweight and obesity. Self-reported dietary intake data has known limitations, particularly in overweight or obese adolescents, who are more likely to under-report.[11] Savory snacks, soft drinks, and sweets are typically mis-reported,[12] making sodium and added sugar intake even more challenging to quantify. Biomarkers can serve as objective indicators of dietary intake, eliminating the biases associated with self-reported data.[13] There is a need for the development of cost-effective biomarkers that can indicate specific consumption of food categories emphasized by the Dietary Guidelines.[14] Urinary biomarkers can provide information about specific dietary components, such as added sugar, sodium, and protein, and urine samples are minimally invasive and relatively easy to process.[15]

Urinary sodium is excreted at a known value of approximately 90% of dietary intake,[16] and sodium excretion has been associated with diet quality in 6-year-olds,[17] and body weight/fat in 3-18-year-olds,[18] but controlled feeding studies have not been performed to establish urinary sodium as a biomarker of sodium intake in adolescents. Physical activity, environmental temperature, and the onset of puberty could affect the amount of sodium excreted through sweat,[16,19] which would consequently impact urinary sodium excretion. Urinary nitrogen is excreted at a known rate of about 80% of dietary nitrogen intake for adults in nitrogen balance,[20,21] but this data is also lacking in growing adolescents. In fact, the Recommended Dietary Allowances for protein intake in adolescents are estimated from data in younger children

and adults, with "an additional amount" added to promote growth.[22] Weighed food records have been used to estimate protein intake, but estimations were based on one 24-hour urine collection, nitrogen excretion was assumed to reflect 80% of dietary intake, and validity was lower among adolescents.[23] Urinary nitrogen excretion may be affected by the type and amount of dietary fiber intake.[21,24,25] Urinary excretion of sucrose and fructose is minimal relative to dietary intake,[26] but sucrose excretion occurs when dietary sucrose passes through gastrointestinal mucosa without being hydrolyzed into glucose and fructose, and fructose excretion occurs when dietary fructose or hydrolyzed sucrose are absorbed in the small intestine but not taken up by other tissues.[27] Urinary sugar excretion is more strongly correlated with extrinsic (added) sugars than intrinsic (natural) sugars when added sugars comprise a larger portion of total sugar intake.[28] Urinary sugars are capable of reflecting changes in sugar consumption regardless of BMI,[29] but controlled feeding studies are needed to establish them as a biomarker of added sugar intake.[27] The current study aims to use a randomized controlled crossover feeding study approach to establish urinary sodium, nitrogen, sucrose, and fructose as objective indicators of dietary intake. It is hypothesized that urinary sodium and nitrogen will reflect sodium and protein intake, respectively, while sucrose, fructose, and urinary total sugars (sucrose+fructose) will be valid indicators of added sugar intake in a non-obese adolescent population.

MATERIALS AND METHODS

Subjects and Design

The Virginia Tech Institutional Review Board approved all study procedures prior to the on-set of study recruitment. A convenience sample of non-obese adolescents was recruited via flyers and word of mouth for participation in a randomized controlled crossover design feeding study. Interested adolescents were included if they met the criteria for age (12-18 years) and BMI

(<95th BMI percentile[30]), had no special dietary needs or restrictions, obtained parental permission (age 12-17) or provided informed consent (age 18), and agreed to comply with all study procedures. Individuals were excluded if they did not meet the inclusion criteria or declined to participate due to the time commitment. The investigation is registered at ClinicalTrials.gov (NCT02455388).

Procedures

The schedule of study visits is depicted in Figure 1. Baseline measures were obtained, including a questionnaire to assess health history and demographic characteristics, the use of a wall-mounted stadiometer (Seca Model 216, Seca; Chino, CA) to measure height, the use of a digital scale (Scale-Tronix 5002, Welch Allyn; Skaneateles Falls, NY) to assess weight, and evaluation of BMI percentile.[30] Female participants completed a menstrual calendar so that feeding periods could be scheduled around the same time of each participant's menstrual cycle, to the extent possible. Four 24-hour dietary recalls (three weekdays and one weekend day) were completed to determine habitual intake, with parents providing additional information related to recipes, cooking method, and food product brands if necessary. Dietary intake during baseline (four 24-hour dietary recalls), the washout period (four 24-hour dietary recalls), and each controlled feeding period (menus for controlled diets) were collected and analyzed using NDS-R (Version 2013; Minneapolis, MN). The Institute of Medicine equations for Boys and Girls ages 9-18 were used to assess resting energy expenditure[31] and multiplied by an activity factor derived from the Physical Activity Questionnaire for adolescents (PAQ-A survey)[32] to estimate total energy needs. Subjects were randomly assigned to a diet sequence to eliminate any bias associated with the order in which the controlled diets were received. Each participant completed either one week of the low added sugar diet (5% AS) or one week of the high added sugar diet

(25% AS) and four weeks of washout, followed by the second feeding period. A washout period was included to allow participants to resume their habitual dietary intake, eliminating the potential for any effects from the first feeding period to be carried over to the second feeding period. During the washout period, dietary intake was re-assessed using four 24-hour dietary recalls to evaluate if habitual dietary intake had resumed. Energy needs were also re-assessed using height, weight, and PAQ-A data collected during the washout phase, prior to the start of the second feeding period.

Controlled Feeding

As seen in Table 1, both diets were matched for macronutrient content (55% carbohydrate, 15% protein, 30% fat) and the form of added sugar (67% solid, 33% liquid), which is in accordance with typical US intake.[33] Animal protein and non-sweetener corn intake levels were also maintained across diets to account for the confounding effects of non-sweetener corn intake on other biomarkers being investigated.[34] Diets were isocaloric as evidenced by weight stability; body weight was assessed every morning while subjects were on the controlled diet. Two optional snack modules (150 calories each) were offered daily to promote weight stability, and they matched the nutrient composition of the controlled diet. Subjects who weighed in above or below their weight stability range (±1 lb from their first weigh-in that week) for three consecutive days had their calorie level or snack module allotment adjusted accordingly.

All food was prepared in a metabolic kitchen using a digital benchtop scale (Practum 5101-1S, Sartorius; Goettingen, Germany). Provided amounts were ±0.8 grams of menu amounts. Caloric intake was calculated using standardized values for the metabolizable energy of macronutrients,[35] and menu sodium content was adjusted based on package labels to reflect the specific brands of food items provided. Breakfast was consumed in the dining laboratory and

supervised daily by research staff; all other meals, snacks, and beverages were provided, packed in portable coolers for consumption throughout the day. Subjects were instructed to consume all provided foods and beverages, with the exception of optional snack modules, three plain water bottles (provided daily to encourage adequate hydration), and the three optional sucralose (Splenda®) packets (provided to increase palatability). Nutrients from the consumption of optional snack modules were included in data analysis. Menus were provided with meal and snack suggestions and microwave instructions for heating frozen or refrigerated items. Participants were instructed to return food and beverage containers unwashed so they could be weighed back to record actual consumption; they were also asked to report any food or drink consumption that was inconsistent with the provided diet.

24-Hour Urine Collections

24-hour urine collections were completed on two consecutive days at the end of each controlled feeding period (Day 6 and Day 7). Subjects were provided with plastic 3-liter urine collection containers, each containing 6.75mL of thymol to prevent bacterial growth and preserve urinary sugars. The para-amino benzoic acid (PABA) test was not used to assess diet compliance due to the compound's carbon content,[36] which would interfere with other biomarkers being investigated. Subjects (and their parents if present) were provided verbal and written instructions for flushing their first morning urine sample, recording the corresponding time as the start time, and then collecting their urine for a complete 24-hour period. Subjects were asked to report missed collections, spills, and plain water intake on the worksheets provided. Worksheets were reviewed the following morning when the urine samples were returned. Stop times were recorded or confirmed at that time, and subjects were provided with new containers and instructions to begin collecting their urine for a second consecutive 24-hour

period. Dietary intake during each urine collection day was recorded separately for each 24-hour period, and as an average of both 24-hour periods to represent 48 hours.

Urine Processing and Analysis. Total urine volume was measured for each 24-hour sample and reported as the total volume of urine minus the volume of thymol added to the container(s) prior to collection, with the exception of the urinary sugars assays which accounted for the dilution with thymol. Urine volumes were measured upon delivery, and samples were either refrigerated for 1-2 days or kept on ice packs and sent directly to SOLSTAS Lab Partners, a CLIA- and CAP-certified lab in Roanoke, VA, for processing. Trained SOLSTAS technicians ran all assays on the Roche Cobas Model 8000. 24-hour sodium content was assessed by the indirect ion selective electrode assay, a method known to have less than 3% variation.[16] 24-hour nitrogen content was assessed by a kinetic test for urease and glutamate dehydrogenase. 24-hour creatinine was assessed using a kinetic calorimetry assay based on the Jaffe method.

Urinary sucrose and fructose assays were completed using mass spectrometry. The urine samples were preserved in 2 milliliter cryotubes, frozen at -81 degrees Celsius during initial processing, and thawed once prior to completing the assays. Urinary sugars were measured as described by Camilleri et al.[37] with modifications. 200 μL urine were combined with 800 μL of internal standard [0.25 mg/mL $^{13}C_6$-glucose in acetonitrile/water (94:6)], vortexed, and centrifuged. Following centrifugation (5 min, 17,000 x g), 500 μL supernatant was analyzed by UPLC-MS/MS (5 μL injected). UPLC separation was performed on a Waters Acquity H-class (Milford, MA) equipped with an Acquity UPLC BEH Amide column (2.1 mm × 50 mm, 1.7 μm particle size). Elution was performed at 0.7 mL/min using a binary mobile phase system: Phase A- acetonitrile/water (65/35) with 0.2% v/v triethylamine (TEA) and Phase B-0.1% v/v formic acid in acetonitrile. The linear elution gradient was 1% A (starting), 99%A (3 min), 1% A (3.05

min), and 1% A (5.5 min). Column and sample temperatures were ambient (25°C and 10°C, respectively). Detection by MS/MS was performed on a Waters Acquity Triple Quadrupole Detector (TQD). Negative-mode electrospray ionization [(−)-ESI] was performed with capillary voltage of −4 kV, and source and desolvation temperatures of 150 and 450°C, respectively. Desolvation and cone gasses were at flow rates of 900 and 100 L/h, respectively. For MS/MS, the collision gas was Ar. The cone voltages, collision energy, and Multiple Reaction Monitoring (MRM) transitions for each compound are listed in Table 2. Peak widths were ~5 seconds, and AutoDwell was employed with required points-per-peak set at 12. The interscan delay time will be 0.02 seconds. Data acquisition, processing, and quantification was performed using Waters MassLynx v4.1 software. Urine sugar concentrations were calculated from standard curves of sucrose and fructose and then multiplied by 24-hour urine volume to represent daily excretion.

Urine Collection Completeness Criteria. Urine collections were deemed incomplete if any one of the following criteria applied: a) creatinine excretion < 0.1 mmol/kg body weight/day,[38] b) reported collection time outside the time frame of 20-28 hours,[39] c) reported as missing two or more collections,[40,19] and d) urine volume <500 milliliters.[40,19] If data was missing, urine specimens were evaluated for completeness based on the remaining criteria. Incomplete urine samples were excluded. Samples considered complete by these criteria were time-adjusted to 24-hours.[39] Samples missing duration times were included and assumed to be 24 hours unless deemed incomplete by one of the other criteria.

Statistical Analysis

Data was analyzed using statistical analysis software (IBM SPSS Statistics Version 24 Armonk, NY). Descriptive statistics were used to characterize participant demographics, estimated energy needs, and habitual dietary intake. Average self-reported dietary intake at baseline and during the washout period were compared to average caloric intake during both

controlled feeding periods using paired t-tests, and Bland-Altman plots were used to analyze the agreement between self-reported energy intake and energy intake during controlled feeding in a non-obese, adolescent population. Weight stability was analyzed using paired t-tests to compare each subject's weight from the beginning (Day 1) to the end (Day 8) of each feeding period. Dietary compliance during each feeding period was assessed from metabolic kitchen diet records kept throughout the study, and paired t-tests were used to evaluate if dietary compliance differed between feeding periods. Correlations were used to evaluate urinary creatinine excretion as an indicator of body weight and height. Intra-class correlations were used to evaluate the reliability of urinary creatinine excretion, which can also indicate urine collection compliance if body mass is maintained throughout the duration of the study. Urinary creatinine excretion was compared across all four 24-hour urine collections. For all urinary data, either 24-hour or 48-hour collections were compared to corresponding 24-hour or 48-hour dietary intake, respectively, based on the amount of complete samples available per participant per feeding period. For the low added sugar feeding period, 25 subjects had two complete 24-hour urine collections and 5 subjects had one complete 24-hour urine collection; the 2 subjects with no complete 24-hour urine collections were excluded from data analysis. For the high added sugar feeding period, 27 subjects had two complete 24-hour urine collections and 4 subjects had one complete 24-hour urine collection; the subject with no complete 24-hour urine collections was excluded from data analysis. One outlier was excluded from data analysis involving the variable of urinary nitrogen recovery due to SPSS identification as an extreme value (IQR>3).[41]

Evaluating Validity. Descriptive statistics were used to evaluate the percentage of dietary sodium intake recovered by urinary sodium excretion. Paired t-tests were used to evaluate the validity of urinary sodium as an objective indicator (i.e. biomarker) of dietary sodium intake by

comparing urinary sodium excretion to the amount of excretion expected (90%)[16] based on corresponding dietary sodium intake. Correlations were used to determine the strength of the relationship between urinary nitrogen excretion and dietary protein intake. Paired t-tests were used to evaluate the validity of urinary nitrogen as an objective indicator of dietary protein intake by comparing urinary nitrogen excretion to the amount of excretion expected (80%)[20,21] based on corresponding dietary nitrogen intake. To address the assumption that our adolescent population was in nitrogen balance, Pearson's correlations were conducted between dietary protein intake (per kilogram of body weight) and urinary nitrogen recovery for the full sample, as well as male and female subsets. To evaluate the impact of dietary fiber intake on the urinary nitrogen biomarker, paired t-tests were run to assess for differences between diet periods. Correlations were used to evaluate the validity of urinary fructose, urinary sucrose, and urinary total sugars as objective indicators of dietary added sugar and total sugar intake. Urinary excretion of each sugar was compared to dietary intake of the corresponding sugar and dietary added sugar intake from corresponding days during each controlled feeding period, and the amount of dietary sugar intake recovered through urinary excretion was calculated. The relationships between urinary excretion of each sugar and dietary added sugar or total sugar intake were also plotted. Regression equations were developed to predict dietary added sugar and total sugar intake from urinary total sugar excretion based on data from the 25% added sugar feeding period, due to its validity. Bland-Altman plots were used to analyze the agreement between sugar intake predicted from urinary sugar excretion and actual sugar intake. Finally, a paired t-test was used to evaluate how urinary sucrose, fructose, and total sugars biomarkers respond to changes in dietary added sugar intake. Urinary excretion of sucrose, fructose, and total sugars during the low added sugar feeding period was compared to excretion during the high added sugar feeding period.

