OBJECTIVE DIETARY ASSESSMENT IN NUTRITION EPIDEMIOLOGY STUDIES

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PUBLISHED IN: Frontiers in Nutrition

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-731-6
DOI 10.3389/978-2-88971-731-6

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OBJECTIVE DIETARY ASSESSMENT IN NUTRITION EPIDEMIOLOGY STUDIES

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Citation: McCrory, M. A., Tasevska, N., Draper, J., eds. (2021). Objective Dietary Assessment in Nutrition Epidemiology Studies. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-731-6

Table of Contents

frontiers in Nutrition

ORIGINAL RESEARCH
published: 06 May 2020
doi: 10.3389/fnut.2020.00062

Comparing Self-Reported Sugar Intake With the Sucrose and Fructose Biomarker From Overnight Urine Samples in Relation to Cardiometabolic Risk Factors

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OPEN ACCESS

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *09 February 2020*
Accepted: *17 April 2020*
Published: *06 May 2020*

Citation:
Ramne S, Gray N, Hellstrand S, Brunkwall L, Enhörning S, Nilsson PM, Engström G, Orho-Melander M, Ericson U, Kuhnle GGC and Sonestedt E (2020) Comparing Self-Reported Sugar Intake With the Sucrose and Fructose Biomarker From Overnight Urine Samples in Relation to Cardiometabolic Risk Factors. Front. Nutr. 7:62. doi: 10.3389/fnut.2020.00062

Studies on sugar intake and its link to cardiometabolic risk show inconsistent results, partly due to dietary misreporting. Cost-effective and easily measured nutritional biomarkers that can complement dietary data are warranted. Measurement of 24-h urinary sugars is a biomarker of sugar intake, but there are knowledge gaps regarding the use of overnight urine samples. We aim to compare (1) overnight urinary sucrose and fructose measured with liquid chromatography-tandem mass spectrometry, (2) self-reported sugar intake measured with web-based 4-day food records, (3) their composite measure, and (4) these different measures' (1–3) cross-sectional associations with cardiometabolic risk factors in 991 adults in the Malmö Offspring Study (18–69 years, 54% women). The correlations between the reported intakes of total sugar, added sugar and sucrose was higher for urinary sucrose than fructose, and the correlations for the sum or urinary sucrose and fructose (U-sugars) varied between $r \approx 0.2$–0.3 ($P < 0.01$) in men and women. Differences in the direction of associations were observed for some cardiometabolic risk factors between U-sugars and reported added sugar intake, as well as between the sexes. In women, U-sugars, but not reported added sugar intake, were positively associated with systolic and diastolic blood pressure and fasting glucose. Both U-sugars and added sugar were positively associated with BMI and waist circumference in women, whereas among men, U-sugars were negatively associated with BMI and waist circumference, and no association was observed for added sugar. The composite measure of added sugars and U-sugars was positively associated with BMI, waist circumference and systolic blood pressure and negatively associated with HDL cholesterol in women ($P < 0.05$). Conclusively, we demonstrate statistically significant, but not very high, correlations between reported sugar intakes and U-sugars. Results indicate that overnight urinary sugars may be used as a complement

to self-reported dietary data when investigating associations between sugar exposure and cardiometabolic risk. However, future studies are highly needed to validate the overnight urinary sugars as a biomarker because its use, instead of 24-h urine, facilitates data collection.

Keywords: added sugar intake, nutritional biomarkers, urinary sucrose and fructose, overnight urinary sugars, cardiometabolic risk factors

INTRODUCTION

Sugars have received increasing attention in recent decades and have been linked to metabolic syndrome and related conditions and diseases (obesity, type 2 diabetes and cardiovascular disease) (1–3). However, the strength of the available evidence is weak (4), and the inconsistent results might partly be explained by difficulties in measuring sugar intake as an exposure. Misreporting through self-reported dietary assessment methods is a challenge that complicates the conclusions of epidemiological investigations of health risks associated with high sugar consumption. Hence, there is a need to identify objective measurements of dietary intake in the form of nutritional biomarkers to complement subjective self-reported data (5). It should also be emphasized that with this need for nutritional biomarkers follows an almost equally important need for these biomarkers to be relatively cost-effective and easily measured.

The measurement of 24-h urinary sucrose and fructose as a predictive biomarker for sugar intake was first recognized after its dose-response relationship was demonstrated through controlled sugar intake and its validity to estimate sugar intake (after *ad libitum* intake) was confirmed (6). Thereon, this biomarker has been compared against several different dietary assessment methods (7, 8), e.g., correlation of $r = 0.21$ with a 4-day food record (8). As compared to the predictive 24-h urinary sugar biomarker, the concentration biomarker from spot or overnight urinary sugar samples (9, 10) is substantially easier to collect but has only been compared with reported sugar intake in three previous studies (two in the same cohort) (11–13). Only one of these studies, which was performed in children, reported correlation coefficients between the spot morning urinary sugar levels and reported sugar intake ($r = 0.25$) (13). In the other cohort, higher urinary sucrose levels (from any time spot urine samples) were associated with an increased risk of being overweight, whereas higher self-reported sugar intake was associated with a decreased risk (12).

The principle behind this biomarker is based on the understanding that very small amounts of sucrose can evade hydrolysis by sucrase and be absorbed in the jejunum as a disaccharide instead of being cleaved into glucose and fructose (10, 14). Fructose, either directly from the diet or as hydrolyzed sucrose, is transported to the liver and only small amounts can evade the hepatic metabolism and remain in the circulation. In the circulation, sucrose and fructose, unlike glucose, are not hormonally regulated by insulin, and hence, non-metabolized sucrose and fructose are excreted in the urine (15). At most, ∼0.05% of consumed sucrose and fructose is excreted in the urine and detected in 24-h samples, but this small amount correlates very well with sugar intake under controlled dietary intake and urination conditions ($r = 0.88$) (6). This correlation exists even though the dietary and urinary sugars reflect somewhat different factors: consumed and absorbed sugars. However, there is a lack of knowledge on the performance of this biomarker in free living populations and in overnight urine instead of 24-h urine samples, which means that both the biomarker and self-reported dietary data in this study are subject to individual uncertainties.

