Nutritional and Health-Related Environmental Studies (NAHRES)

Optimising nuclear techniques to assess accurate quantitative biomarkers of added sugar intake in adults

Brief summary

The proposed CRP will contribute to a better understanding of the appropriate use of natural abundance stable isotope ratios of carbon ($^{13}C/^{12}C$, hereafter the "CIR") to develop a biomarker to assess free sugar intake in different populations. This is relevant in terms of the emerging evidence for the role of carbohydrate and free sugar intake in chronic disease, and the recent WHO guidelines which recommend restricting free sugar in the diet to less than 5% of total energy intake.

In many countries, the intake of refined carbohydrates and cane sugar is high in both adults and children. A biomarker of, for example, cane sugar intake, could offer very useful information in dietary surveys. It would allow to assess the association of added sugars with chronic disease, within the framework of body fat accumulation. However, it has become clear in recent years, based on comparisons with the few established dietary intake biomarkers, that available self reported dietary assessment approaches have limitations. Objective biochemical measurements provide a useful approach to characterizing dietary exposures; however, to be acceptable for scientific use, the objective measure must provide a suitably accurate estimate of intake variation in study populations.

The natural abundance carbon isotope ratio (CIR, 13C/12C) has great potential as a biomarker for sugar intake due to the elevated CIR characteristic of sugar cane. Worldwide, there are many countries for which cane sugar is the predominant source of free sugars and for which corn is not a major component of either processed foods or animal feeds. In those countries, the CIR is likely to have higher validity and specificity as a biomarker of free sugar intake. The specificity of the CIR for free sugar intake can be improved when it is measured in molecules that favour the incorporation of glucose carbon, such as the amino acid alanine (CIR-ala). Studies in the USA have confirmed that CIR-ala has improved validity and specificity for free sugars/sugar sweetened beverage intake when compared with total CIR. However, measurement of CIR-ala is considerably more expensive and time consuming than measurement of total CIR. Whether those additional costs are justified depends on the relative performance of total CIR and CIR-ala as biomarkers of free sugars in a given population. This is unknown for populations outside the USA.

This CRP aims at testing the utility of total CIR and CIR-ala as biomarkers of free sugar intake in adults, and to identify and gain knowledge of interfering dietary practices and other limitations. Therefore, adults from different populations outside the USA with different dietary background, including those who eat corn, millet or sorghum as a staple, will be assessed by comparing the biomarker with accurate measurements of the daily added sugar intake. The Doubly Labelled Water method will be used to validate the accuracy of the dietary recalls.

Background

Burden of disease and need for better biomarkers of refined carbohydrate intake

The recent Global Burden of Disease report has shown that an estimated 72·3% of all deaths in the world in 2016 were from non-communicable diseases (NCD) [1]. Since 2006, deaths from ischaemic heart disease (IHD) have increased by 19% globally. IHD was the leading cause of premature mortality in all socioeconomic development categories apart from those countries in which it was very low. While levels of obesity continued to rise worldwide, diabetes caused 1.43 million deaths in 2016, an increase of 31.1% from the rate in 2006. In these mortality figures, smoking and poor diet remained the leading risk factors of ill health.

The 'poor diet' association with NCD has been considered for decades, with different components of the diet being implicated at different times. While it is rational to think that a balanced and diverse diet will cover the intake of most needed nutrients, and that a diet with plentiful fruits and vegetables, nuts and milk is likely to be healthy, the fact is that globally, the intake of macronutrients such as carbohydrate and fats have dominated the 'risk-foods' debate. Current healthy diet guidelines recommend a low-fat diet (<30% of energy) and limiting saturated fatty acids to less than 10% of energy intake by replacing them with unsaturated fatty acids [2], and the WHO guidelines restrict free sugar in the diet to less than 5% of total energy intake [3].