Evaluating Reliability. Intra-class correlations were used to evaluate the reliability of the percentage of urinary sodium recovered from the four diet days corresponding to 24-hour urine collection. The percentage of recovery was used to account for differences in the sodium content for each diet day. Intra-class correlations and paired t-tests (between Day 6 and Day 7) were used to evaluate the reliability of urinary fructose, sucrose, and total sugars when dietary added sugar intake was held constant. Fructose and sucrose excretion were only assessed for subjects with two complete 24-hour urine samples collected during the same controlled feeding period.

RESULTS

As shown in Figure 2, 58 adolescents were assessed for eligibility, 33 were enrolled and then randomized to a dietary sequence, and 32 completed both controlled feeding periods; 1 subject was disqualified during the study due to poor compliance with the controlled diet (Figure 2). Another subject repeated one of the controlled feeding periods (following a second washout period) after reporting non-compliance following completion of the first attempt. Participant demographic characteristics, estimated energy needs, and average baseline self-reported dietary intake are provided in Table 3. The population was representative of both genders and all ages within the 12-18 year-old range, in addition to being white, primarily healthy according to BMI-for-age percentile, and low-active according to PAQ-A scores (Table 3). Self-reported dietary intake was not significantly different between baseline and the washout period values reported here, with respect to energy intake (2475±604kcal, p=0.663), macronutrient distribution (carbohydrate: 50.3±4.9%, p=0.642, protein: 15.8±3.3%, p=0.064, fat: 33.8±4.4%, p=0.472), added sugar intake as a percentage of total energy intake (12.4±4.4%, p=0.832), and sodium intake (3757±1310mg, p=0.713). However, average self-reported caloric intake from baseline and washout differed significantly from average caloric intake during the controlled feeding

periods (2796±624kcal, p=0.003). Figure 3 shows the Bland-Altman plot for the full study sample; subjects reported consuming 89% of their measured dietary intake (about 300 calories less) from both controlled feeding periods, which were isocaloric as evidenced by weight stability. Subject weight did not change significantly from the beginning to the end of either feeding period (5% AS: -0.063±0.69kg, p=0.613; 25% AS: -0.016±0.58kg, p=0.879), and weight change did not differ between feeding periods (p=0.879). Recorded intake data from the feeding period indicates that subjects were compliant with both diets, consuming 99% (5% AS) and 98% (25% AS) of the food and beverage items researchers intended for them to consume during each feeding period, with no difference in dietary compliance between feeding periods (p=0.107).

Urinary creatinine excretion, the indicator of urine collection compliance, was strongly correlated with measures of body weight (r=0.867, n=29, p<0.001) and height (r=0.755, n=29, p<0.001) for the full sample during both feeding periods (creatinine data was missing for n=3). For body weight, the correlation with creatinine excretion became slightly stronger when the sample was restricted to participants with two complete urine samples during the 5% AS feeding period (r=0.916, n=25, p<0.001) and 25% AS feeding period (r=0.951, n=26, p<0.001), and slightly weaker when the sample was restricted to participants with at least one complete 24-hour urine collection during the 5%AS feeding period (r=0.845, n=30, p<0.001) and the 25% AS feeding period (r=0.849, n=29, p<0.001). For height, the correlation with creatinine excretion varied slightly when the sample was restricted to participants with two complete urine samples during the 5% AS feeding period (r=0.682, n=25, p<0.001) and 25% AS feeding period (r=0.760, n=26, p<0.001), and when the sample was restricted to participants with at least one complete 24-hour urine collection during the 5%AS feeding period (r=0.732, n=30, p<0.001) and the 25% AS feeding period (r=0.720, n=29, p<0.001). Intra-class correlation coefficients (ICC)

for urinary creatinine excretion for the full sample (n=29, 4 items) indicate good to excellent[42] test-retest reliability (ICC=0.887, 95% CI=0.802 – 0.942, p<0.001). Reliability was similar when the sample was restricted to participants with two complete 24-hour urine samples during the 5% AS feeding period (n=25, 2 items, ICC=0.892, 95% CI=0.753 – 0.952, p<0.001) and the 25% AS feeding period (n=26, 2 items, ICC=0.979, 95% CI=0.921 – 0.992), p<0.001). ICC estimates and their 95% confident intervals were calculated based on an average measures, absolute-agreement, 2-way mixed-effects model.

Biomarker Validity. Dietary sodium intake was recovered through urinary sodium excretion at rates of 85-90% for each feeding period as seen in Table 4, including the full sample and the sample restricted to participants with complete 24-hour urine collections. The average amount of dietary sodium intake recovered through urinary sodium excretion did not differ significantly from the expected recovery of 90% for the full sample (87.9±17.8%, p=0.502), or any subset of the data (urine collection completeness, PAQ-A physical activity status, age) with the exception of gender; females recovered significantly more sodium (95.5±10.5%, p=0.045) as seen in Table 5. Males recovered significantly less urinary sodium than females (79.2±20.6%, p=0.012), but this only remained significant for the 5% added sugar feeding period (Day 6: p=0.009; Day 7: p=0.010). Dietary sodium intake was significantly different between controlled feeding periods (5%: 3551±691mg; 25%: 3657±724mg; p<0.001), but urinary sodium excretion was not significantly different between feeding periods for the full sample (5%: 2995±748mg; 25%: 3280±1052mg; p=0.089) or the sample restricted to participants with four complete 24-hour urine samples (5%: 3077±727mg; 25%: 3461±1060mg; p=0.063).

Average 24-hour urinary nitrogen excretion was significantly correlated to average 24-hour dietary protein intake for the full sample (n=29, r=0.694, p<0.001) and every subset of the

data, regardless of grouping by urine collection completeness, yielding moderate to strong r-values (Table 6). However, the average amount of dietary nitrogen recovered through urinary nitrogen excretion was significantly different from the expected excretion of 80% for the full sample (62.2±6.8%, $p<0.001$), and every subset of the data, regardless of grouping by urine collection completeness, physical activity status, gender, or age, as seen in Table 7. The correlation between dietary protein intake (g/kg body weight) and urinary nitrogen recovery (%) was significant and negative for the full sample (n=28, r=-0.468, p=0.012), and this correlation was stronger for males (n=12, r=-0.564, p=0.056) than females (n=16, r=-0.162, p=0.548). To determine if the negative correlation was due to growth, height change between baseline and the washout period was evaluated. The correlation between height change (cm) and urinary nitrogen recovery (%) was significant and negative for the full sample (n=28, r=-0.470, p=0.012). This correlation was also significant for males (n=12, r=-0.666, p=0.018), who averaged a height increase of 0.53±0.53cm, but it was not significant for females (n=16, r=-0.232, p=0.387), who averaged a height increase of 0.23±0.60cm. Although dietary nitrogen intake was not significantly different between controlled feeding periods (5%: 17.1±3.6g, 25%: 16.9±3.7, p=0.322), urinary nitrogen excretion (5%: 9.9±2.1g/d, 25%: 11.1±2.5g/d, $p<0.001$) and the percentage of dietary nitrogen recovered as urinary nitrogen (5%: 58±7%, 25%: 66±8%, $p<0.001$) were significantly lower during the 5% added sugar feeding period. In addition, dietary fiber intake, which may affect nitrogen excretion, was significantly different between controlled feeding periods (5%: 35.5±7.8g/d, 25%: 22.1±4.5, $p<0.001$) and between males (5%: 42.4±5.6g/d, 25%: 25.3±3.8g/d) and females (5%: 30.6±3.8g/d; 25%: 19.1±2.0g/d) for both controlled feeding periods ($p<0.001$).

During the 5% added sugar feeding period, urinary sucrose, fructose, and total sugars excretion were not correlated with dietary intake of the corresponding sugar (Table 8), or with added sugar intake (Table 9). However, during the 25% added sugar feeding period, all urinary sugar measures yielded significant correlations with dietary intake of the corresponding sugar (Table 8) as well as added sugar intake (Table 9). Average urinary sugar excretion (sucrose, fructose, total sugars) for each controlled feeding period and the percentage of dietary sugar intake recovered through urinary excretion are presented in Table 10, including results for the full sample and subsets based on urine collection completeness. Urinary total sugars recovery varied by sex (F: 0.0002±0.00005%, M: 0.0001±0.00004%, p=0.003) but not age (≥15y: 0.0002±0.00006%, <15y: 0.0001±0.00004, p=0.194). Graphical representations of the relationships between urinary excretion of each sugar, dietary added sugar intake, and total sugar intake are depicted in Figures 4-9. All slopes were significantly different from zero ($p<0.001$) and R^2 values for the comparisons between urinary sugar excretion and dietary added sugar intake showed a better fit with the linear regression equation. Data from the 25% added sugar feeding period was used to develop regression equations for predicting added sugar intake ($y=126.108 + 132.3x$, $r^2 = 0.296$, $p=0.001$) and total sugar intake ($y=154.610 + 171.510x$, $r^2 = 0.298$, $p=0.001$) from urinary total sugar excretion. Bland-Altman plots (Figures 10-11) analyze the agreement between sugar intake predicted from urinary sugar excretion and actual sugar intake. Urinary sucrose, fructose, and total sugars were responsive to the change in dietary intake for both the full sample and subsets based on urine collection completeness (Table 11). For the full sample, significant difference was evident between controlled feeding periods for urinary sucrose excretion (5%: 0.016±0.01mg/d, 25%: 0.034±0.03mg/d, p=0.001), urinary fructose

excretion (5%: 0.209±0.09mg/d, 25%: 0.331±0.15, p<0.001), and total sugars excretion (5%: 0.226±0.37, 25%: 0.365±0.16, p<0.001).

Biomarker Reliability. Intra-class correlation coefficients for urinary sodium recovery of the full sample (n=32, 4 items) indicate moderate[42] test-retest reliability (ICC=0.523, 95% CI=0.216 – 0.737, p=0.001). Sodium recovery for the full sample was more reliable during the 25% added sugar diet (ICC=0.490, 95% CI=-0.015–0.748, p=0.010) than the 5% added sugar diet (ICC=0.201, 95% CI=-0.334 – 0.562, p=0.204). As seen in Table 12, the intra-class correlation coefficient for urinary sucrose excretion on the 5% added sugar diet indicates poor[42] test-retest reliability (n=24, ICC=0.011, 95% CI=-1.089 – 0.554, p=0.488). Continuing on the 5% added sugar diet, intra-class correlation coefficients for urinary fructose excretion (n=25, ICC=0.501, 95% CI=-0.043 – 0.772, p=0.027) and urinary total sugars excretion (n=24, ICC=0.521, 95% CI=--0.030 – 0.786, p=0.029) indicate moderate [42] test-retest reliability. For the 25% added sugar diet, urinary sucrose excretion (n=25, ICC=0.665, 95% CI=0.234 – 0.853, p=0.005) indicates moderate[42] test-retest reliability, whereas urinary fructose excretion (n=27, ICC=0.798, 95% CI=0.409 – 0.919, p<0.001) and urinary total sugars excretion (n=25, ICC=0.837, 95% CI=0.520 – 0.936, p<0.001) indicate good[42] test-retest reliability.

DISCUSSION

This investigation represents the first controlled feeding study in adolescents to establish urinary sodium urinary sodium, nitrogen, sucrose, fructose, and total sugars as objective indicators of dietary sodium, protein, and added sugar intake. Our findings indicate that urinary sodium is a valid indicator of dietary sodium intake for adolescents since excretion was not significantly different from the expected excretion rate of 90%[16] of dietary sodium intake. Urinary nitrogen excretion was significantly different from the expected excretion of 80% of

dietary nitrogen intake,[20,21] but it may still have potential as a protein intake biomarker. Urinary sucrose, fructose, and total sugars excretion are correlated with dietary added sugar intake at higher levels of added sugar intake, so urinary sugars appear to be a valid biomarker at high added sugar intake levels within our target population. Urinary sugars also appear to be a better biomarker for dietary added sugars than dietary total sugars, although our study was not designed to determine this outcome. Finally, urinary sugars are reliable at higher levels of intake and they are responsive to changes in dietary added sugar intake.

A study examining self-reported dietary sodium intake and urinary sodium excretion in children and adolescents reported median sodium intakes between 1968mg/d and 3059mg/d for male and female subsets between 10-18 years old, and corresponding median sodium excretion between 2170mg/d and 3190mg/d,[18] which is comparable to our findings. Male subjects may have recovered less sodium due to poorer compliance with the 24-hour urine collections, increased sweat excretion due to physical activity that was not captured by the PAQ-A, or increased sweat excretion due to differences in body surface area or seasonality. During the 5% added sugar diet, 47% of the female subset completed urine collections during the spring and summer months compared to 73% of male subjects. During the 25% added sugar diet, 53% of the female subset completed urine collections during the spring and summer months compared to 73% of male subjects. Finally, our findings suggest that urinary sodium recovery was only moderately reliable when all four samples for each participant were included, further justifying the need for multiple 24-hour collections.