This biomarker from non-24-h urine samples classifies as a so-called concentration biomarker, and therefore lacks the ability to predict true sugar intake and to use for regression calibration (10, 16). However, Freedman et al. (17, 18) have proposed that combining self-reported intake with concentration biomarkers into composite measures is a way to improve investigation of diet-disease relationships.

The objective of this study was to compare the measurement of sucrose and fructose in overnight urine samples, self-reported sugar intake and their combination, as well as to assess and compare their associations with cardiometabolic risk factors.

MATERIALS AND METHODS

Study Design and Subjects

The Malmö Offspring Study (MOS) is a prospective cohort comprised of adult children and grandchildren of participants from the Malmö Diet and Cancer-Cardiovascular Cohort (MDC-CC), which was conducted in the 1990s. The individuals comprising MOS were recruited through invitation letters beginning in 2013. The eligibility criteria were a minimum of 18 years of age and at least one parent or grandparent who participated in the MDC-CC. The participants visited the research clinic twice (∼1 week apart) for clinical examinations, collection of biological samples and instructions on how to fill in a lifestyle questionnaire and maintain a 4-day web-based food record at home (19). Participants started recording their diet prospectively the day after their first visit and brought their overnight urine samples on the morning of the second visit. For the present cross-sectional study, from the first 1,532 urine samples collected in the MOS, we selected those from all the non-diabetic participants with complete dietary data

Abbreviations: BMI, body mass index; BP, blood pressure; e-GFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LTPA, leisure-time physical activity; MDC-CC, malmö diet and cancer-cardiovascular cohort; MOS, malmö offspring study; PC, principal component; QC, quality control; SSB, sugar-sweetened beverage; U-fructose, urinary fructose; U-osm, urine osmolality; U-sucrose, urinary sucrose; U-sugars, sum of urinary sucrose and fructose.

and a reported energy intake within the range of 500–6,000 kcal/d (**Figure 1**). The MOS received ethical approval from the Regional Ethics Committee in Lund (Dnr.2012/594) and all the participants signed a written informed consent form prior to participation, all in accordance with the Declaration of Helsinki.

Dietary Data

Dietary data were collected using the Riksmaten2010 method, developed by the Swedish National Food Agency, which involves an online 4-day web-based food record (20). Each participant started recording their diet on the day after the first visit to the research clinic to ensure representation of all days of the week among the studied population. The participants were instructed to record everything they consumed for four consecutive days. The portion size was estimated using photographs of different portion sizes, and the food record was linked to the food database of the Swedish National Food Agency.

Data on total mono- and disaccharides and sucrose were obtained from the food database. Total sugars (g/d) were calculated by summing all mono- and disaccharides (which includes glucose, fructose, galactose, sucrose, lactose and maltose), and the total sugar density (g/1,000 kcal) was calculated by dividing the total sugar intake by the energy intake/1,000. The level of added sugars, as defined by the European Food Safety Authority and the Nordic Nutrition Recommendations: "The term "added sugars" refers to sucrose, fructose, glucose, starch hydrolysates (glucose syrup, high-fructose syrup) and other isolated sugar preparations used as such or added during food preparation and manufacturing." (21, 22) (including isolated sugar preparations such as honey and syrup), was estimated by subtracting naturally occurring monosaccharides and sucrose in fruits and berries (10 g/100 g), vegetables (3 g/100 g), and juices (8 g/100 g) from the sum of the reported intake of monosaccharides and sucrose (assuming that lactose and maltose are not added to foods). The following formula was used for the estimation (all intake variables are expressed in g/day): added sugar = monosaccharides + sucrose—(fruit and berries × 0.1 + vegetables × 0.03 + juice × 0.08). The resulting value was transformed into the percentage of non-alcoholic energy intake (E%). The total sugar density is expressed in g/1,000 kcal, whereas the added sugar density is expressed in E% to facilitate comparisons with previous studies. The investigated sugar sources were desserts (desserts, cakes, cookies, pastries and ice cream), sweets (sweets, chocolate and bars), toppings expressed in servings/day (1 serving of table sugar, syrup or honey = 10 g, 1 serving of jam, marmalade or jelly = 20 g), sugar-sweetened beverages (SSBs; soft drinks, non-carbonated sugar-sweetened drinks, chocolate drinks), fruits (fruits and berries including dried and preserved) and juices (fruit and vegetable juices). One subject outlier was excluded from the statistical analysis due to an extremely high reported juice intake, which resulted in an unreasonable estimation of added sugar intake; and thus, the total study sample comprised 991 subjects (**Figure 1**). The added sugar intake (E%) was the dietary variable primarily investigated in this study because that is what is of most interest in terms of cardiometabolic risk. This is despite the fact that the measured urinary biomarker cannot distinguish between naturally occurring and added sucrose and fructose, but the alternative, i.e., investigation of the total sugar intake, does not perfectly reflect the biomarker either because both lactose and maltose form a substantial part of the total sugars.