The guidelines for fat and carbohydrate intake are now a subject of intense debate. For example, in a recent epidemiological cohort study of slightly over 135,000 individuals aged 35–70 years, enrolled in 18 countries, with a median follow up of 7.4 years, it was found that a high carbohydrate intake was associated with higher risk of total mortality, whereas total fat and individual types of fat were related to lower total mortality [4]. Indeed, isocaloric (5% of energy) replacement of carbohydrate with polyunsaturated fatty acids was associated with an 11% lower risk of mortality, whereas replacement of carbohydrate with saturated fatty acids, monounsaturated acids, or protein was not significantly associated with risk of total mortality. In earlier analyses from the same cohort [5], increased carbohydrate intake was associated with lower LDL cholesterol but also with lower HDL cholesterol and higher triglycerides, total cholesterol-to-HDL cholesterol ratio, and ApoB-to-ApoA1 ratio. The latter is particularly noteworthy as ApoB-to-ApoA1 ratio is the strongest lipid predictor of myocardial infarction and ischaemic stroke. An important feature that was missing in the food intake was details about the type of carbohydrate eaten. A biomarker of refined carbohydrate intake, particularly free sugars, would have been very useful. Added free sugars are defined as sugars that are added to foods during food processing, manufacturing, or preparation, and also include sugars naturally present in unsweetened fruit juices. At present, the relative contribution of free or added sugar to the incidence or prevalence of obesity is unknown, even though the relationship of fructose and sugar to risk factors for cardiovascular disease and fatty liver has been described [6]. This is also because strong, objective data on the exposure to free sugar is unavailable.

In many countries, the intake of refined carbohydrates and cane sugar is high in both adults and children. A biomarker of, for example, cane sugar intake, could offer very useful information in dietary surveys. It would allow to assess the association of added sugars with chronic disease, within the framework of body fat accumulation. However, it has become clear in recent years, based on comparisons with the few established dietary intake biomarkers, that available self reported dietary assessment approaches have limitations [7]. Systematic biases can occur when people are asked to self report their dietary intake of what is considered to be a 'harmful' nutrient. There is a large body of evidence that there is systematic bias related to underreporting (approximately 30–50%) of energy intake among overweight and obese women and that younger postmenopausal women underreport energy more than do older women [8]. This bias attenuates associations between dietary intakes and disease risk.

Objective biochemical measurements provide a useful approach to characterizing dietary exposures; however, to be acceptable for scientific use, the objective measure must provide a suitably accurate estimate of intake variation in study populations. Although progress is being made in this field, several clear needs remain including: testing in controlled feeding studies, evaluation of a variety of foods and dietary patterns across diverse populations, improved reporting standards to support study replication, standardized approaches for biomarker validation, comprehensive and accessible food composition databases, a common ontology for dietary biomarker literature and methodologic work on statistical procedures for intake biomarker discovery. Dietary intake biomarker development is best approached as an iterative process, involving a well-integrated methodologic strategy from biomarker discovery through validation. Biomarker development should also rely on sufficiently robust study designs to identify candidate biomarkers that subsequently can be successfully validated [9]. While controlled feeding studies are particularly informative for both biomarker discovery and validation, other study designs, such as cross-sectional studies, may be employed to capture the characteristics of dietary variation and identify candidate dietary biomarkers for a wide diversity of foods.

Potential biomarker of free sugar intake

The natural abundance carbon isotope ratio (CIR, $^{13}C/^{12}C$) has great potential as a biomarker for sugar intake due to the elevated CIR characteristic of sugar cane. Plants convert or "fix" atmospheric CO_2 into sugars by the process of photosynthesis, which has a strong preference for $^{12}CO_2$ over $^{13}CO_2$ ([10] and references therein). However, some plants that are native to arid environments have evolved physiological mechanisms to reduce leaf water loss that decrease the discrimination against $^{13}CO_2$. These arid resistant "C4" plants have a markedly higher CIR relative to the more common "C3" plants. Although most of the plants in the human food supply are C3, including wheat, rice, soy, potatoes and the vast majority of fruits and vegetables, sugar cane is C4, as are corn and some minor grains such as millet and sorghum. The vast majority of worldwide sugar production is from sugar cane, with lesser contributions from sugar beets (a C3 plant used predominantly in Europe) and corn syrup (a C4 plant used heavily in the USA). The isotopic difference in CIR between sugar cane and most other components of the diet could provide a reliable, objective biomarker for sugar intake, because the dietary carbon incorporated into human tissues retains the isotope ratio of its dietary source. However, validation of this promising biomarker is at a relatively early stage.