The urinary nitrogen excretion reported (about 62%) was significantly different from the expected excretion of 80% of dietary nitrogen intake in adults.[20,21] Previous studies in adolescents reveal similar, but slightly lower values for 24-hour urinary nitrogen excretion

(following conversion of reported amounts to grams per day), along with substantially lower estimates of dietary protein intake.[23,43] However, these studies relied upon three-day weighed food records, so protein intake estimates may be under-reported.[20] If dietary protein intake is under-reported and urinary nitrogen excretion is valid and reliable, then the amount of dietary nitrogen recovered as urinary nitrogen would be falsely inflated, making data appear more comparable to adult levels of nitrogen recovery.[21,44]

Lower levels of nitrogen recovery overall can be attributed to the positive nitrogen balance in growing children and adolescents.[23] A study in adolescent sprint athletes indicated that 1.46g/kg/d of protein intake in girls and 1.35g/kg/d of protein in boys was adequate to achieve positive nitrogen balance, regardless of growth or increases in fat-free mass, but dietary intake was self-reported.[45] During the current study, only one female subject consumed less protein than this amount (1.43g/kg/d) and average protein intake across all subjects was 1.85±0.25g/kg/d for urine collection days, indicating that study participants were likely in positive nitrogen balance. The association between higher levels of protein intake (g/kg body weight) and lower levels of urinary nitrogen recovery (%) supports this idea, and suggests that some subjects were in greater positive nitrogen balance than others. Subjects with the highest protein-calorie requirements were likely experiencing the most rapid growth and development, so although they were consuming more nitrogen, they were excreting less, relative to their dietary intake. Peak growth typically occurs between ages 11-14 for females and ages 15-18 for males,[46] so the age range we recruited (12-18 years) overlapped peak growth rates, particularly for our male participants, who experienced more vertical growth during the study. This accumulation of lean mass partially explains why males recovered less nitrogen; females also may have excreted additional nitrogen if they were menstruating during the urine collections.

Younger children may have recovered less nitrogen due to high growth rates or poorer compliance with the 24-hour urine collections.

The difference in urinary nitrogen excretion and nitrogen recovery between feeding periods could be attributed to the dietary fiber content of each diet. Dietary fiber intake can increase the amount of nitrogen excreted in the feces relative to urinary excretion.[21,24,25] A controlled feeding study conducted on four medical school students showed an increase in fecal nitrogen excretion from 1.4 grams per day to 2.49 grams per day with the addition of 30 grams of dietary fiber to the diet.[24] Studies in pigs explain this mechanism; since fermentable carbohydrates provide energy to gut microflora, dietary intake stimulates the transfer of urea from the blood to the large intestine for use in microbial protein synthesis prior to excretion in the feces,[47,48] which would subsequently result in less urinary nitrogen excretion. As a result, consumption of 36 grams of dietary fiber during the 5% added sugar feeding period resulted in significantly less urinary nitrogen excretion than consumption of 22 grams of dietary fiber during the 25% added sugar feeding period. Furthermore, since males consumed more dietary fiber than their female counterparts on both controlled diets (as a result of higher estimated energy needs), they could have excreted even more nitrogen in the feces relative to the urine.

Strong correlations between excretion of urinary sugars (sucrose, fructose, and total sugars) and 25% dietary added sugar intake demonstrate that urinary sugar biomarkers are likely a valid measure of added sugar intake for adolescents, who consume over 16% of their energy from added sugars.[6] Urinary sugars also appear to be a better biomarker for dietary added sugars than dietary total sugars due to slightly higher R-squared values indicating a better fit with the linear regression equation, although this cannot be confirmed with the current study design. Upon graphing the relationships between urinary sugar excretion and dietary added and total

sugar intake the slopes were significantly different from zero indicating some degree of sensitivity. However, the regression equations for predicting dietary added sugar and total sugar intake from urinary total sugars excretion indicate urinary sugar excretion have low predictive value, only accounting for 29.6% (added sugars) and 29.8% (total sugars) of the variation around the mean values. Furthermore, urinary sugars biomarkers may not be valid indicators of dietary added sugar intake at lower levels of intake (5% added sugar) since sucrose and fructose are already excreted in such small amounts.[26] Urinary sugar excretion values for the current study were much smaller than prior research suggests, relative to our controlled dietary intake levels of 5% and 25% added sugar (23% and 31% total sugar). One controlled feeding study in adults found a urinary total sugars excretion of 98.3mg/d (0.21%) in response to an 18% added sugar diet and 29% total sugars diet.[28] Another controlled feeding study in adults found excretion of 10.3mg/d total sugars (0.61%), 8.3mg/d sucrose (0.26%), and 2mg/d fructose in response to a 10% total sugar diet, and 42.4mg/d total sugars (0.34%), 25.9mg/d sucrose (0.21%), and 16.5mg/d fructose in response to a 22% total sugar diet.[49] Fructose intake was not reported.[49] A third controlled feeding study in adults found similar values, including excretion of 69.4mg/d total sugars, 21.4mg/d sucrose (0.01% recovery), and 48.0mg/d fructose (0.03% recovery) at a dietary intake of 30% total sugars.[29] The only study known to assess fructose excretion in pre-pubertal males and females was not a controlled feeding study, but sugar intake was estimated at 15% added sugar and 23% total sugar, yielding urinary fructose excretion of 19.8mg/d in males, and 20.7mg/d in females.[50] Fructose intake was not estimated from the weighed food records.[50] These studies analyzed urinary sugars using enzymatic analysis[28,49,50] or a combination of enzymatic analysis and mass spectrometry.[29] A previous study validated the detection and quantification of urinary sucrose via LC/MS analysis using a similar method, and found intra-

batch precision (%CV) ranging from 2-11% and inter-batch precision of 4-10% using a sequence of known concentrations (0, 2µM, 20µM, 70µM, 450µM).[51] The highest concentrations of urinary sucrose (70µM and 450µM) were within 1% and 2%, respectively, of their corresponding theoretical results, whereas the lowest concentrations (2µM and 20µM) were within 15% and 7% of their respective accurate values.[51] Lower values in our adolescent population could also be a result of reduced intestinal permeability relative to adults. Aging is associated with deteriorating intestinal barrier function in aged monkeys, independent of dietary intake, and these results may apply to aging humans.[52] If intestinal permeability increases with age, fewer sugars would be able to leak out of the adolescent gut, subsequently decreasing urinary excretion. Participants age 15 and older likely excreted more urinary total sugars due to higher calorie assignments yielding higher total sugars intake, particularly since there were no difference by age for urinary sugars recovery, which factors in dietary intake. It has been reported that urinary sugars excretion was unaffected by age, gender, race, BMI, or renal function in healthy participants,[53] so male participants likely recovered less urinary sugars than females due to poorer compliance with the urine collections, particularly since male participants also recovered less urinary sodium and nitrogen. The responsiveness of urinary sucrose, fructose, and total sugars to a dietary change demonstrates the potential for these biomarkers to serve as objective indicators of dietary added sugar; however additional research is needed to determine the sensitivity of urinary sugars between levels of 5% and 25% of added sugar intake. Less reliability at the lower level of added sugar intake may again be a result of the already low levels of excretion,[26] in combination with the many factors known to affect intestinal permeability (e.g. inflammation, gut microbiota,[54] medication use, running[55]). However, good reliability at a high level of added sugar consumption is promising, given the current dietary intake of adolescents.[6]

The validation of urinary sodium, urinary nitrogen, and urinary sugars as objective indicators of dietary intake in adolescents is a novel topic. A limited amount of research has focused on this population even though adolescent obesity rates continue to increase,[1] adolescents are the highest consumers of added sugars,[6] and targeting overweight and obese adolescents appears to protect their cardiometabolic health.[56,57] Additional strengths include the use of a randomized controlled crossover feeding approach with a high completion rate, a metabolic kitchen for food preparation, and the combination of subjective and objective measures to indicate compliance with the controlled diets and 24-hour urine collections. Collecting two 24-hour urine samples per feeding period was also a strength, since two to three 24-hour urine collections yield a reliability index of 0.8 for most biomarkers.[58] Furthermore, urinary biomarkers are minimally invasive, urinary sugar measures reflect all sources of dietary sugar,[15] and multiple biomarkers can be assessed from the same urine sample. Finally, subjects arrived fasted each morning (for fingerstick blood samples related to other study objectives), which made urine collections more likely to reflect all nutrients consumed during each 24-hour collection. Since sodium intake for a given day is thought to be excreted within the next 18-31 hours,[16] fasting after dinner and overnight before excreting the final urine sample the next morning improves the ability of a 24-hour urine sample to reflect all sodium consumed the previous day, although this is still a limitation.

Regarding other limitations, the study population lacks diversity, so additional research is needed to determine if these findings apply to racially diverse and obese adolescents. Diet calculations may be limited by the potential inaccuracy of values representing the metabolizable energy of macronutrients,[35] and the need to calculate sodium intake from package labels. Urinary measurement error is possible, including the subtraction of the thymol preservative from the total

urine volume for measures of urinary sodium, nitrogen, and creatinine, and the inability to use PABA to determine sample completeness. Seasonality was not accounted for during scheduling, which may have unsystematically impacted the amount of sodium excreted through sweat. In addition, height was only measured at baseline and washout as opposed to throughout the duration of the study, and feces were not collected to confirm that dietary fiber intake shifts nitrogen excretion from the urine to the feces. Finally, the need for multiple 24-hour urine collections is burdensome to subjects which could have impacted compliance; however, spot urine samples are being evaluated for validity,[15,27,16,40] which would reduce subject burden in future studies.

CONCLUSION

Consistently high levels of sodium intake in this non-obese, adolescent population justify the need for accurate measures of sodium intake assessment. Urinary sodium was validated as an objective indicator of dietary sodium intake for adolescents across the age range and physical activity levels of the study population, although older adolescents (15-18 years) and females may be more capable of collecting 24-hour urine samples, and multiple collections are likely needed as a result of moderate reliability. Strong correlations between urinary nitrogen excretion and dietary protein intake suggest that urinary nitrogen may be a valid biomarker for protein intake in some populations, but the varying growth rates of adolescents put them in positive nitrogen balance, decreasing the percentage of dietary nitrogen recovered through urinary excretion. The extent of positive nitrogen balance remains unknown, but it appears to vary by gender in accordance with the timing of peak growth and the amount of lean body mass accrued. Furthermore, a 14 gram difference in dietary fiber intake significantly impacted urinary nitrogen excretion, meaning that dietary fiber intake would have to be accounted for when estimating

dietary protein intake using the urinary nitrogen biomarker. Therefore, the urinary nitrogen biomarker may not be suitable for determining dietary protein intake in an adolescent population given the challenge of establishing prediction equations that are specific to gender and growth rate, paired with the current lack of understanding regarding how dietary fiber intake (amount, type, etc.) impacts urinary nitrogen excretion, which has implications beyond an adolescent population. Urinary fructose, sucrose, and total sugars biomarkers appear to be more valid at 25% added sugar intake, and they may be a better indicator of dietary added sugar intake than total sugar intake. Only fructose and total sugars excretion can be considered reliable at levels of 5% added sugar intake, but all sugars were reliable at 25% added sugar intake. All urinary sugars measured were responsive to the 20% change in dietary added sugar, but the sensitivity of each urinary sugar biomarker to smaller dietary changes has not yet been established and predictive value appears low. Further research is warranted to a) confirm the validity of urinary sodium as an objective indicator of sodium intake, b) examine the effects of adolescent growth rate and dietary fiber intake (type, amount, etc.) and gender differences on urinary nitrogen excretion, c) validate urinary sucrose, fructose, and total sugars biomarkers at lower levels of added sugar intake (<25% AS), and d) determine the sensitivity of urinary sugar biomarkers to smaller dietary changes, all with larger, more diverse samples.

FIGURES

Figure 1. Schedule of study visits.