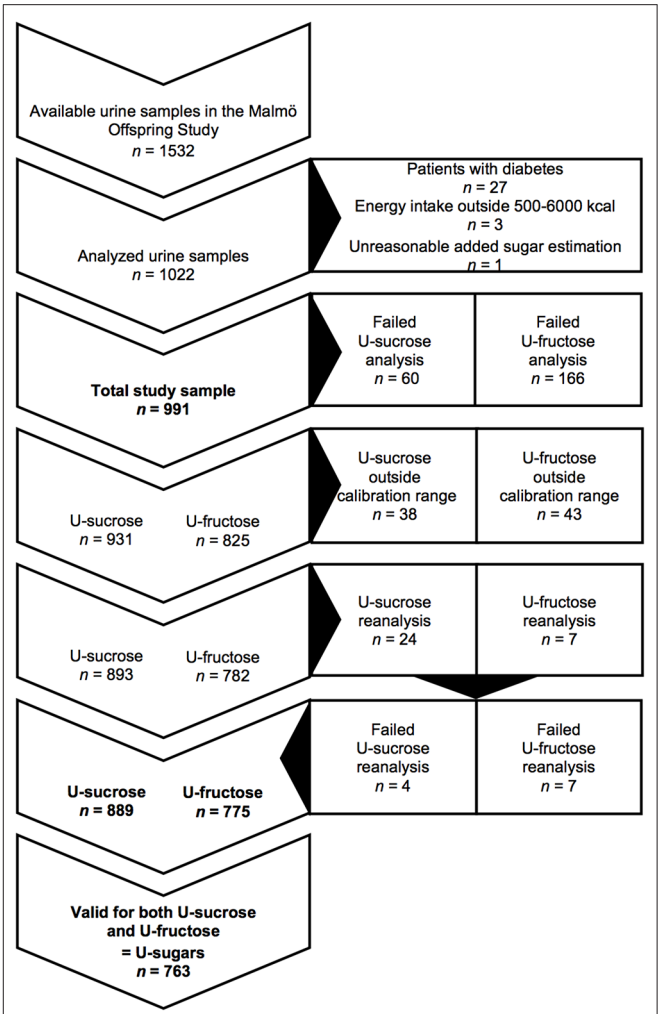

FIGURE 1 | Flow chart of participants and urine samples in the Malmö Offspring Study. U-sucrose, urinary sucrose; U-fructose, urinary fructose; U-sugars, sum of urinary sucrose and fructose.

Urinary Data

Collection of Overnight Urine Samples

Comprehensive instructions to ensure a standardized urine collection procedure were provided on the first visit to the research clinic. Overnight urine was collected on the morning of the second research visit. The instructions were to empty the bladder before bedtime and collect all urine thereafter during the night and all of the first morning urine in a plastic bottle while fasting. At the clinic, the urine samples were stored in a

refrigerator for a maximum of 4 h before being transferred to the laboratory, where they were aliquoted and relocated to a −80°C freezer.

Preparation of Calibration Standards, Internal Standards and Quality Control

Calibration standards of sucrose and fructose (Sigma Aldrich, Gillingham, UK) were prepared ranging from 0.1 to 500 μmol/L. Stable isotope-labeled internal standard solution was prepared in acetonitrile containing $^{13}C_{12}$-sucrose at 4 μg/mL and $^{13}C_6$-fructose at 10 μg/mL. Quality controls (QCs) of 1 μmol/L (low QC), 7.5 μmol/L (medium QC), and 75 μmol/L (high QC) were analyzed in duplicate throughout each batch of samples. The precision and accuracy of the analysis were assessed by determining the replicates of the low, medium and high QC samples across all batches of samples.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The urine samples were stored at−80°C until, thawed at 4°C and diluted with the internal standard mix. LC-MS/MS analysis was performed using an Acquity UPLC system (Waters, Milford, MA, USA), coupled to a Quattro Ultima tandem quadrupole mass spectrometer (Micromass, Manchester, UK). The mass spectrometer was operated through electrospray ionization in positive ion mode using multiple reaction monitoring mode. In total, 226 samples were not successfully analyzed, and 81 samples were outside the calibration range and were thus excluded from the analysis. Out of those 81 samples, those above the calibration range (24 sucrose samples and 7 fructose samples) were reanalyzed at a 4-fold-higher dilution and a calibration range extending to 1,000 μmol/L. These re-analyses were successfully performed and resulted in re-inclusion for the sucrose samples (with the exception of 4 samples), but not with fructose (**Figure 1**).

Adjustment for Urine Dilution

To adjust for urine dilution, the urinary sugars concentrations were expressed as ratios to the urine osmolality (U-osm, mOsm/kg H_2O), i.e., in units of $(\mu mol \cdot L^{-1})/(mOsm \cdot kg^{-1})$. U-osm was selected for dilution adjustment over urinary creatinine because the latter could be associated with body mass index (BMI) and could consequently induce a false association between urinary sugars and BMI. U-osm was measured using an i-Osmometer basic (Löser, Germany). The osmolality-adjusted urinary sucrose (U-sucrose) and fructose (U-fructose) were also added together and investigated as their sum (U-sugars). U-sugars was correlated ($r = 0.95$, $P < 0.001$) with the sum of urinary sucrose and fructose adjusted for creatinine. The osmolality-adjusted urinary sugar variables are used throughout this paper unless stated otherwise.

Data on Cardiometabolic Risk Factors

During the visits to the research clinic, anthropometrics and blood pressure were measured, and fasting blood samples were collected by a research nurse. Weight was measured using either a calibrated balance beam or a digital scale with the participant wearing light clothing. Height was measured to the nearest centimeter using a stadiometer. BMI was calculated as weight (kg)/height $(m)^2$ (19). Waist circumference was measured between the lowest rib margin and the iliac crest with the participant in a standing position. Resting blood pressure (BP) was assessed while lying after 10 min rest as the mean from two measurements. Fasting blood samples were drawn and plasma was analyzed directly for fasting glucose using HemoCue Glucose 201^+ (HemoCue AB, Sweden) and within 4 h for total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol using the Cobas system (Roche Diagnostics, Germany). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation.