Studies validating the CIR as a biomarker of free sugar intake have been centered in the USA, where about 75-80% of free ("added") sugars derive from either sugar cane or corn syrup [11]. Several studies have demonstrated low to moderate associations between added sugar or sugar sweetened beverage (SSB) intake and blood CIR [12-17] with coefficients ranging from 0.18 [12] to 0.39 [14]. The CIR can easily be measured in hair [18], nails [19], blood [20] and blood spots [13], making it a practical biomarker for dietary assessment even in remote populations. There are two major limitations to the CIR as a biomarker of sugar intake in the USA. The first is that about 20-25% of added sugars derive from C3 sources: primarily beet sugar, but also including honey and maple syrup [11]. The second is that corn is widely used as animal feed, giving meats an elevated CIR [21-23], and it is an important ingredient in many US foods including cereals and snack foods. Most studies have found that the CIR has stronger associations with animal-based foods than with free sugars in US populations [12, 24-26] which is likely to be a key factor limiting its validity as a biomarker of free sugars. Worldwide, there are many countries for which cane sugar is the predominant source of free sugars and for which corn is not a major component of either processed foods or animal feeds. In those countries, the CIR is likely to have higher validity and specificity as a biomarker of free sugar intake.

Another approach to improving the specificity of the CIR for free sugar intake is to measure it in molecules that favour the incorporation of glucose carbon, thus reducing or eliminating the influence of dietary confounders. The pattern of CIR enrichment of such molecules should be more discriminant for free sugars. For example, the amino acid alanine has a close metabolic link to glucose via the glucose-alanine cycle [27, 28] suggesting that its CIR should reflect glucose deriving from free sugars. The CIR of individual amino acids can be measured using gas chromatography isotope ratio mass spectrometry (GC-C-IRMS). The first study of this potential biomarker was conducted with an Alaska Native study group (n = 68) whose diet was carefully characterized with four 24-h dietary recalls [29]. In that study the CIR of total Red Blood Cells (RBC) was only weakly associated with free sugar and SSB intake (r = 0.20 and 0.26, respectively) whereas the CIR of RBC alanine (CIR-ala) was strongly associated with intake of SSBs, free (added) sugar, and total sugar (r = 0.70, 0.59, and 0.57, respectively; $P < 0.0001$) [29]. Importantly, the CIR of total RBC was associated with other foods having elevated CIR (including meats and corn products), whereas the CIR-ala was not. Thus, CIR-ala had greatly improved validity and specificity for free sugars than did total CIR. This study also assessed SSB and sugar intake by using hair CIR-ala in a subset of their population (n = 30), and found similar associations with SSB intake.

The finding that CIR-ala has improved validity and specificity for free sugars/SSB intake when compared with total CIR has been confirmed by preliminary results from two other USA studies. One study examined dietary associations with serum CIR and CIR-ala in the Women's Health Initiative (WHI) Nutrition and Physical Activity Study – Feeding Study (NPAAS-FS) cohort of 153 postmenopausal women. In the NPAAS-FS, the total serum CIR was not associated with measures of sugar intake but was significantly associated with animal protein [25], whereas the CIR-ala was significantly associated with added sugar but not animal protein [30]. These findings are further supported by the Developing Biomarkers of Diet (DBD) Study, a 12-week, inpatient dietary intervention of 32 men maintained on diets differing in exposure to meat, fish and SSB. In the DBD Study, the plasma CIR-ala was strongly associated with SSB intake at 16% of energy but not with meat intake at 19% of energy. In contrast, the whole-plasma CIR was more strongly associated with meat than with SSB intake [26]. These findings suggest that CIR-ala offers generally better validity and specificity for free sugars/SSB than total CIR. However, measurement of CIR-ala is considerably more expensive and time consuming than measurement of total CIR. Whether those additional costs are justified depends on the relative performance of total CIR and CIR-ala as biomarkers of free sugars in a given population. This is completely unknown for populations outside the USA.

Methodological considerations and requirements for this CRP

This CRP aims at testing the utility of total CIR and CIR-ala as biomarkers of added sugar intake in different adult populations outside the USA with different dietary background, including those who eat corn, millet or sorghum as a staple, by comparing the biomarker with accurate measurements of the daily sugar intake. There has been no careful and systematic study of CIR in the blood of adults from different parts of the world, with varying dietary carbohydrate intakes and a careful discrimination of the sources of carbohydrates that are eaten. This CRP aims to test the utility of these biomarkers of added sugar intakes in adults first. If successful, the biomarkers' utility should be further investigated in children since the origins of adult chronic disease might lie in childhood, in particular in the first 1000 days of children from conception to 2 years of age. Therefore, it will be important to have better tools to evaluate added sugar intake in early childhood.