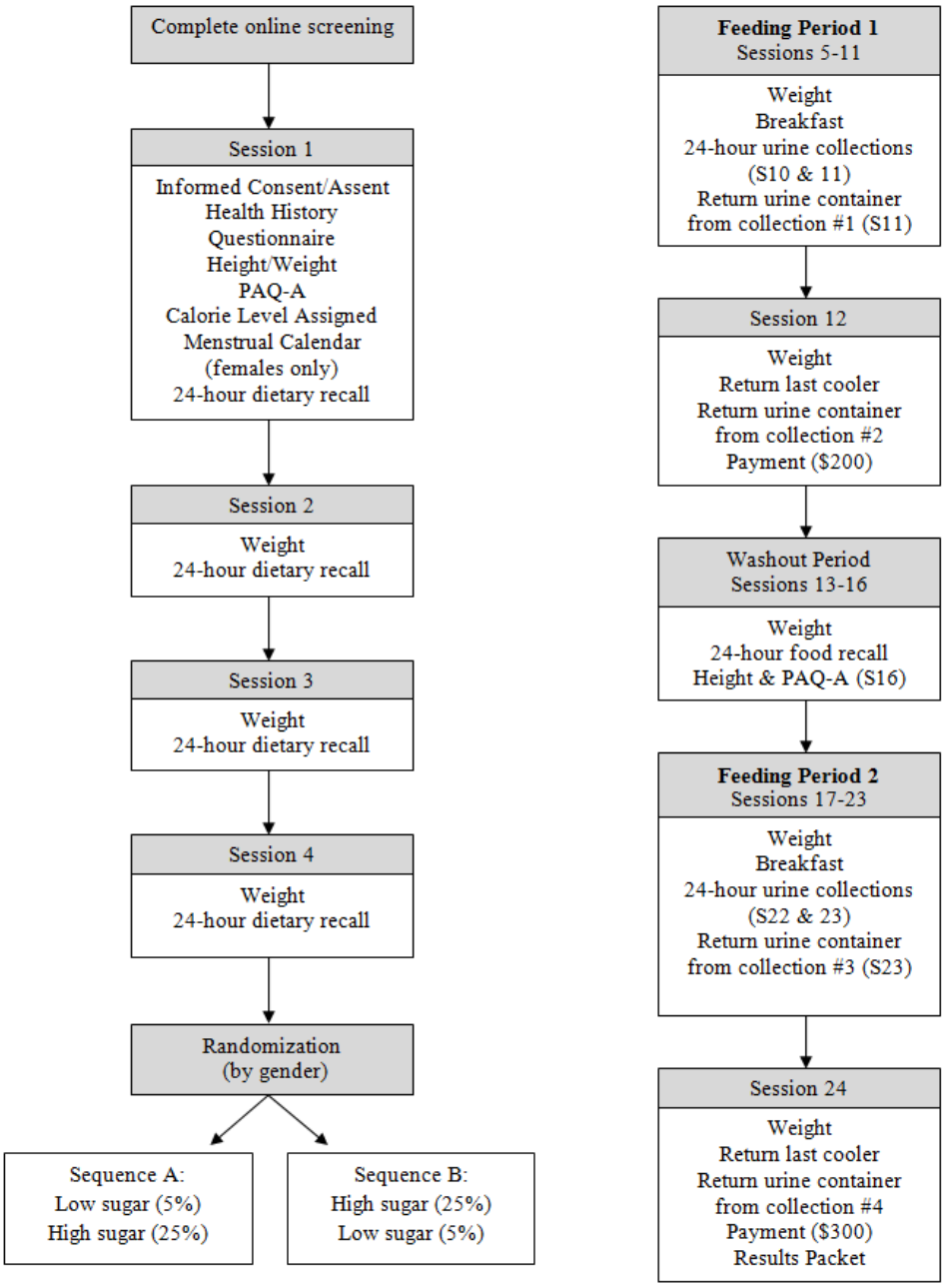
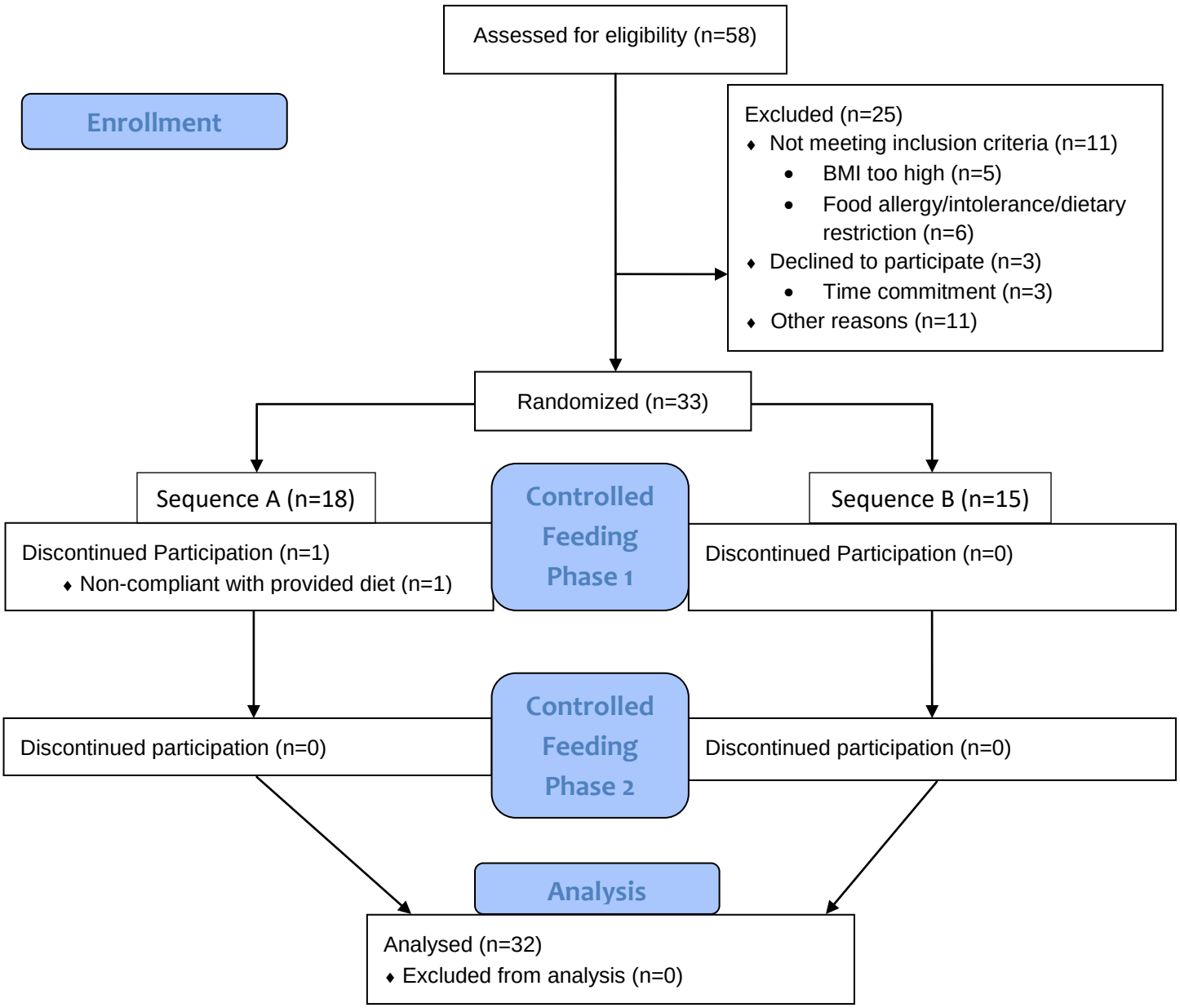


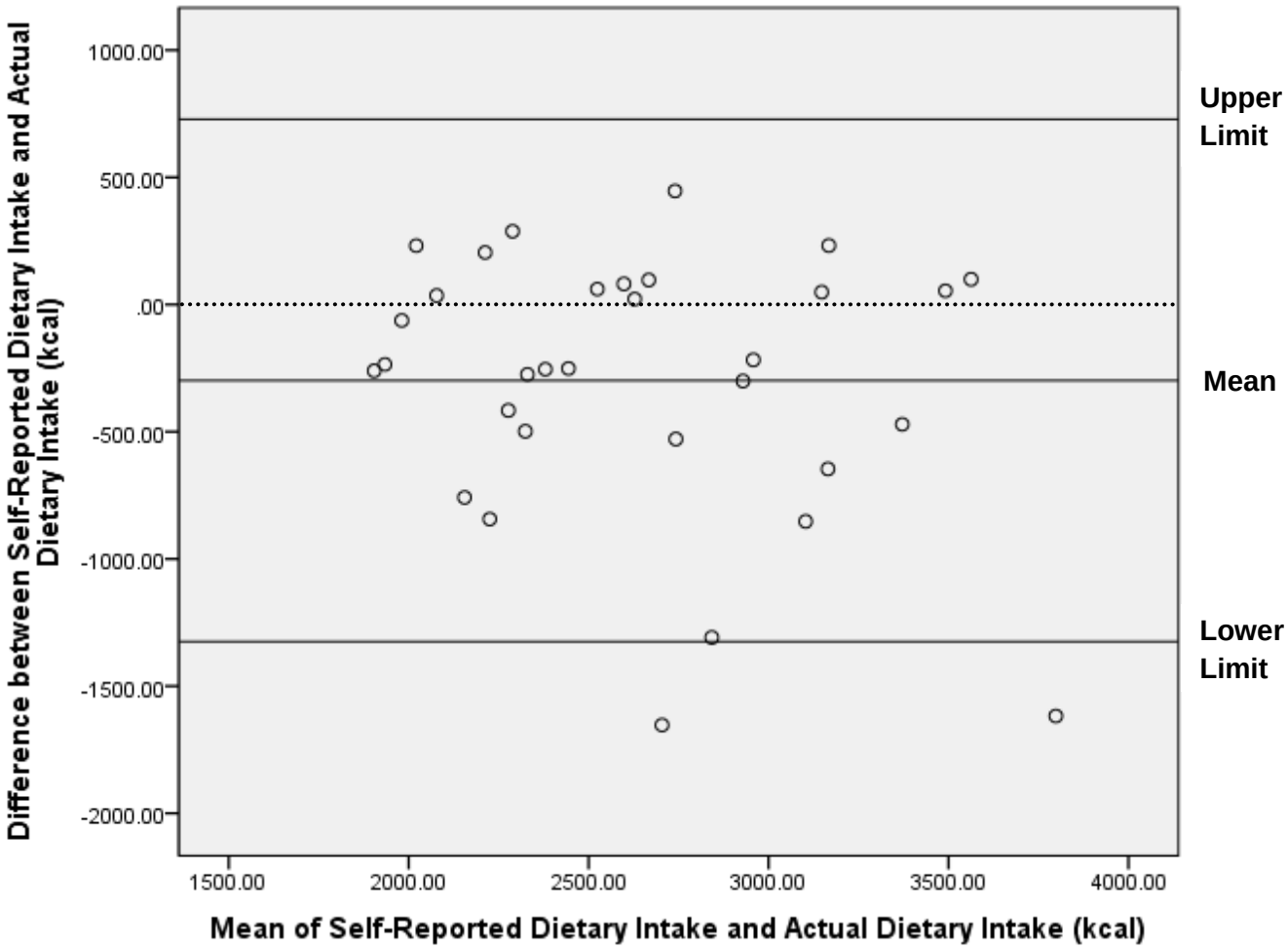
Figure 2. CONSORT Diagram



Sequence A = Low-added sugar diet in controlled feeding phase 1 and high-added sugar diet in phase 2

Sequence B= High-added sugar diet in controlled feeding phase 1 and low-added sugar diet in phase 2

Figure 3. Bland Altman Plot of caloric intake for the full study sample.



Caloric intake data are presented for the full study sample (n=32). The horizontal dotted line (y=0) represents ideal agreement, where the difference between self-reported caloric intake and actual caloric intake equals zero. The inside solid line represents the mean difference between measures (mean=-298.5kcal), and the upper and lower solid lines indicate the upper and lower 95% limit of agreement (±1.96 x SD) where SD= 523.7.

Figure 4. The relationship between urinary sucrose excretion and total sugar intake.

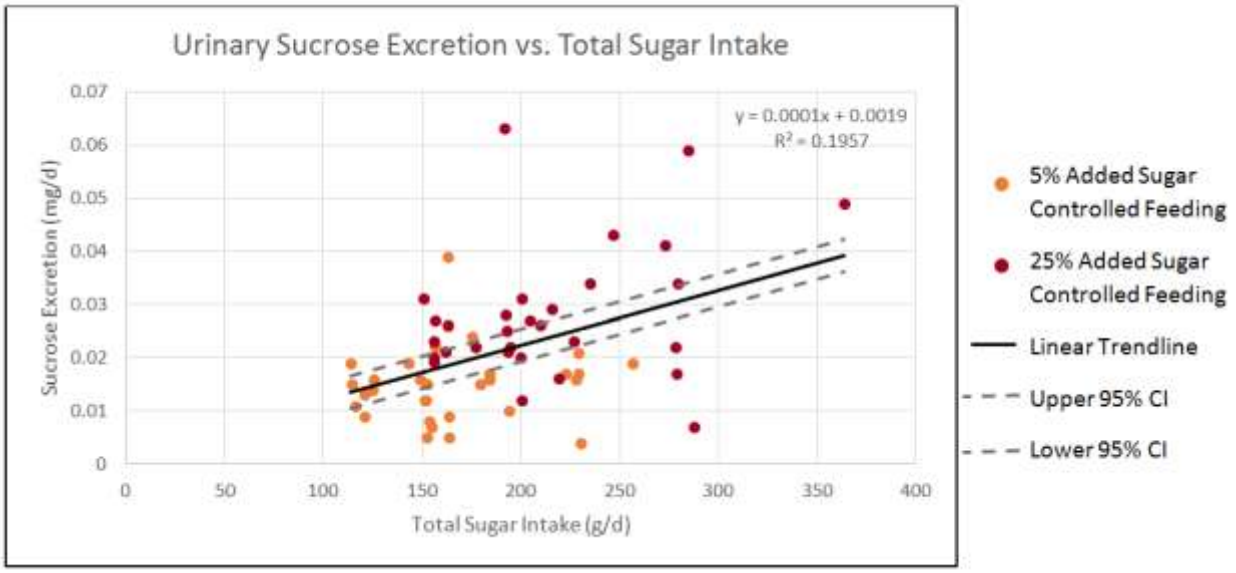


Figure 5. The relationship between urinary sucrose excretion and added sugar intake.