Data on Confounding Factors

Data on potential confounders were collected via a lifestyle questionnaire. Leisure-time physical activity (LTPA) was assessed using a four-level scale ranging from sedentary to regular activity (≥3 × 30 min/week). Smoking status was categorized as never smoked, ex-smoker, irregular smoker, and regular smoker. Alcohol consumption habits were assessed on a five-level scale from never to ≥4 times/week, and their education level was categorized as compulsory school, upper secondary school and university degree. Data on medication use were also self-reported via the lifestyle questionnaire. The relative estimated glomerular filtration rate (e-GFR) was estimated using the revised Lund-Malmö equation (23).

Misreporting of energy intake was evaluated according to Goldberg and Black's cutoffs for misreporting of energy intake based on a two-standard-deviations discrepancy between individual physical activity levels and the ratio of energy intake to the basal metabolic rate (24). Individual physical activity levels were obtained from the Riksmaten2010 based on physical activity at work and LTPA (both assessed using a four-level scale ranging from sedentary to heavy manual labor/exercise ≥3 × 30 min/week). To enable comparisons with the ratio of energy intake to basal metabolic rate, basal metabolic rate was calculated using the Oxford equations by taking sex, age, and weight into account (22, 25).

Statistical Analysis

All statistical analyses were performed using Stata/SE (version 15; StataCorp LLC, USA). The urinary sugar variables were skewed even after adjustment for U-osm and were therefore $\log_{10}$-transformed. Since sex differences were observed, the statistical analyses were mainly performed divided by sex.

The baseline characteristics were evaluated separately for men and women over quintiles of U-sugars and 6 categories of added sugar intake, namely, ≤5E, >5-≤7.5E, >7.5-≤10E, >10-≤15E, >15-≤20E, and >20E%, which were previously investigated in relation to mortality in the Malmö Diet and Cancer Study (26). Categorical variables were expressed as percentages and continuous variables were expressed and mean (SD) or median (IQR), dependent on their distribution.

Partial correlation analysis adjusting for energy intake, age, sex, and BMI was performed between the different urinary sugar variables and the reported dietary sugar variables to evaluate the

agreement between the two measurement methods. An alluvial plot was created to visualize the agreement between the 6 added sugar intake categories and the quintiles of U-sugars. To assess misclassification, equal groups are necessary and for this purpose sex-specific quartiles were used for crosstabulation of the U-sugars and reported added sugar intake.

We assume no mediation through the urinary sugar biomarker in the potential association between sugar intake and cardiometabolic disease. Therefore, according to reasoning by Freedman et al. (17) the combination of U-sugars and reported added sugar intake (E%) into one composite measure of exposure was obtained using both the principal component (PC) method and the Howe's method. In the PC method, in the case of only two variables that are positively correlated (such as in this situation), the first PC is proportional to the sum of the two variables, each divided by their own standard deviations. In Howe's method, all U-sugar values and added sugar values were ranked, and the ranks were summed (17).

Linear regression was used to examine the associations of U-sugars, reported added sugar intake (E%) and their composite measures with cardiometabolic risk factors. In the regression models, model 1 was adjusted for age and sex [and total energy intake for the analyses of added sugar intake (E%) and the composite measures, i.e., the multivariate nutrient density model was used for energy adjustment (27)], and model 2 was additionally adjusted for educational level, LTPA, smoking status, alcohol consumption habits and fiber density (g/1,000 kcal). The regressions with total cholesterol, triglycerides, HDL and LDL cholesterol were also adjusted for the usage of lipid-lowering drugs, and the regressions with systolic and diastolic BP were also adjusted for the usage of antihypertensive drugs. The interaction with sex was evaluated in all regression analyses. In sex-specific analyses, interactions with obesity (BMI$\geq$30 or BMI$<$30) were evaluated for U-sugars, and interactions with energy underreporting (yes or no) and obesity were evaluated for added sugar intake.

To further understand the relationships, an attempt to identify and map out all potential and measured predictors of U-sugars was performed through partial correlation analyses for men and women separately. The multivariate partial correlation model was determined through stepwise backward linear regression. All covariates were added simultaneously to the linear regression model, and the covariate with the highest *P*-value was excluded from the model in a stepwise manor until all covariates were deemed statistically significant. The investigated variables included added sugar intake; intake of desserts, sweets, toppings, SSBs, fruit, and berries, and juice; educational level; smoking status; alcohol consumption habits; LTPA; BMI; waist circumference; systolic BP; fasting glucose; U-osm; and e-GFR. We investigated U-sugars unadjusted for U-osm. Instead, U-osm was included as a covariate. All of these sex-specific partial correlations and multivariate linear regressions were adjusted for energy intake and age. A significance level of $\alpha = 0.05$ was applied and corrections for multiple testing were not performed. Therefore, the presented *P*-values should be interpreted with caution.

RESULTS

Baseline Characteristics

Among the total study sample of 991 participants with complete dietary data, we obtained valid measurements within the calibration range for U-sucrose [median 32.7 μmol/L (12.6–85.7)] from 889 participants and for U-fructose [median 18.0 μmol/L (7.4–44.0)] from 775 participants (not adjusted for U-osm). In total, 763 participants presented valid measurements for both U-sucrose and U-fructose (**Figure 1**). The mean age of the cohort was 39 years (range 18–69). As observed in **Tables 1**, **2**, the lowest mean age was seen in the highest groups of both added sugar and U-sugars. A higher percentage of women were seen among the low groups of both added sugar and U-sugars. Among those reporting high added sugar intake, we observed lower proportions of energy underreporters, high consumers of alcohol and individuals with regular LTPA $\geq$3 $\times$ 30 min/week in both men and women. In those with high U-sugars, the proportions with a university degree appeared to be lower. High reported energy intake was observed among those reporting high added sugar intake, but not in those with high U-sugars. Higher U-osm was observed in women with higher U-sugars. Intake of most sugar-rich foods and beverages appears to increase with increasing U-sugars, while intake of fruit appears to decrease. Although, a substantial part of zero-consumption has been reported for some of the sugar-rich foods and beverages (**Table 2**).