Theoretically, diet-biomarker validations are best performed in tightly controlled feeding experiments, such as in a controlled metabolic ward, with different doses of the nutrient. However, the length of time for equilibration between the biomarker and the nutrient or diet depends on the turnover of the marker, and the time to cover multiple (at least

3) turnovers of that pool under steady state diet conditions. Thus, the time required within restrictive metabolic ward conditions may be 2 weeks or more, if the turnover of the marker takes 4-5 days. A pragmatic and empirically tested alternative is to recruit a free-living group of subjects, and to provide them with all their meals (with known nutrient and sugar content) in a ready-cooked manner, to eat at home [25]. The meals can be provided for about 2 weeks, and their energy and nutrient content is calculated such that they remain weight stable and compliant to the feeding schedule. The energy content of the meals is based on the prediction of the subject's Basic Metabolic Rate (BMR) and a questionnaire based evaluation of their habitual physical activity patterns, to compute the total daily energy expenditure, assuming weight stability. Meals and schedules are planned based on prior weighed records of intake, and diets are also carefully designed to mimic habitual patterns and preferences of food consumption. Subjects would visit the laboratory to collect food for home consumption 2-3 times a week, while also consuming a meal at the laboratory. This visit allows for the patient to be weighed to ensure weight stability; if the weight changed, the food provided would changed appropriately, assuming all the weight change was due to a positive or negative energy balance. While this method has been shown to work [25], it is still a very demanding protocol, and requires great care and expertise. To carry out such a demanding protocol in low- and middle-income countries (LMICs) will be difficult.

Therefore, an even more pragmatic method to testing the utility is to recruit adult subjects who are weight stable, and accurately measure their dietary (and added sugar) intake through repeated non-consecutive daily dietary recalls (quantitative 24 hour recalls). The key is to recruit study participants with a sufficiently wide range of sugar intake; as in the Choy et al experiment [29], one should aim for a range that is double the least intake. During the recall period, a measurement of total daily energy expenditure is also made using the Doubly Labelled Water (DLW) method, to validate the accuracy of the dietary recalls. A (fasting) blood sample and, if possible, a hair sample will be taken for the measurement of the total CIR and CIR-ala biomarkers. In addition, if possible, a complete 24 hour urine sample will be collected for the measurement of urinary sucrose, as a biomarker of total sugar intake, and other metabolites that can yield other biomarkers of interest.

The recruitment of study participants must be carefully considered, and can be performed in two stages. First, healthy subjects within the normal BMI range, not on any medication (see below for statistical considerations) should be screened for weight stability over the last two weeks (within a CV to be defined at the first RCM) and for habitual diet stability (based on energy and added sugar; metric to be defined at the first RCM). In weight and diet stable subjects, tertiles of sugar intake can be constructed, and the final number of subjects should be recruited based on stratified sampling; sex can be an additional stratum.

The country should also be able to undertake detailed dietary recalls. This requires countries to have in-house diet to nutrient conversions; this in turn requires that databases of food portions to weight conversions, and recipes should be available, to allow for raw food conversion into nutrients (specifically added or intrinsic sugar). Repeated daily dietary recalls (at least 4 non consecutive recalls, including at least one weekend day recall) will be conducted to measure the added sugar intake. During the recall period, a venous blood sample (and a hair sample if possible) will be taken, with subsequent separation of the packed cell fraction and serum. Both will be aliquoted (at least 3 separate aliquotes for each fraction) and stored at -80C. In addition, a complete 24 hour urine sample will be collected.

A sub-sample of study participants will undergo a measurement of DLW (as soon as possible to ensure continued weight and diet stability), with the standard 10-14 day protocol (depending on their habitual water turnover). Individual doses will be prepared from separate solutions of deuterium oxide (99.8 at.% ^{2}H) and oxygen-18 labeled water (10 at.% ^{18}O). Study participants will drink the doses using a straw, after collecting one basal urine sample. Post-dose urine samples will be collected for several days: at 4-6 hours post dosing, and several times during the protocol period (to be decided at the first RCM). The exact time of dose administration and urine collection will have to be recorded. Urine samples will be stored at -20°C in tightly sealed containers until analysis.