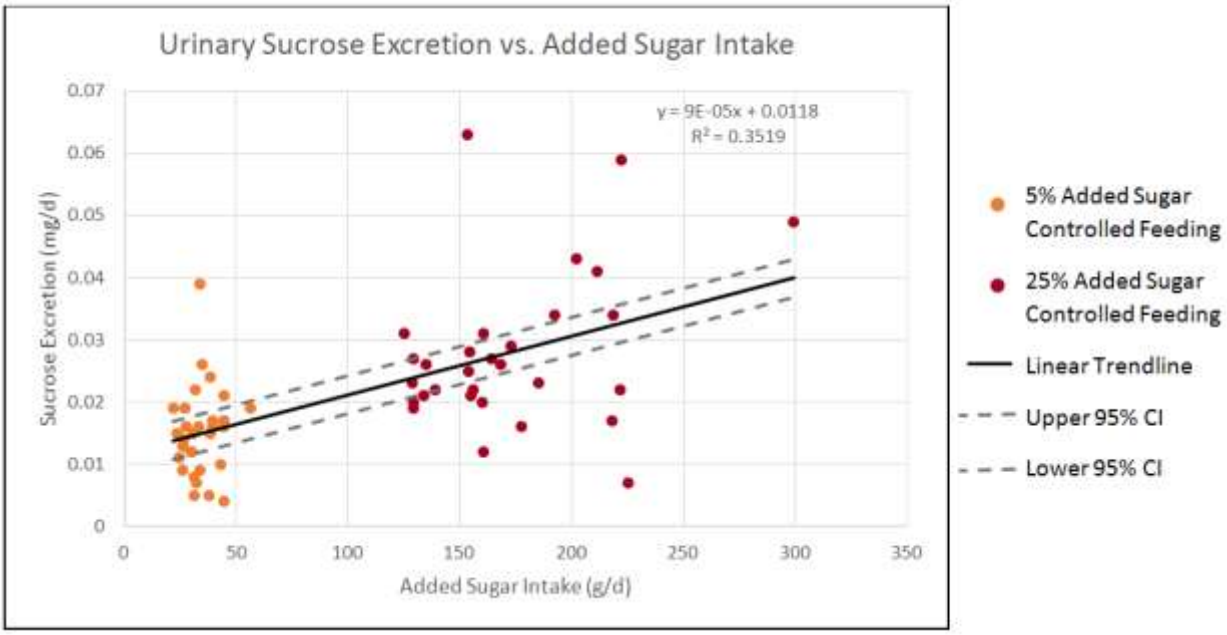


Figure 6. The relationship between urinary fructose excretion and total sugar intake.

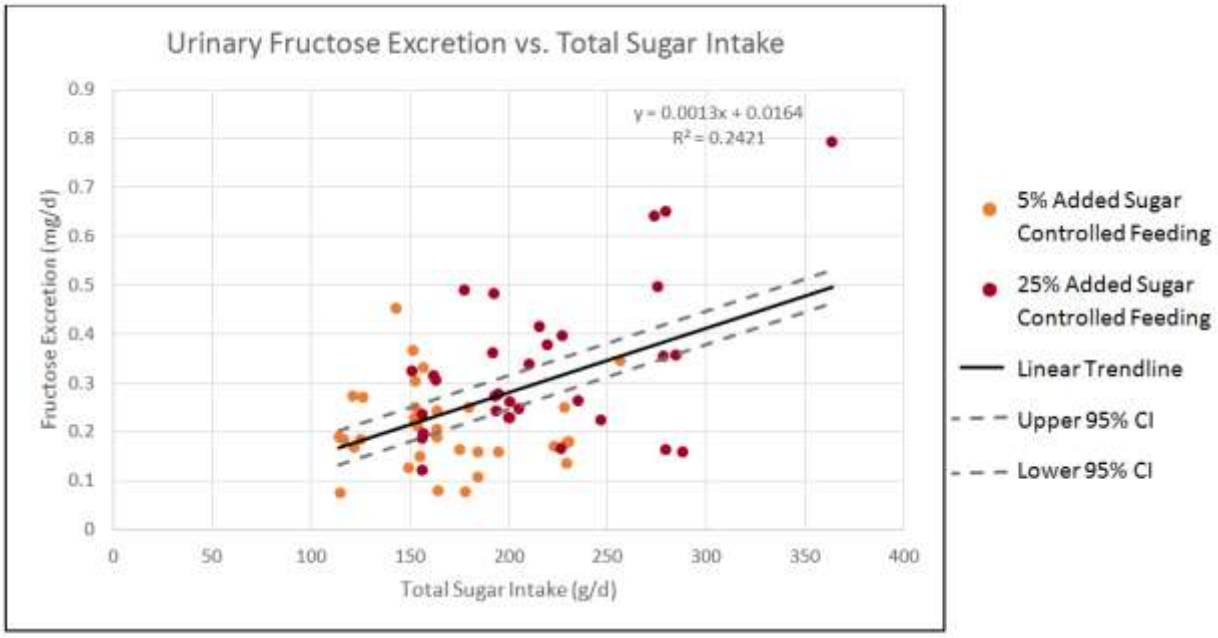


Figure 7. The relationship between urinary fructose excretion and added sugar intake.

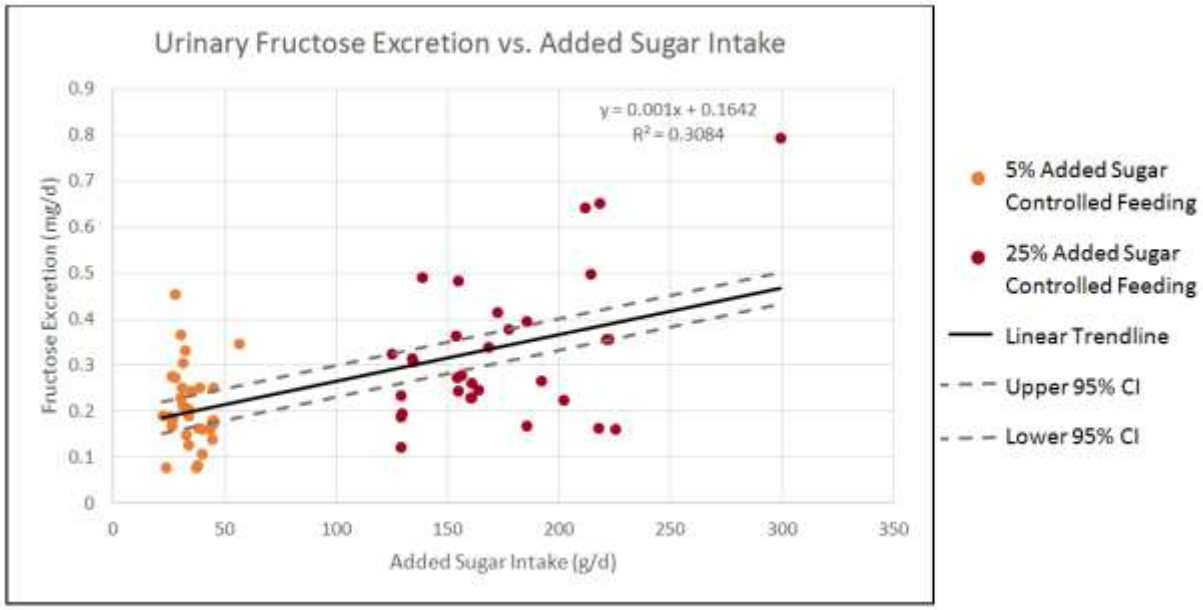


Figure 8. The relationship between urinary sucrose and fructose (total sugar) excretion and total sugar intake.

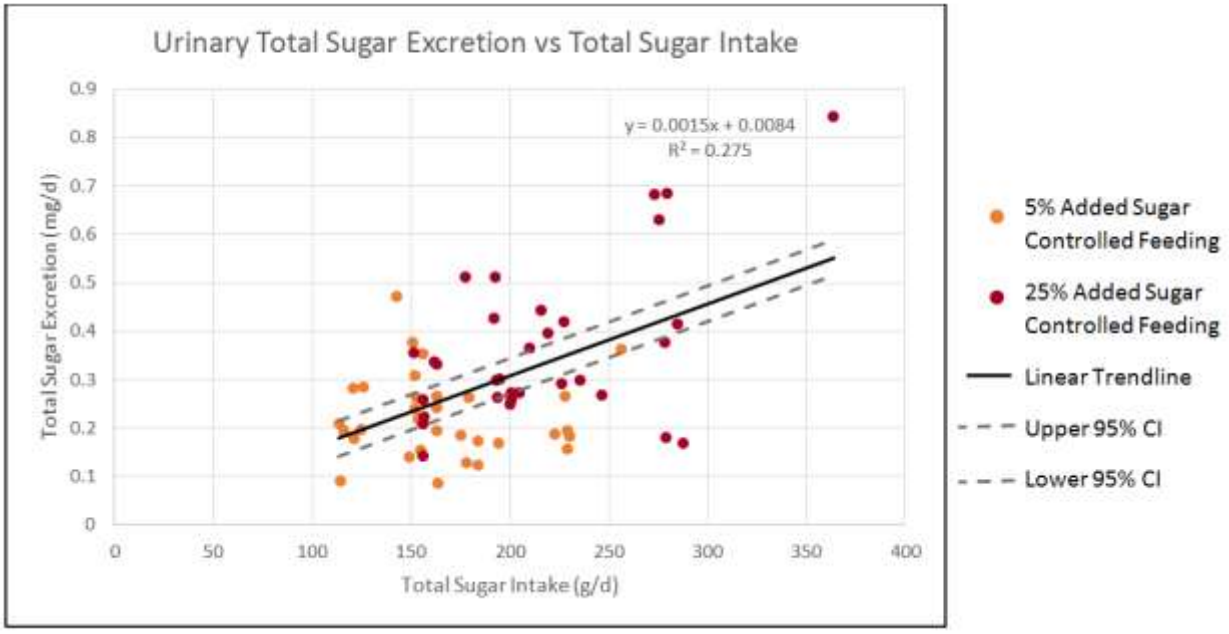


Figure 9. The relationship between urinary sucrose and fructose (total sugar) excretion and added sugar intake.

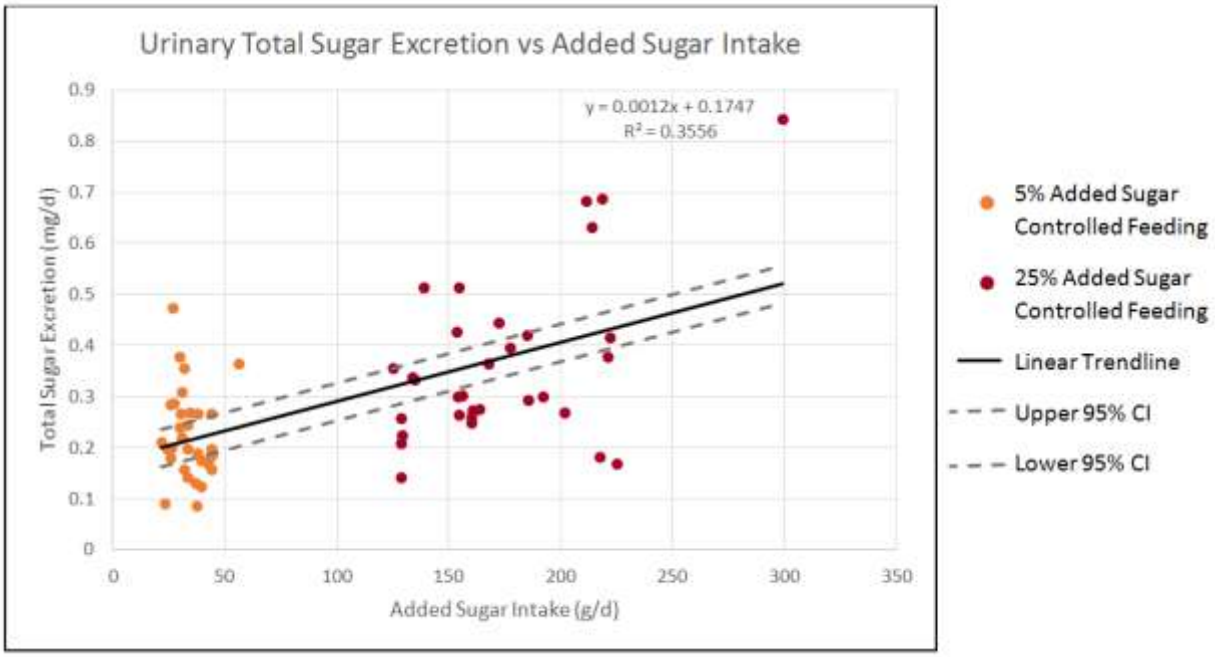


Figure 10. Bland-Altman plot comparing predicted added sugar intake from urinary total sugar excretion with actual added sugar intake during the 25% added sugar feeding period.