Correlations Between Urinary and Dietary Sugars

The alluvial plot in **Figure 2** displays the agreement between the 6 categories of added sugar intake and quintiles of U-sugars based on the proportion of participants belonging to each category of the two different variables. In assessment of misclassification, the percentage of gross misclassification equaled 8% for women and 7% for men, while 32 and 34%, respectively, of the values were correctly classified (**Supplementary Figure 1**).

Sucrose intake (g/d), total sugar intake (g/d), total sugar density (g/1,000 kcal) and added sugar intake (E%) showed a higher statistically significant correlation with both U-sucrose and U-sugars ($r \approx 0.20$–0.30, $P < 0.01$), than with U-fructose ($r \approx 0.11$–0.14, $P < 0.03$), after adjusting for energy intake, age, sex and BMI (**Table 3**). Overall, the correlations were slightly weaker with total sugar and total sugar density ($r = 0.21$, $P < 0.01$ and $r = 0.20$, $P < 0.01$ with U-sugars, respectively) than with reported intake of sucrose and added sugar (both $r = 0.24$, $P < 0.01$ with U-sugars), and were weaker for women than for men e.g., ($r = 0.20$, $P < 0.01$ and $r = 0.27$, $P < 0.01$ between added sugar intake and U-sugars, respectively). U-sugars correlated with intake of desserts, sweets and SSBs, but not with toppings, juice and fruits. Additionally, among men but not women, SSB intake correlated with all the different urinary sugars, and U-fructose was positively correlated with juice intake and negatively correlated with fruit intake.

TABLE 1 | Baseline characteristics of women and men in the Malmö Offspring Study across 6 categories of added sugar intake, E%.

Added sugar intake, E%	≤5E%	>5E% to ≤7.5E%	>7.5E% to ≤10E%	>10E% to ≤15E%	>15E% to ≤20E%	>20E%
Women (*n* = 533)						
n (% women)	13 (34.2)	50 (53.2)	100 (56.5)	192 (52.3)	119 (58.1)	59 (53.6)
University degree, %	60.0	45.8	50.0	48.1	41.7	49.1
LTPA ≥3 × 30 min/wk, %	46.2	38.0	21.0	35.9	35.3	20.3
Current smokers, %	0.0	14.6	11.0	8.15	13.8	19.6
Alcohol consumed >twice/wk, %	40.0	41.7	47.8	26.1	22.9	8.93
Energy underreporters, %	69.2	36.0	31.0	30.2	20.2	28.8
Age, y	39.3 (15)	45.7 (13)	43.2 (12)	39.7 (13)	37.6 (14)	32.5 (11)
BMI, kg/m^2	22.2 (1.8)	24.7 (3.2)	25.2 (5.0)	25.0 (4.9)	24.1 (4.3)	26.1 (6.8)
Energy intake, kcal/d	1348 (402)	1569 (398)	1782 (450)	1833 (486)	1894 (493)	1920 (564)
U-osm, mOsm/kg	556 (325)	535 (218)	563 (233)	578 (231)	597 (229)	675 (255)
Men (*n* = 458)						
n (% men)	25 (65.8)	44 (46.8)	77 (43.5)	175 (47.7)	86 (42.0)	51 (43.4)
University degree, %	26.1	23.1	41.8	41.8	21.1	31.3
LTPA ≥3 × 30 min/wk, %	36.0	34.1	36.4	29.7	22.1	19.6
Current smokers, %	8.33	15.4	7.46	9.87	17.8	12.5
Alcohol consumed >twice/wk, %	25.0	53.9	46.3	39.7	28.8	21.7
Energy underreporters, %	64.0	45.5	46.8	30.3	24.4	25.5
Age, y	39.4 (11)	42.2 (15)	41.1 (13)	38.5 (13)	38.2 (13)	37.7 (13)
BMI, kg/m^2	27.2 (4.1)	25.9 (3.3)	26.2 (2.9)	25.9 (4.2)	25.9 (4.3)	26.3 (4.1)
Energy intake, kcal/d	1991 (779)	2014 (532)	2127 (532)	2389 (672)	2440 (664)	2497 (669)
U-osm, mOsm/kg	695 (260)	660 (236)	683 (249)	737 (249)	689 (264)	764 (286)

The categorical variables are expressed as percentages. The continuous variables are expressed as the means (SDs).
LTPA, leisure-time physical activity; BMI, body mass index; U-osm, urine osmolality.

Cardiometabolic Risk Factors

Differences between men and women were observed in the associations between sugar exposure (both added sugar intake and U-sugars) and several cardiometabolic risk factors. U-sugars, but not reported added sugar intake, were positively associated with systolic BP, diastolic BP and fasting glucose only in women. However, added sugar intake, but not U-sugars, was negatively associated with fasting glucose in men. Additionally, in women, both U-sugars and added sugar intake associated positively with BMI and waist circumference, whereas among men, U-sugars were negatively associated with BMI and waist circumference, and no association was observed for added sugar intake. Statistically significant interactions with sex were found for the associations of both U-sugars and added sugar intake with BMI and waist circumference. Added sugar intake was negatively associated with HDL cholesterol in both men and women. No associations were found with total cholesterol, triglycerides or LDL cholesterol (**Table 4**).