The statistical power of this planned study is based on individual power within each country. Each country has two components to their study: first, the main study in which the association of the total CIR and CIR-ala biomarkers will be tested against the habitual sugar intake of a group of subjects, and second, a validation of the accuracy of their dietary recalls, which will be performed in a subset of subjects from the main study.

For the main study, Choy et al [29] reported a correlation coefficient of 0.57 between the biomarker of CIR-ala and the total sugar intake, in 68 subjects. The variation of the CIR-ala was between -20 to -12‰. The range of the energy and sugar intake was about 1000-3000 kCal/d and 77-104 g/d respectively. It is important that subjects with a wide range of sugar intakes are recruited, such that there is adequate variation ($\pm$ 15% of the mean) in the sugar intake exposure.

Assuming a smaller correlation coefficient of 0.4 and the same variability in the CIR-ala, a sample size of n=62 per country (n=70 with drop-outs) would be sufficient to observe a significant correlation with 90% power and 5% level of significance.

The second component of this study is to validate the accuracy of the historical (daily recall based) reporting of sugar intake. This is performed against an external 'gold standard' validating instrument, such as the measurement of daily energy expenditure (EE) by the DLW method. The DLW method has a precision of 2-8% under controlled conditions and is well suited for this purpose, although the precision is dependent on good laboratory and clinical practice, as well as the accurate measurement of the food quotient of the diet during the study (to calculate the energy equivalent of the measured CO_2 output). There are two important assumptions to the validation. First, that the daily energy intake (EI) is equal to the daily EE, which should happen in a weight stable subject. Weight stability among selected subjects in the validation study is vital; typically, weight may vary by about 3-5%. If there is a change of weight during the protocol, a correction can be made for change in the water pool size. Second, the daily total sugar intake should correlate with the daily EI; in the study by Choy et al [29], this correlation coefficient was 0.5.

It is difficult to decide on an acceptable statistical method of the validation of EI against EE. This could be through different approaches, including correlation, or the identification of the number of under- and over-reporters, or regression analysis of EE against EI and Bland-Altman analyses. However, the number of subjects required to confer adequate power in an EI validation study is difficult to assess.

For correlation with an external validation measurement, earlier studies have accepted correlation coefficients of 0.3 to 0.5 to represent successful validations of the 24-hr dietary recall [31]. A specific example from a reputable group found the correlation between dietary recall EI and EE-DLW to be 0.425 [32]. If a similar correlation coefficient (with 5% level of significance and 80% power) is expected in the proposed study, it would require a sample size of n=43 study participants. The number of study participants can be reduced by improving the precision of the measurement of EI and therefore improving the expected correlation coefficient; a more precise measure of EI can be expected by taking more non-consecutive recalls. The expected correlation coefficient is also important: depending on the degree of correlation expected, the sample size would vary. With the precision afforded by 3 non-consecutive recalls, a smaller sample size of n=15 (per site) would allow for the measurement of a correlation coefficient of 0.7 with 5% level of significance and 80% power. A sample size of n=20 would be sufficient to measure a correlation coefficient of 0.6, while n=25 would allow for the measurement of a correlation coefficient of 0.55, with 5% level of significance and 80% power. Conservatively, a sample size of n=25 per site is ideal for a controlled and precisely measured DLW-EI validation study. However, the low correlation coefficients that have been deemed acceptable in the foregoing discussion could still limit the satisfactory interpretation of the results.