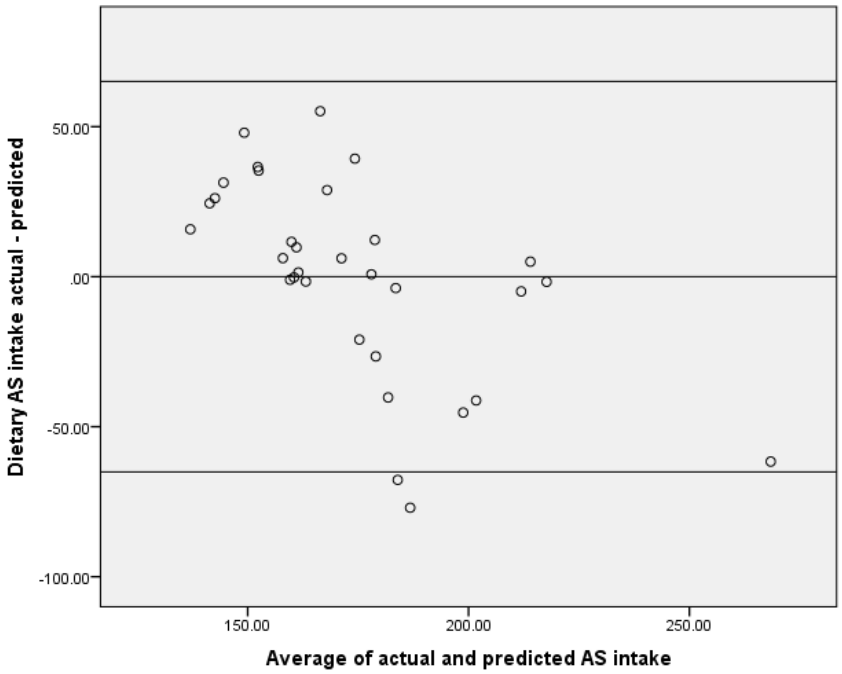
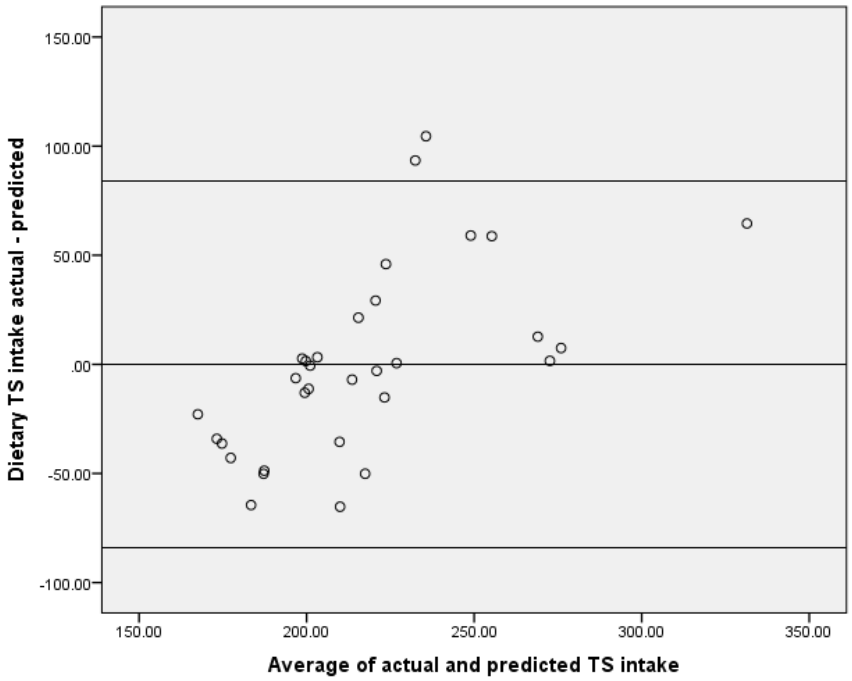


Figure 11. Bland-Altman plot comparing predicted total sugar intake from urinary total sugar excretion with actual total sugar intake during the 25% added sugar feeding period.



TABLES

Table 1. Nutrient composition of the 7-day controlled feeding period diets.

Nutrient	Quantity	
	5% Added Sugar	**25% Added Sugar**
Energy, kilocalories (range)	2000-4500	2000-4500
Carbohydrates (% kcal)	55	55
Added Sugar[a] (% kcal)	5	25
Liquid Added Sugar (%AS)	33	33
Solid Added Sugar (%AS)	67	67
Total Sugars[b] (% kcal)	23	31
Sucrose (%kcal)	6.1	16.5
Fructose (%kcal)	6.6	5.5
Fat (% kcal)	30	30
Protein (% kcal)	15	15
Animal Protein (% protein)	42	42
Dairy Protein (% protein)	20	20
Vegetable/Other (% protein)	38	38
Sodium (mg/day)	3549±745 (2713-5960)	3785±746 (2959-6170)

[a]The NDSR variable "Added Sugars by Total Sugars" was used[59]
[b]NDSR defines Total Sugars as the sum of glucose, fructose, galactose, sucrose, lactose, and maltose[59]

Table 2. UPLC-MS/MS transitions for detection of urine sugars

Compound	$[M–H]^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
glucose + fructose	178.6	88.8	18	8
sucrose	340.7	178.9	34	12
$^{13}C_6$-glucose	184.6	91.8	16	8

Table 3. Participant characteristics of the full sample.	
Participant Characteristics	**mean±SD (range)** (unless otherwise indicated)
Total number of participants, n	32
Gender Male, n (%) Female, n (%)	 15 (46.9%) 17 (53.1%)
Age (y) 12, n (%) 13, n (%) 14, n (%) 15, n (%) 16, n (%) 17, n (%) 18, n (%)	15.3 ± 1.6 (12-18) 2 (6.3%) 2 (6.3%) 4 (12.5%) 11 (34.4%) 6 (18.8%) 3 (9.4%) 4 (12.5%)
Race/Ethnicity White	 32 (100%)
BMI-for-age[a] (percentile) Obese, n (%) Overweight, n (%) Healthy Weight, n (%) Underweight, n (%)	47 ± 25% (2-93) 0 (0.0%) 1 (3.1%) 28 (87.5%) 3 (9.4%)
PAQ-A[b]	2.08±0.52 (1.09-3.19)
Estimated Energy Needs[c]	2916±695 (1939-4771)
Baseline Self-Reported Dietary Intake Energy (kilocalories) Added Sugar (% of total kcal) Sodium (mg) Carbohydrates (% of total kcal) Protein (% of total kcal) Fat (% of total kcal)	 2519±577 (1678-3774) 12.2±3.3 (6.2-18.2) 3820±1123 (1916-7082) 50.8±3.5 (43.1-58.3) 14.9±2.6 (9.4-18.8) 34.4±3.3 (27.0-40.0)
[a]According to the CDC, weight status categories are as follows[30]: obese (≥95th percentile), overweight (85th to <95th percentile), healthy weight (5th percentile to <85th percentile), underweight (<5th percentile) [b]The PAQ-A is scored on a scale of 1-5 (1=low physical activity, 5=high physical activity[32] [c]Energy needs were estimated based on IOM equations[31] and PAQ-A scores[32]	

Table 4. Percentage of dietary sodium recovered by urinary excretion.

	Full Sample Sodium Recovery [a] (%) n=32	**Two Complete 24-Hour Urine Samples** Sodium Recovery [b] (%) n=25 (5% AS) n=27 (25% AS)	**≥1 complete 24-Hour Urine Sample** Sodium Recovery [c] (%) n=30 (5% AS) n=31 (25% AS)
5% Added Sugar Feeding Period, mean±SD (range)	85.8±20.4 (45.6-120)	87.8±19.0 (45.6-111.8)	89.8±24.3 (25.3-129.2)
25% Added Sugar Feeding Period, mean±SD (range)	85.6±18.6 (30.3-112)	89.6±13.1 (68.4-111.8)	88.00±17.9 (31.9-114.6)

[a]Percentage of urinary sodium recovered on days 6 and 7 (or corresponding) of each controlled diet based on additive dietary compliance-adjusted sodium intake and additive, 24-hour time-adjusted sodium excretion for the full sample; sodium values were recorded from food labels.

[b]Percentage of urinary sodium recovered on days 6 and 7 (or corresponding) of each controlled diet based on additive dietary compliance-adjusted sodium intake and additive, 24-hour time-adjusted sodium excretion for subjects with two "complete" 24-hour urine samples per feeding period based on established criteria

[c]Percentage of urinary sodium recovered on days 6 and/or 7 (or corresponding) of each controlled diet based on additive dietary compliance-adjusted sodium intake and additive, 24-hour time-adjusted sodium excretion for subjects with at least one "complete" 24-hour urine sample per feeding period based on established criteria

Table 5. Percentage of dietary sodium recovered through excretion compared to expected excretion.				
	Average Percentage of Sodium Recovered (mean±SD)	**Percentage of Sodium Excretion Expected**[c]	**Paired T-Test p-value**[d]	**Independent Sample T-Test p-value**
Both feeding periods, full sample[a] (n=32)	87.9±17.8	90	p=0.502	
Male (n=15)	79.2±20.6	90	p=0.061	p=0.012*
Female (n=17)	95.5±10.5	90	p=0.045*	
Activity subgroups				
Both feeding periods, more physically active[b] participants (n=15)	88.0±21.8	90	p=0.733	p=0.942
Both feeding periods, less physically active[b] participants (n=17)	87.7±14.1	90	p=0.510	
Age subgroups				
Both feeding periods, older participants (≥15y, n=24)	89.7±17.5	90	p=0.928	p=0.327
Both feeding periods, younger participants (<15y, n=8)	82.4±18.9	90	p=0.294	
24-hour urine completeness subgroups				
5% AS, sample restricted to 2 complete 24-hr urine collections[e] (n=25)	91.6±21.7	90	p=0.716	p=0.587[g]
25% AS, sample restricted to 2 complete 24-hr urine collections[e] (n=27)	91.0±13.7	90	p=0.716	
5% AS, sample restricted to at least 1 complete 24-hr urine collection[f] (n=30)	89.8±24.3	90	p=0.973	
25% AS, sample restricted to at least 1 complete 24-hr urine collection[f] (n=31)	88.0±17.9	90	p=0.541	

[a] Average for all subjects regardless of urine sample completeness
Average of D6 and D7 Sodium Recovery for subjects with two "complete" 24-hour urine samples (n=25 for LAS, n=27 for HAS)
[b] Physical activity determined by average of PAQ-A scores from baseline and washout; participants with average PAQ-A score at or above mean sample PAQ-A score of 2.08 were considered more physically active
[c] 90% excretion was expected[16]
[d] Paired t-tests between average percentage of sodium recovered and 90% expected excretion
[e] All subjects included had 2 complete 24-hour urine collections based on established urinary completeness criteria, and average 24-hour urinary sodium recovery is reported.
[f] All subjects included had at least 1 complete 24-hour urine collection. For subjects with only 1 complete sample (5% AS: n=5, 25% AS: n=4), 24-hour urinary sodium excretion from the complete sample is compared to 24-hour dietary intake from the corresponding day. For subjects with 2 complete samples (5% AS: n=25, 25% AS: n=27) average urinary sodium recovery is reported.
[g] Paired t-test between average sodium recovery during the 5% AS diet and 25% AS diet for the full sample (n=32)

Table 6. Average nitrogen excretion relative to dietary protein intake.				
	Urinary Nitrogen Excretion (g/24 hours)[a]	**Corresponding Dietary Protein Intake (g/d)[b]**	**Pearson's Correlation**	**p-value**
Both feeding periods, full sample (n=29)	10.4±2.3	105.3±22.6	0.694	p<0.001*
5% AS, sample restricted to complete urine collections (n=25)	9.7±2.1	105.2±23.0	0.739	p<0.001*
25% AS, sample restricted to complete urine collections (n=26)	11.0±2.5	103.2±23.0	0.825	p<0.001*
5% AS, sample restricted to at least 1 complete 24-hr urine collection (n=30)	9.7±2.7	106.7±22.1	0.528	p=0.003*
25% AS, sample restricted to at least 1 complete 24-hr urine collection (n=29)	10.9±2.5	104.7±23.2	0.688	p<0.001*
[a] Urinary nitrogen excretion was averaged from the two 24-hour urine samples collected during each controlled feeding period [b] Dietary protein intake was measured from the 48-hours of dietary intake corresponding to the urine collections, including snack modules. *Urinary nitrogen excretion is significantly correlated with dietary protein intake.				

Table 7. Percentage of dietary nitrogen recovered through excretion compared to expected excretion.				
	Average Percentage of Nitrogen Recovered (mean±SD)	**Percentage of Nitrogen Expected**[c]	**Paired T-Test p-value**[d]	**Independent Samples T-Test p-value**
Both feeding periods, full sample[a] (n=28)	62.2±6.8	80	p<0.001*	
Males (n=12)	60.2±7.8	80	p<0.001*	p=0.108
Females (n=16)	63.7±5.6	80	p<0.001*	
Activity subgroups				
Both feeding periods, more physically active[b] participants (n=12)	62.8±6.7	80	p<0.001*	p=0.725
Both feeding periods, less physically active[b] participants (n=16)	61.8±7.2	80	p<0.001*	
Age subgroups				
Both feeding periods, older participants, ≥15 years (n=21)	62.9±6.9	80	p<0.001*	p=0.345
Both feeding periods, younger participants, <15 years (n=7)	60.1±6.8	80	p<0.001*	
24-hour urine completeness subgroups				
5% AS, sample restricted to 2 complete 24-hr urine collections[e] (n=25)	57.5±8.5	80	p<0.001*	p<0.001*
25% AS, sample restricted to 2 complete 24-hr urine collections[e] (n=26)	66.0±8.1	80	p<0.001*	
5% AS, sample restricted to at least 1 complete 24-hr urine collection[f] (n=29)	57.8±10.7	80	p<0.001*	
25% AS, sample restricted to at least 1 complete 24-hr urine collection[f] (n=28)	65.6±8.0	80	p<0.001*	

[a] Average for all subjects regardless of urine sample completeness; n=3 subjects missing some nitrogen data, n=1 excluded as outlier

[b] Physical activity determined by average of PAQ-A scores from baseline and washout; participants with average PAQ-A score at or above mean sample PAQ-A score of 2.08 were considered more physically active

[c] Nitrogen excretion was expected to be 80%[20]

[d] Paired t-tests run show that average percentage of sodium recovered is significantly different from 80% expected excretion

[e] All subjects included had 2 complete 24-hour urine collections based on established urinary completeness criteria, and average 24-hour urinary nitrogen recovery is reported

[f] All subjects included had nitrogen data recorded and at least 1 complete 24-hour urine collection. For subjects with only 1 complete sample (5% AS: n=5, 25% AS: n=3), 24-hour urinary nitrogen excretion from the complete sample is compared to 24-hour dietary intake from the corresponding day. For subjects with 2 complete samples (5% AS: n=25, 25% AS: n=26) average urinary nitrogen recovery is reported.

*Significant difference detected between average nitrogen recovery during the 5% AS diet and 25% AS diet based on paired t-test for the full sample (n=28)

Table 8. Correlations between sucrose, fructose, and total sugar excretion and corresponding sugar intake.