The combination of U-sugars and reported added sugars into a composite measure of sugar exposure using the PC method revealed statistically significant positive associations with BMI, waist circumference and systolic BP, and a statistically significant negative association with HDL cholesterol in women, whereas none of the cardiometabolic risk factors were associated with the PC of sugar exposure in men (**Table 4**). Combining by Howe's method (**Supplementary Table 1**) showed resembling associations with the same cardiometabolic risk factors as when using the PC method, but overall yielded lower coefficients and only the associations with HDL were statistically significant.

Energy underreporting was identified as a statistically significant effect modifier in the associations between reported added sugar intake and BMI and waist circumference in women. These positive associations were attenuated after the removal of energy underreporters. A statistically significant interaction between obesity and U-sugars was obtained in the regression with systolic BP in women: obese individuals exhibited a markedly stronger positive association than the non-obese individuals (BMI≥30: $\beta = 3.15$, $P = 0.03$; BMI<30: $\beta = 11.9$, $P < 0.01$; **Supplementary Table 2**).

Potential Predictors of Overnight Urinary Sugars

We used stepwise backward linear regression in an attempt to identify the major predictors of U-sugars (not adjusted for U-osm) (**Table 5**). After taking possible and measured predictors into account, the main predictors of the various sugar intake variables were added sugar intake for men ($r = 0.31$) and intake of desserts ($r = 0.10$) and sweets ($r = 0.21$) for women. U-osm was found to be a strong predictor in both women and men ($r = 0.41$ and $r = 0.40$, respectively). Systolic BP and fasting glucose also exhibited positive associations with U-sugars in women, whereas education level and waist circumference showed negative associations

TABLE 2 | Baseline characteristics of women and men in the Malmö Offspring Study across quintiles (Q1–Q5) of U-sugars, (μmol·L^{-1})/(mOsm·kg^{-1}).

	Q1	Q2	Q3	Q4	Q5
U-sugars, mean (range), (μmol·L^{-1})/(mOsm·kg^{-1})	**0.02 (0.01–0.04)**	**0.05 (0.04–0.07)**	**0.09 (0.07–0.12)**	**0.17 (0.12–0.26)**	**0.43 (0.26–3.85)**
Women (*n* = 412)					
n (% women)	75 (49.0)	76 (50.0)	70 (45.8)	96 (62.8)	95 (62.5)
University degree, %	50.7	60.0	44.8	50.0	36.0
LTPA≥3 × 30 min/wk, %	29.3	35.5	31.4	25.0	26.3
Current smokers, %	7.04	8.45	10.5	17.4	13.5
Alcohol consumed >twice/wk, %	29.6	32.4	32.8	39.5	21.4
Energy underreporters, %	32.0	25.0	22.9	25.0	27.4
Age, y	41.1 (14)	42.2 (12)	40.1 (13)	39.8 (13)	36.9 (13)
BMI, kg/m^2	24.4 (4.17)	25.2 (4.76)	24.6 (4.18)	24.6 (4.27)	25.5 (5.63)
U-osm, mOsm/kg	540 (236)	568 (229)	636 (250)	592 (239)	653 (244)
Energy intake, kcal/d	1,782 (508)	1,869 (481)	1,840 (443)	1,835 (500)	1,864 (536)
Sucrose, g/d	33.0 (20.3)	38.2 (23.7)	35.7 (19.2)	41.2 (23.9)	45.9 (27.8)
Total sugar, g/d	73.7 (30.4)	83.1 (31.2)	76.2 (30.5)	84.3 (35.2)	90.5 (38.4)
Total sugar density, g/1,000 kcal	41.3 (11.3)	44.6 (12.4)	41.1 (12.8)	45.5 (14.5)	48.3 (14.6)
Added sugar, E%	11.6 (4.98)	12.8 (4.50)	12.1 (4.92)	13.6 (5.85)	15.0 (5.42)
Desserts, g/d[a]	25.3 (10.0, 53.1)	28.1 (10.6, 69.8)	35.4 (21.3, 59.5)	38.1 (11.9, 73.1)	37.5 (12.8, 75.0)
Sweets, g/d[a]	10.0 (0.5, 20.8)	13.4 (3.5, 28.9)	13.8 (3.5, 37.0)	15.5 (4.3, 35.9)	13.8 (0.0, 50.0)
Toppings, servings/d[a]	0.24 (0.0, 0.54)	0.05 (0.0, 0.84)	0.0 (0.0, 0.59)	0.26 (0.0, 0.71)	0.0 (0.0, 0.54)
SSBs, g/d[a]	0.0 (0.0, 50.0)	0.0 (0.0, 75.0)	0.0 (0.0, 125)	25.0 (0.0, 129)	0.0 (0.0, 150)
Juice, g/d[a]	0.0 (0.0, 75.0)	0.0 (0.0, 90.6)	0.0 (0.0 (75.0)	0.0 (0.0, 87.5)	0.0, 0.0, 75.0)
Fruits, g/d[a]	103 (39.0, 170)	95.1 (55.8, 169)	81.1 (47.2, 128)	82.3 (32.6, 152)	75.6 (24.5, 155)
Men (*n* = 351)					
n (% men)	78 (51.0)	76 (50.0)	83 (54.3)	57 (37.3)	57 (37.5)
University degree, %	33.3	50.8	30.0	25.5	22.7
LTPA ≥3 × 30 min/wk, %	32.1	26.3	28.9	28.1	29.8
Current smokers, %	7.04	8.82	18.1	6.38	15.9
Alcohol consumed >twice/wk, %	40.3	48.5	40.9	36.2	27.3
Energy underreporters, %	39.7	36.8	33.7	31.6	21.1
Age, y	39.7 (11.0)	41.5 (12.8)	39.9 (14.5)	39.1 (13.7)	36.9 (14.3)
BMI, kg/m^2	27.5 (4.52)	25.9 (3.53)	25.8 (3.61)	26.4 (3.98)	25.5 (4.23)
U-osm, mOsm/kg	740 (264)	698 (262)	692 (245)	689 (260)	781 (230)
Energy intake, kcal/d	2,278 (707)	2,297 (670)	2,370 (624)	2,285 (575)	2,479 (698)
Sucrose, g/d	36.6 (29.3)	47.4 (31.7)	47.0 (25.9)	50.3 (33.7)	66.8 (39.9)
Total sugar, g/d	79.9 (40.6)	93.7 (46.3)	96.7 (41.0)	97.7 (47.4)	113 (49.3)
Total sugar density, g/1,000 kcal	34.2 (12.0)	40.5 (13.6)	40.5 (12.9)	42.0 (16.3)	45.3 (14.5)
Added sugar, E%	10.6 (4.94)	12.5 (5.06)	13.0 (5.06)	13.8 (5.51)	15.4 (6.15)
Desserts, g/d[a]	27.5 (0.0, 57.5)	30.0 (6.3, 63.5)	38.0 (10.0, 72.5)	33.8 (10.0, 72.5)	56.3 (15.0, 94,8)
Sweets, g/d[a]	0.0 (0.0, 20.5)	7.8 (0.0, 20.8)	11.3 (0.0, 26.3)	9.0 (0.0, 35.0)	12.5 (0.0, 49.0)
Toppings, servings/d[a]	0.0 (0.0, 0.39)	0.09 (0.0, 0.67)	0.31 (0.0, 0.89)	0.0 (0.0, 0.80)	0.0 (0.0, 0.76)
SSBs, g/d[a]	0.0 (0.0, 100)	75.0 (0.0, 175)	75.0 (0.0, 200)	125 (0.0, 275)	200 (0.0, 375)
Juice, g/d[a]	0.0 (0.0, 100)	0.0 (0.0, 150)	0.0 (0.0, 100)	0.0 (0.0, 150)	0.0 (0.0, 150)
Fruits, g/d[a]	42.7 (5.7, 84.5)	57.1 (14.5, 103)	42.5 (12.5, 98.0)	31.7 (4.4, 100)	28.0 (2.0, 64.5)