An alternative method to assess the validity, or judge the accuracy, of the EI measurement is through the evaluation of the proportion of under- and over-reporters of EI. This would permit the self-reported recalls to be categorized into one of the two groups, following which, the agreement between self-reported recalls and the biomarker could be compared between these two groups. Given the primary assumption that habitual EI must equal habitual EE when subjects are in energy balance and stable in weight, the expected EI:EE ratio should be 1. The limits for which this ratio should not be exceeded depends on assumptions of the CV of each measurement, and the limits within which the differences between EI and EE would have arisen due to random error. This can be determined by calculating the 95% confidence limits of the ratio [32] as

$$95\%\ Confidence\ limits = 2[CV_{wEI}^2/d) + CV_{wEE}^2 - 2r.(CV_{wEE}/d).CV_{wEE}]$$

where CV_{wEI} is the within-subject CV for daily EI (taken as 23%), d is the number of days of diet records (7 days), CV_{wEE} is the within-subject CV for repeat EE-DLW measurements (taken as 8.2%) and r is the correlation between EI and EE, taken as 0.425, based on Black [33]. Substituting these values yields a figure of ±18%. Thus, subjects with EI:EE < 0.82 or > 1.18 would be deemed under- or over-reporters, respectively. In this review, it was stated that a value of ~15% is the lowest that could be expected. One study [34] observed 20% under-reporting by 24h recalls compared to DLW using these criteria. To assess this degree of under- or over-reporting, the data will have to be pooled across countries to give the required sample size (total sample size of ≥120), that is, to observe 20% under- or over-reporting of EI with 95% confidence interval and 30% relative precision.

Finally, a regression analysis or Bland-Altman approach could be undertaken in this validation. While not providing any set value for the number of subjects required, beyond what is calculated for the power of the correlation analysis (above), the Bland-Altman approach can provide 95% confidence limits for the agreement between the methods (calculated as a difference, or bias), and also provide information about the degree of systematic and random errors between the methods. It can also evaluate if the bias is associated with the magnitude of the measurement.

Future studies based on the pilot data that will be generated from this CRP could be designed, which sought the analysis of the CIR signature in serum samples from existing large cohorts that are evaluating the diet-disease/mortality relationship, where the intake of sugar might be of interest as a risk factor for all-cause, as well as NCD mortality. One could then collaborate with existing cohorts that seek to evaluate risks for NCD, and that have serum or RBC samples, to assess their interest in evaluating CIR-ala against their food intake data, as well as outcomes.

Key elements required for the studies in this CRP will be the ability to recruit adult subjects with a range of habitual added sugar intakes, the ability to perform accurate and detailed dietary recalls, and the ability to collect urine samples and a single blood sample. The ability to work with stable isotopes, and the careful attention to detail in these studies will be critical. Finally, storage for sample aliquots and the ability to ship samples is important. Therefore, successful proposals will have to indicate the sources of added sugar, the range of added sugar intakes and potential dietary confounders (e.g. corn-fed animals), as well as the team's experience with conducting dietary recalls and using isotope techniques.

Analytical techniques to be used

The $\delta^{13}C$ signatures will be measured in serum samples and if possible in hair samples as non-invasive alternative. The aim is to evaluate the carbon isotope ratio of whole serum and of alanine as biomarkers of added free sugar intake in adults. The Doubly Labelled Water technique will be used to assess total energy expenditure to correct for bias in self-report of energy intake assessed by repeated 24hr recall. If possible in some sites, urinary sucrose will be measured as an estimate of total sugar intake (intrinsic, milk and added sugars).

Whole serum CIR: Stable isotope ratio measurements use isotope ratio mass spectrometry (IRMS), which is designed to measure small differences in the low natural abundance of heavy stable isotopes very precisely [35, 36]. Because all of the samples in this study are at natural abundance, none approach the limit of detection (LOD) for IRMS. This analysis requires < 5µl of serum. Samples will be prepared in duplicate by pipetting into pre-weighed tin capsules, autoclaved, oven-dried for 48 hours and analyzed using elemental analysis – isotope ratio mass spectrometry (EA-IRMS) at the Alaska Stable Isotope Facility (ASIF) at the University of Alaska Fairbanks (UAF). Isotope ratios of each element are measured as delta values in permil (‰) relative abundance of heavy isotope, as follows: $\delta X = (R_{sample} - R_{standard})/(R_{standard}) \times 1000‰$, where X is the heavy isotope, R is the ratio of heavy to light isotope and the standards are IAEA-certified reference materials calibrated (by convention) to Vienna Pee Dee Belemnite ($^{13}C/^{12}C_{V\text{-}PDB} = 0.01124$). Reproducibility is typically within 0.2‰.