		Corresponding Dietary Intake (g/24 hours)	**Urinary Excretion** (mg/24 hours)	**Pearson's Correlation**	**p-value**
5% Added Sugar Feeding Period, mean±SD (range)	**Sucrose**[a] n=24	44.3±10.1	0.015±0.01	0.17	p=0.422
	Fructose n=25	46.4±13.2	0.199±0.07	0.10	p=0.620
	Total Sugars[a] n=24	165.2±42.0	0.21±0.07	0.16	p=0.467
25% Added Sugar Feeding Period, mean±SD (range)	**Sucrose**[b] n=25	121.2±27.9	0.028±0.01	0.69	p<0.001*
	Fructose n=27	36.0±9.7	0.348±0.15	0.74	p<0.001*
	Total Sugars[b] n=25	212.6±52.4	0.369±0.16	0.77	p<0.001*

[a]One outlier excluded due to SPSS identification as extreme value for sucrose excretion. 2nd outlier identified was already excluded via incomplete sample.
[b]Two outliers excluded due to SPSS identification as extreme values for sucrose excretion. 3rd outlier identified was already excluded via incomplete sample.

Table 9. Correlations between sucrose, fructose, and total sugar excretion and dietary added sugar intake.

		Dietary Added Sugar Intake (g/24 hours)	**Urinary Excretion**[c] (mg/24 hours)	**Pearson's Correlation**	**p-value**
5% Added Sugar Feeding Period, mean±SD (range)	**Sucrose**[a] n=24	34.3±8.3	0.015±0.01	0.16	p=0.422
	Fructose n=25		0.199±0.07	0.14	p=0.512
	Total Sugars[a] n=24		0.213±0.07	0.15	p=0.493
25% Added Sugar Feeding Period, mean±SD (range)	**Sucrose**[b] n=25	172.4±40.4	0.028±0.01	0.66	p<0.001*
	Fructose n=27		0.348±0.15	0.75	p<0.001*
	Total Sugars[b] n=25		0.369±0.16	0.77	p<0.001*

[a]One outlier excluded due to SPSS identification as extreme value for sucrose excretion. 2nd outlier identified was already excluded via incomplete sample.
[b]Two outliers excluded due to SPSS identification as extreme values for sucrose excretion. 3rd outlier identified was already excluded via incomplete sample.
[c]Subjects with two complete 24-hour urine collections per feeding period

Table 10. Sucrose, fructose, and total sugar excreted in the urine relative to dietary intake.					
		Dietary Intake (g/24 hours) **Full Sample** n=32	**Urinary Sugar Excretion** mg/24 hours (% recovery) **Full Sample**[a] n=32	**Urinary Sugar Excretion,** mg/24 hours (% recovery) **Two Complete 24-Hour Urine Samples**[b] n=24, 25[d] (5% AS) n=25, 27[e] (25% AS)	**Urinary Sugar Excretion** mg/24 hours (% recovery) **≥1 complete 24-Hour Urine Sample**[c] n=30 (5% AS) n=29, 31[f] (25% AS)
5% Added Sugar Feeding Period, mean±SD	**Sucrose**	44.5±9.4	0.016±0.01 (0.00004)	0.015±0.01 (0.00003)	0.015±0.01 (0.00004)
	Fructose	46.9±12.6	0.209±0.09 (0.00049)	0.199±0.07 (0.00046)	0.205±0.08 (0.00047)
	Total Sugars	166.8±38.7	0.226±0.09 (0.00014)	0.213±0.07 (0.00014)	0.221±0.08 (0.00014)
25% Added Sugar Feeding Period, mean±SD (range)	**Sucrose**	123.7±27.4	0.034±0.03 (0.00003)	0.028±0.01 (0.00002)	0.028±0.01 (0.00002)
	Fructose	36.7±9.5	0.331±0.15 (0.00091)	0.348±0.15 (0.00096)	0.328±0.15 (0.00090)
	Total Sugars	217.3±51.2	0.365±0.16 (0.00017)	0.369±0.16 (0.00017)	0.354±0.16 (0.00016)

[a]Urinary sugar excretion and recovery on days 6 and 7 (or corresponding) of each controlled diet based on average dietary intake and average 24-hour time-adjusted excretion for complete population
[b]Urinary sugar excretion and recovery on days 6 and 7 (or corresponding) of each controlled diet based on average dietary intake and average 24-hour time-adjusted excretion for subjects with two "complete" 24-hour urine samples per feeding period based on established criteria
[c]Urinary sugar excretion and recovery on days 6 and 7 (or corresponding) of each controlled diet based on average dietary intake and average 24-hour time-adjusted excretion for subjects with at least one "complete" 24-hour urine sample per feeding period based on established criteria
[d]Outlier for sucrose excretion excluded from sucrose and total sugars (n=24), but not fructose (n=25)
[e]Two outliers for sucrose excretion excluded from sucrose and total sugars (n=25), but not fructose (n=27)
[f]Two outliers for sucrose excretion excluded from sucrose and total sugars (n=29), but not fructose (n=31)

Table 11. Responsiveness of urinary sugar biomarkers to a change in dietary intake.				
			Average Excretion of Corresponding Sugar (mg/24 hours) mean±SD	**Paired T-Test p-value**
Sucrose	**Full Sample[a] n=32**	5% AS	0.016±0.01	p=0.001*
		25% AS	0.034±0.03	
	2 Complete 24-Hour Urine Collections[b] n=23	5% AS	0.016±0.01	p<0.001*
		25% AS	0.030±0.01	
	≥1 Complete 24-Hour Urine Collection[c] n=30	5% AS	0.015±0.01	p=0.006*
		25% AS	0.038±0.04	
Fructose	**Full Sample n=32**	5% AS	0.209±0.09	p<0.001*
		25% AS	0.331±0.15	
	2 Complete 24-Hour Urine Collections n=23	5% AS	0.203±0.07	p<0.001*
		25% AS	0.343±0.16	
	≥1 Complete 24-Hour Urine Collection n=	5% AS	0.205±0.08	p<0.001*
		25% AS	0.333±0.15	
Total Sugars	**Full Sample n=32**	5% AS	0.226±0.37	p<0.001*
		25% AS	0.365±0.16	
	2 Complete 24-Hour Urine Collections n=	5% AS	0.220±0.07	p<0.001*
		25% AS	0.373±0.165	
	≥1 Complete 24-Hour Urine Collection n=	5% AS	0.221±0.08	p<0.001*
		25% AS	0.371±0.16	

[a]Average for all subjects regardless of urine sample completion
[b]Average for subjects with two complete 24-hour urine samples during each controlled feeding period
[c]Average for subjects with at least one complete 24-hour urine sample during each controlled feeding period

Table 12. Reliability of urinary sugar excretion.

		Cronbach's Alpha	ICC Average Measures	ICC 95% Confidence Interval	p-value
5% Added Sugar Feeding Period[a]	**Sucrose** n=24[b]	0.012	0.011	-1.089-0.554	p=0.488
	Fructose n=25	0.553	0.501	-0.043-0.772	p=0.027*
	Total Sugars n=24[b]	0.554	0.521	-0.030-0.786	p=0.029*
25% Added Sugar Feeding Period[a]	**Sucrose** n=25[c]	0.659	0.665	0.234-0.853	p=0.005*
	Fructose n=27	0.851	0.798	0.409-0.919	p<0.001*
	Total Sugars n=25[c]	0.877	0.837	0.520-0.936	p<0.001*

[a]Urinary sugar excretion between D6 and D7 (2 items) of each controlled feeding period for subjects with two complete urine collections.
[b]One outlier excluded due to SPSS identification as extreme value for sucrose excretion. 2nd outlier identified was already excluded via incomplete sample.
[c]Two outliers excluded due to SPSS identification as extreme values for sucrose excretion. 3rd outlier identified was already excluded via incomplete sample.

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CHAPTER 3: Conclusions and Future Directions

Urinary sodium, nitrogen, fructose, sucrose, and total sugars excretion are capable of reflecting dietary sodium, protein, and added sugar intake in non-obese adolescents. Urinary sodium appears to be a valid biomarker in adolescents, but additional research is needed to confirm its validity in a more diverse adolescent population. Then, urinary sodium could be used to objectively assess adolescent sodium intake, which would improve current understanding of the relationship between dietary sodium and health outcomes. Documentation of health outcomes (weight status, adiposity, cardiovascular disease risk) at different levels of sodium intake could inform dietary recommendations and policies to reduce levels of sodium in the food supply, supporting National School Lunch Program standards to gradually decrease the sodium content of school meals between 2012 and 2022.[1]

Urinary nitrogen may become a valid indicator of dietary protein in adolescents, but since excretion levels differ from adults, further research is warranted to understand the extent of positive nitrogen balance that occurs during growth, and to explore any sex-based differences. This research would inform dietary protein recommendations for male and female adolescents, which currently lack evidence,[2] in addition to supporting the development of protein recommendations based on biological age rather than chronological age.[3] Additional research is also needed to understand the impacts of dietary fiber type and amount on urinary nitrogen excretion in adolescents and other age groups. A metabolomics approach recently identified metabolites that were associated with fiber intake.[4] If validated, a dietary fiber biomarker could be used in conjunction with urinary nitrogen to determine dietary protein intake. Prediction equations could be developed for use with the urinary nitrogen biomarker in consideration of biological age, sex, and fiber intake.

Urinary fructose, sucrose, and total sugars appear to be valid and reliable indicators of added sugar consumption, but only at higher levels of intake. Future research should determine the range of dietary added sugar intake that urinary sugar biomarkers are able to reflect. A prediction equation should also be developed with consideration of the various factors affecting intestinal permeability of adolescents. It was determined that urinary sugars are responsive to dietary changes in this population, but additional research is warranted to determine the sensitivity of urinary sugar biomarkers to smaller, more gradual dietary changes. The ability to objectively assess dietary added sugar intake would resolve current controversy related to the impact of added sugars on health outcomes, such as weight status, adiposity, and cardiometabolic consequences.[5] Documentation of the contribution of dietary added sugar to adverse health effects at various levels of intake could align recommended limits for added sugar intake and promote implementation of policies related to the inclusion of added sugars on nutrition facts labels[6] and health-related warnings on food items that are high in added sugars such as sugar-sweetened beverages, a strategy that appears to positively affect adolescents' choices.[7]

Overall, the validity, reliability, and sensitivity of urinary sodium, nitrogen, fructose, sucrose, and total sugars biomarkers should be evaluated in a larger, more diverse sample of adolescents, and spot urine samples should continue to be evaluated[8–11] for effectiveness to reduce subject burden associated with 24-hour urine collections.

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APPENDIX A: List of Additional Figures and Tables

Appendix Figures:

Appendix Tables:

APPENDIX B: Additional Figures and Tables

Figure 1. Urine collection completeness flowchart.

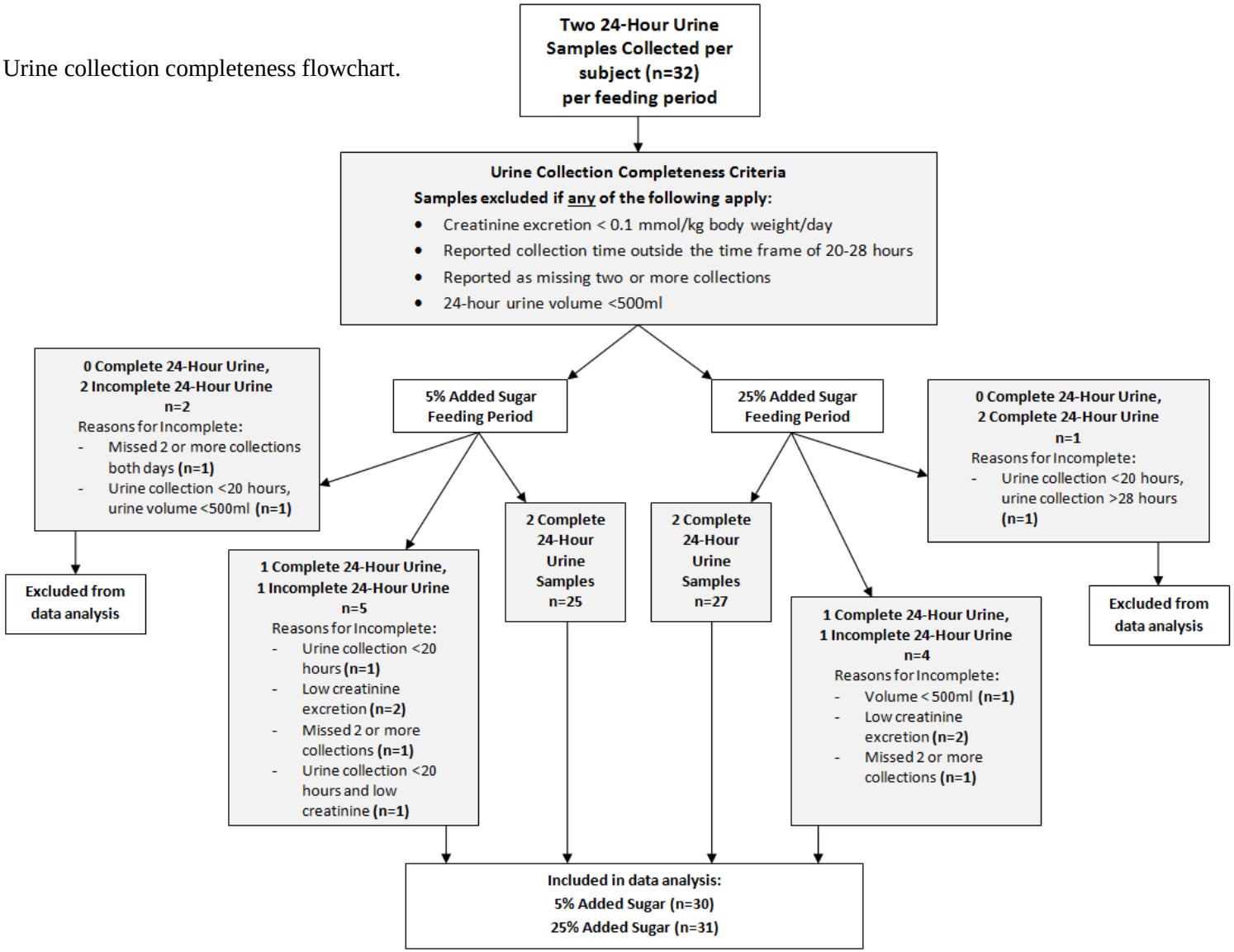

Table 1. Habitual self-reported dietary intake at baseline and washout in adolescents (n=32) compared to current recommendations.