The categorical variables are expressed as percentages. The continuous variables are expressed as the means (SDs) unless stated otherwise.

[a] *data is expressed as median (IQR) due to skewed distribution.*

U-sugars, sum of urinary sucrose and fructose; LTPA, leisure-time physical activity; BMI, body mass index; U-osm, urine osmolality, SSB, sugar-sweetened beverage.

with U-sugars in men. When examining osmolality-adjusted U-sugars instead, excluding U-osm in the multivariate model, the same predictors remained to a similar extent (data not shown).

DISCUSSION

We showed statistically significant correlations of $r \approx 0.20–0.30$ between reported sugar intakes and overnight urinary sugars

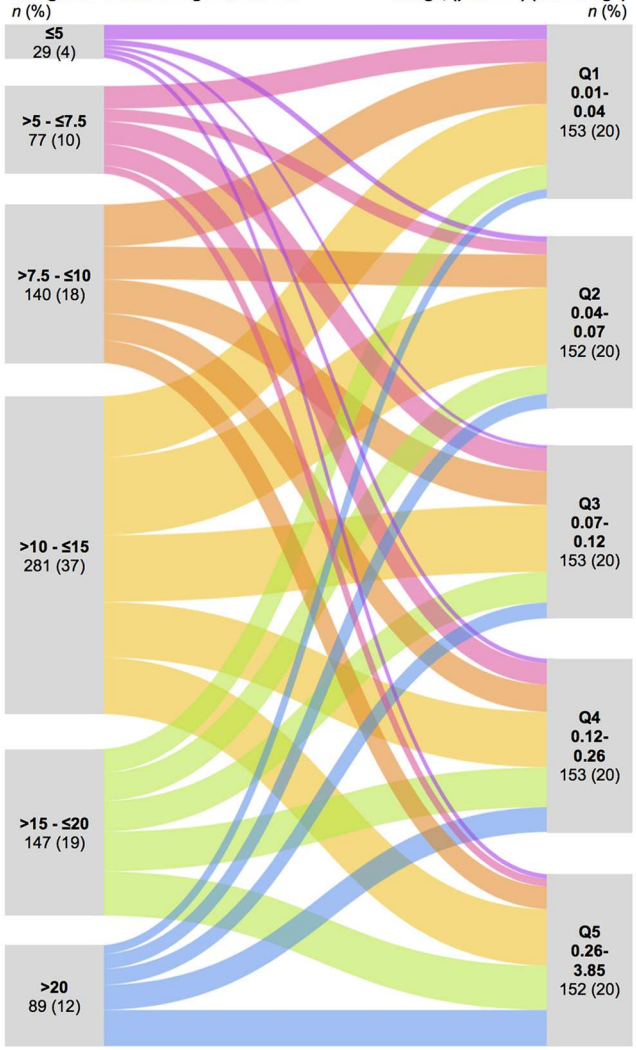

FIGURE 2 | Alluvial plot demonstrating the agreement based on crosstabulation of the 6 categories of reported added sugar intake (≤5E, >5-≤7.5E, >7.5-≤10E, >10-≤15E, >15-≤20E, and >20E%) and quintiles of U-sugars (Q1–Q5). U-sugars, sum of urinary sucrose and fructose.

after adjusting for age, sex, energy intake, and BMI. The relatively low coefficients of these correlations and the modest agreement observed in the alluvial plot may reflect that both these measures of sugar intake are subject to random variation and measurement error. However, importantly, such possible errors are completely unrelated (misreporting and potential unknown determinants), which therefore indicates a potential and need for the combination of these two measurements. As discussed and shown in both data simulations and in real world examples by Freedman et al. (17, 18), even when the correlation between reported intakes and the biomarker is not very high, combination of the two measurements is motivated. Hence, these two measurements could potentially complement each other to improve the assessment of the associations between added sugar intake and cardiometabolic risk.