Serum amino acid CIR: Analyses of serum amino acid (free + bound) CIR will be conducted at the UAF and the ASIF using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). Serum samples will be hydrolyzed in 6N HCl, lipid-extracted with hexane/dichloromethane and derivatized to amino acid N-acetyl methyl esters [37] following modified methods developed by the O'Brien lab and collaborators. The $\delta^{13}C$ values of amino acid derivatives will be measured in triplicate using the GC IsoLink II IRMS system (ThermoFisher Scientific). Corrections for carbon added during derivatization are based on amino acid standards that will be prepared and analyzed concurrently. Internal standards will be added to both samples and mixed amino acid standards both before (norleucine) and after (caffeine and nonadecane) derivatization. Isotope ratios will be reported as delta values in permil (‰) relative abundance of heavy isotope as described above. With these methods we achieve good peak heights and chromatographic separation for 10 amino acids in serum: alanine, aspartate/asparagine, glutamate/glutamine, glycine, isoleucine, leucine, phenylalanine, proline, serine and valine.

DLW: The urine samples will be analysed using IRMS or an off-axis laser spectroscopy liquid water isotope analyser**.** Samples will be run alongside laboratory standards of known enrichment, which are traceable to the international standards. For standardisation, isotope enrichments will be converted to daily CO_2 production using a two pool model equation and a measured food quotient from the daily dietary recalls. This will be converted to total energy expenditure using the Weir equation.

CIR in hair: Hair samples will be cleaned with triplicate 30-min rinses in 2:1 methanol: chloroform. Hairs will be chopped into small pieces, placed into 11*8 mm tin capsules, and oven dried at 50°C for 24 h. Capsules will be crushed into balls and loaded into an autosampler for isotope analysis. The hair samples will be analyzed for carbon isotope ratios by using continuous-flow isotope ratio mass spectrometry as described for whole serum CIR.

Urinary sucrose: Urine from study participants will be collected and preserved with boric acid (1.5 g/L). The urine samples will be aliquoted and stored at −20°C. Complete 24 hour urine samples are required. Urinary sucrose can be quantified in biological samples by several methods, including enzymatic kit-based assays, high-performance liquid chromatography (HPLC)–mass spectrometry, HPLC fluorescence detector and gas chromatography with mass spectrometry (GCMS). Urine samples, along with IS (internal standard solution ($^{13}C_{12}$-sucrose) and 50 µL urease will be vortex-mixed and incubated for 1 h at 37°C. Following incubation, 800 µL cold methanol will be added to each sample, and will be vortex-mixed and centrifuged for 10 min at 14 000 *g*. The supernatant will then be transferred into a glass vial and dried under reduced pressure. The samples will then be processed to their silylation derivatives and analysed by GCMS. In high resolution accurate mass spectrometry (HRAM) analysis, full scan mode can be used to allow the identification of additional metabolites that are biomarkers of sugar intake.

Overall Objective

To provide new knowledge and evidence on the application of nuclear methods to define accurate biomarkers of added sugar intake in adults. This is relevant in the context of the rapidly increasing incidence of chronic diseases presently underway in LMIC.

Specific Research Objective (purpose)

Obtain new information on the utility of an isotopic biomarker to assess added sugar intake across different populations and countries.

Expected outcome

The availability of a biomarker for added sugar intake will allow researchers to study dietary risk factors of chronic diseases and will subsequently provide guidance for programme managers and public health nutrition policy makers to optimize the design and evaluation of interventions and policies on healthy eating.

Expected outputs

1. New data on the utility and specificity of the stable isotope biomarkers to assess added sugar intake in adults and its interfering dietary practices and other limitations.
2. New data on added sugar intake in different adult populations.
3. New data on total energy expenditure in different adult populations.
4. Publications in the form of scientific reports and peer-reviewed papers and conference presentations.

Proposal submission forms

Research institutions in Member States interested in participating in this CRP are invited to submit proposals directly to the Research Contracts Administration Section (NACA) of the International Atomic Energy Agency or to Dr Cornelia Loechl. The forms can be downloaded from the CRA website.

For more information about research contracts and research agreements, please visit our web-site.

Deadline for submission of proposal

Proposals must be received **no later than 15 September 2019**. Transmission via Email is acceptable if all required signatures are scanned.

For additional information, please contact:

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