	Current Recommendations[a]	**Average Self-Reported Intake at Baseline**[b]	**Average Self-Reported Intake at Washout**[b]	**p-value**
Added Sugar (% of total kcal), mean±SD (range)	≤10	12.2±3.3 (6.2-18.2)	12.4±4.4 (5.1-21.2)	p=0.832
Sodium (mg), mean±SD (range)	<2200 (ages 9-13) <2300 (ages 14+)	3820±1123 (1916-7082)	3757±1310 (1786-7781)	p=0.713
Carbohydrates (% of total kcal), mean±SD (range)	45-65	50.8±3.5 (43.1-58.3)	50.3±4.9 (43.4-62.9)	p=0.642
Protein (% of total kcal), mean±SD (range)	10-30	14.9±2.6 (9.4-18.8)	15.8±3.3 (9.6-21.3)	p=0.064
Fat (% of total kcal), mean±SD (range)	25-35	34.4±3.3 (27.0-40.0)	33.8±4.4 (23.2-41.1)	p=0.472

[a] According to 2015-2020 Dietary Guidelines for Americans
[b] Average of four 24-hour recalls during baseline and four 24-hour recalls during washout

Table 2. Paired t-test between self-reported caloric intake and measured caloric intake.

	Mean±SD	**p-value**
Self-Reported Caloric Intake at Baseline (kilocalories), mean±SD	2519±577	[c]p=0.663
Self-Reported Caloric Intake during Washout (kilocalories), mean±SD	2475±604	
Average Self-Reported Caloric Intake[a] (kilocalories), mean±SD	2497±518	[c]p=0.003*
Caloric Intake on Controlled Diet[b] (kilocalories), mean±SD	2796±624	

[a] Average of four 24-hour recalls during baseline and four 24-hour recalls during washout
[b] Average caloric intake during controlled feeding periods (including module consumption) while weight stable
[c] Paired t-tests, baseline vs. washout self-reported data, and average self-reported data vs. measured caloric intake on controlled diet
*Significant differences ($\alpha<0.05$) noted between average self-report and measured caloric intake

Table 3. Subject compliance with the intended diets based on recorded dietary intake.		
	Compliance[a]	**p-value**
5% Added Sugar Feeding Period, (% compliance) mean±SD (range)	98.8±0.01 (95-101[b])	[c]p=0.107
25% Added Sugar Feeding Period, (% compliance) mean±SD (range)	98.4±0.02 (89-100)	
[a]Compliance measures were determined by the average measured nutrient consumption for each controlled feeding period divided by the nutrient quantity researchers intended for subjects to consume during that feeding period [b]Compliance measures can exceed 100% because the amount of food provided to each subject could exceed the intended amount (up to 0.9g per ingredient) in accordance with metabolic kitchen protocol		

Table 4. Paired t-test for weight stability of adolescent population (n=32) during controlled feeding periods.					
	Day 1 Weight (kg)	**Day 8 Weight (kg)**	**p-value**	**Weight Stability[a] (kg)**	**p-value**
5% Added Sugar Feeding Period (mean±SD)	57.5±10.2	57.4±10.2	p=0.613	-0.063±0.69	p=0.879
25% Added Sugar Feeding Period (mean±SD)	57.3±10.1	57.3±10.1	p=0.879	-0.016±0.58	
[a] The difference between each subject's weight from Day 1 to Day 8 of each controlled feeding period Paired t-tests used to compare weight stability for both feeding periods					

Table 5. Average creatinine excretion relative to body height and body weight.							
	Urinary Creatinine Excretion (mg/24 hours)	**Body Height[b] (cm)**	**Pearson's Correlation**	**p-value**	**Body Weight[c] (kg)**	**Pearson's Correlation**	**p-value**
Average for both feeding periods, full sample[a] (n=29)	1300±378	167.5±9.8	0.755*	p=0.000	57.6±10.1	0.867*	p=0.000
5% AS, sample restricted to 2 complete 24-hr urine collections[d] (n=25)	1268±369	166.7±9.7	0.682*	p=0.000	56.3±10.4	0.916*	p=0.000
25% AS, sample restricted to 2 complete 24-hr urine collections[d] (n=26)	1370±409	168.0±10.3	0.760*	p=0.000	57.3±10.5	0.951*	p=0.000
5% AS, sample restricted to at least 1 complete 24-hr urine collection[e] (n=30)	1306±444	167.5±9.8	0.732*	p=0.000	57.3±10.3	0.845*	p=0.000
25% AS, sample restricted to at least 1 complete 24-hr urine collection[e] (n=29)	1359±421	167.7±9.7	0.720*	p=0.000	57.5±10.2	0.849*	p=0.000

[a]Full sample (n=29) excludes participants who were missing creatinine excretion data (n=3)
[b]Average height (cm) from baseline and washout
[c]Average body weight (kg) from baseline and washout
[d]All subjects included had 2 complete 24-hour urine collections based on established urinary completeness criteria, and average urinary creatinine excretion is reported for all subjects
[e]All subjects included had creatinine values recorded and at least 1 complete 24-hour urine collection. For subjects with only 1 complete sample (5% AS: n=5, 25% AS: n=3), urinary creatinine excretion from the complete sample is reported. For subjects with 2 complete samples (5% AS: n=25, 25% AS: n=26) average urinary creatinine excretion is reported

Table 6. Reliability of urinary creatinine excretion				
	Cronbach's Alpha	**ICC Average Measures**	**ICC 95% Confidence Interval**	**p-value**
Creatinine Excretion for full sample (n=29, 4 items)[a]	0.890	0.887	0.802 – 0.942	p=0.000
5% AS for complete collections (n=25, 2 items)	0.888	0.892	0.753 – 0.952	p=0.000
25% AS for complete collections (n=26, 2 items)	0.986	0.979	0.921 – 0.992	p=0.000
[a]Urinary creatinine excretion from Day 6 LAS, Day 7 LAS, Day 6 HAS, and Day 7 HAS included for full sample (n=29) with (n=3) excluded due to lack of creatinine data				

Table 7. Impact of dietary fiber intake on urinary nitrogen excretion.			
	5% AS Diet	25% AS Diet	p-value
Average Dietary Nitrogen Intake (g/24 hours)	17.1±3.6	16.9±3.7	p=0.322
Average Dietary Fiber Intake (g/24 hours)	35.5±7.8	22.1±4.5	p=0.000*
Average Urinary Nitrogen Excretion (g/24 hours)	9.9±2.1	11.1±2.5	p=0.000*
Average Nitrogen Recovery (%)	58±7	66±8	p=0.000*

Table 8. Correlations between protein intake per kilogram of body weight, height change, and percentage of nitrogen recovery.									
	Full Sample (n=28)			**Females (n=16)**			**Males (n=12)**		
	mean±SD	Pearson Correlation	p-value	mean±SD	Pearson Correlation	p-value	mean±SD	Pearson Correlation	p-value
Average protein intake (g/kg BW)	1.82±0.24	-0.468	p=0.012*	1.72±0.17	-0.162	p=0.548	1.96±0.25	-0.564	p=0.056
Average nitrogen recovery (%)	62±7			64±6			60±8		
Height change from baseline to washout (cm)	0.36±0.58	-0.470	p=0.012*	0.23±0.60	-0.232	p=0.387	0.53±0.53	-0.666	p=0.018*
Average nitrogen recovery (%)	62±7			64±6			60±8		

Table 9. Standardization and comparison of urinary nitrogen recovery data to the literature.

Study	Population	Study Design	24-Hour Urinary Nitrogen Excretion (in units reported)	24-Hour Urinary Nitrogen Excretion[a] (g/d)	Dietary Protein Intake (g/d)	Dietary Nitrogen Intake (g/d)[b]	Percentage of Dietary Nitrogen Recovered in 24-Hour Urine (%)
Moore et al (2016)	12-18 years Male/Female n=29	Controlled feeding; multiple 24-hr urine collections	10.4±2.3 g/d	10.4	105.3±22.6	16.8	62%
Krupp et al (2012)[12]	12-15 years Male/Female n=120	3-day weighed food records; single 24-hour urine collection	628±170 mmol/d	8.8	66.4±17.3	10.6	83%
Bokhof (2010)[13]	11-13 years Male/Female n=102	3-day weighed food records; single 24-hour urine collection	765mmol/L[c,d]	7.9[d]	62.4[d]	10.0[d]	79%
Bingham (1997)[14]	50-65 years Female n=156	16-day weighed food records; multiple 24-hour urine collections	9.84 ± 1.78 g/d	9.8	69.0±12.0	11.0	89%
Bingham (1985)[15]	24-27 years Male/Female n=8	Weighed food records/observation; multiple 24-hour urine collections	12.7g/d	12.7	98.8	15.8	80%

[a]Conversions based on the relative atomic mass of nitrogen
[b]Nitrogen intake standardized by use of conversion factor 6.25[16]
[c]N per volume multiplied by reported median urine volume (735ml=0.735L)
[d]Only median values reported

Table 10. Total sugars excretion and recovery by gender and age.				
	Average Excretion of Total Sugars[a] (mg/24 hours) mean±SD	**Independent Sample T-Test p-value**	**Average Recovery of Total Sugars[a] (%)** mean±SD	**Independent Sample T-Test p-value**
Both feeding periods, full sample (n=32)	0.296±0.11		0.0002±0.00005	
Male (n=15)	0.301±0.13		0.0001±0.00004	p=0.003*
Female (n=17)	0.290±0.09	p=0.786	0.0002±0.00005	
Both feeding periods, older participants (≥15y, n=24)	0.317±0.12	p=0.008*	0.0002±0.00006	p=0.194
Both feeding periods, younger participants (<15y, n=8)	0.232±0.05		0.0001±0.00004	
[a] Average for all subjects regardless of urine sample completeness				

Table 11. Standardization and comparison of urinary sugars excretion to the literature.

Study	Population	Study Design	Method Used to Measure Urinary Sugars	Added Sugar Intake, (% of total energy intake)	Total Sugar Intake, (% of total energy intake)	24-Hour Urinary Fructose Excretion, mg/24 hours (% recovery)	24-Hour Urinary Sucrose Excretion, mg/24 hours (% recovery)	24-Hour Urinary Total Sugars Excretion mg/24 hours (% recovery)
Moore et al (2016)	12-18 years Male/Female n=29	Controlled feeding; multiple 24-hr urine collections	Mass spectrometry	5	23	0.209 (0.00049)	0.016 (0.00004)	0.226 (0.00014)
				25	31	0.331 (0.00091)	0.034 (0.00003)	0.365 (0.00017)
Johner et al[17] (2010)	Pre-pubertal Male/Female n=114	3-day weighed food records, one 24-hour urine collection	Enzymatic analysis	15	23	19.8 (boys) 20.7 (girls)		
Joosen et al[18] (2008)	20-85 years Male/Female n=10	Controlled feeding; multiple 24-hour urine collections	Enzymatic analysis for urinary fructose; mass spectrometry for urinary sucrose		13	18.4 (0.03)	7.7 (0.01)	26.1
					30	48.0 (0.03)	21.4 (0.01)	69.4
					50	93.2 (0.04)	109.4 (0.04)	202.6
Tasevska et al[19] (2005)	25-77 years Male n=12	Controlled feeding; multiple 24-hour urine collections	Enzymatic analysis		10	2.0	8.3 (0.26)	10.3 (0.61)
					22	16.5	25.9 (0.21)	42.4 (0.34)
					40	22.7	45.5 (0.28)	68.2
Tasevska et al[20] (2009)	23-66 years Male/Female n=13	Controlled feeding; multiple 24-hour urine collections	Enzymatic analysis	18	29			98.3 (0.21)

Figure and Table References

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APPENDIX C: Institutional Review Board Approval

VirginiaTech

Office of Research Compliance
Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
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email irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM

DATE:	January 10, 2017
TO:	Brenda Davy, Madlyn Irene Frisard, Valisa Ellen Hedrick, Tina Savla
FROM:	Virginia Tech Institutional Review Board (FWA00000572, expires January 29, 2021)
PROTOCOL TITLE:	Determining the validity, reliability, and sensitivity of the d13C added sugar biomarker during a controlled feeding study in adolescents
IRB NUMBER:	**15-222**

Effective January 10, 2017, 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.

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) 2,3,4,6,7**
Protocol Approval Date:	**February 9, 2017**
Protocol Expiration Date:	**February 8, 2018**
Continuing Review Due Date*:	**January 25, 2018**

*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.

Date*	OSP Number	Sponsor	Grant Comparison Conducted?
03/03/2015	13173902	National Institute of Child Health & Human Development	Compared on 03/03/2015

* 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.

VirginiaTech

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PROTOCOL INFORMATION:

Approved As:	**Expedited, under 45 CFR 46.110 category(ies) 2,3,4,6,7**
Protocol Approval Date:	**February 9, 2016**
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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.

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03/03/2015	13173902	National Institute of Child Health & Human Development	Compared on 03/03/2015

* 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.

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MEMORANDUM

DATE: March 11, 2015

TO: Brenda Davy, Madlyn Irene Frisard, Valisa Ellen Hedrick, Tina Savla

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

PROTOCOL TITLE: Determining the validity, reliability, and sensitivity of the d13C added sugar biomarker during a controlled feeding study in adolescents

IRB NUMBER: **15-222**

Effective March 10, 2015, the Virginia Tech Institution 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.

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) 2,3,4,6,7**
Protocol Approval Date:	**March 10, 2015**
Protocol Expiration Date:	**March 9, 2016**
Continuing Review Due Date*:	**February 24, 2016**

*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.

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