The observed correlation coefficients in this study agree with the results of a previous study of spot morning urine samples in children ($r = 0.25$) (13). This similarity was obtained even though the collection of urinary and dietary data did not reflect the exact same days in our study, which was the case in the previous study. However, the previous study did not reveal large differences in the comparison of the single 24-h recall from the day before collection of the morning spot urine samples and multiple 24-h recalls (13). The correlation coefficients obtained in our study are also similar to previous findings obtained with the validated predictive 24-h urinary sugar biomarker in free living populations; in the Nutrition and Physical Activity Assessment Study, the correlation with total sugar density from a 4-day food record was $r = 0.21$ (8), which is comparable to that found in our study between total sugar density and U-sugars ($r = 0.20$). Because we only can compare against self-reported sugar intake and not true intake, it is not straightforward to compare these correlations and the observed exact agreement of 32–34% to warranted limits used in biomarker validation studies [$r = 0.5$–0.6 and quartile agreement of at least 50% (28, 29)].

In our study, the correlation of reported added sugar intake with U-fructose was notably weaker than that with U-sucrose (0.14 and 0.27, respectively), even though only monosaccharides, in theory, should be absorbed in the jejunum. In addition, total sugar (g/d) and total sugar density (g/1,000 kcal) were not as strongly correlated with the urinary sugars as sucrose intake (g/d) and added sugar intake (E%). Previous studies have also revealed weaker correlations for intrinsic sugar (included in total sugars) than for extrinsic sugars (mainly added sugars) with the urinary sugar biomarker (13, 30). This effect could be due to the rate of digestion and absorption of the sugars, which is believed to be lower when the sugars are naturally occurring in complex foods compared to simple sugars added to foods (31). Additionally, the relatively high intakes of sugars from dairy (lactose, included in total sugars) in the Swedish diet might contribute to some of these differences.

We observed slightly stronger correlations between dietary sugars and urinary sugars in men than in women, which is supported by the findings of previous studies (7, 12). In addition to biological differences between men and women, another plausible reason for the sex differences could be that women generally tend to underreport their dietary intake more than men (7, 32, 33). However, we do not know the degree of sugar intake underreporting in our study, and energy misreporting does not necessarily reflect misreporting of sugar. However, the percentage of energy underreporters was lower among those with higher added sugar intake and tended to be lower in those with higher U-sugars. Furthermore, the high proportion of zero-reporters of SSB intake among women (57%), might contribute to why we only can see a statistically significant correlation between SSB intake and U-sugars in men but not in women.

No previous studies have evaluated urinary sugar biomarkers in relation to cardiometabolic risk factors other than

TABLE 3 | Partial correlations between U-sucrose, U-fructose and U-sugars and different measures and sources of dietary sugars in all, women and men in the Malmö Offspring Study.

	U-sucrose			U-fructose			U-sugars		
	n	***r***	***P*-value**	***n***	***r***	***P*-value**	***n***	***r***	***P*-value**
All									
Sucrose (g/d)	889	0.27	< 0.01	775	0.13	< 0.01	763	0.24	< 0.01
Total sugar (g/d)	889	0.22	< 0.01	775	0.12	< 0.01	763	0.21	< 0.01
Total sugar density (g/1,000 kcal)	889	0.22	< 0.01	775	0.13	< 0.01	763	0.20	< 0.01
Added sugar (E%)	889	0.27	< 0.01	775	0.14	< 0.01	763	0.24	< 0.01
Desserts (g/d)	889	0.09	< 0.01	775	0.05	0.14	763	0.11	< 0.01
Sweets (g/d)	889	0.20	< 0.01	775	0.07	0.04	763	0.18	< 0.01
Toppings (servings/d)	889	0.03	0.31	775	−0.01	0.78	763	0.02	0.67
SSBs (g/d)	889	0.18	< 0.01	775	0.09	0.01	763	0.16	< 0.01
Juice (g/d)	889	0.04	0.25	775	0.11	< 0.01	763	0.06	0.08
Fruits (g/d)	889	−0.04	0.21	775	−0.04	0.25	763	−0.05	0.15
Women									
Sucrose (g/d)	467	0.23	< 0.01	421	0.11	0.03	412	0.19	< 0.01
Total sugar (g/d)	467	0.19	< 0.01	421	0.13	< 0.01	412	0.18	< 0.01
Total sugar density (g/1,000 kcal)	467	0.15	< 0.01	421	0.12	0.02	412	0.16	< 0.01
Added sugar (E%)	467	0.21	< 0.01	421	0.13	< 0.01	412	0.20	< 0.01
Desserts (g/d)	467	0.05	0.25	421	0.005	0.92	412	0.09	0.07
Sweets (g/d)	467	0.21	< 0.01	421	0.12	0.02	412	0.20	< 0.01
Toppings (servings/d)	467	−0.02	0.65	421	−0.05	0.33	412	−0.04	0.48
SSBs (g/d)	467	0.08	0.08	421	0.01	0.79	412	0.05	0.34
Juice (g/d)	467	0.02	0.71	421	0.05	0.27	412	0.03	0.48
Fruits (g/d)	467	−0.05	0.33	421	0.0007	0.99	412	−0.03	0.60
Men									
Sucrose (g/d)	422	0.30	< 0.01	354	0.14	< 0.01	351	0.28	< 0.01
Total sugar (g/d)	422	0.25	< 0.01	354	0.11	0.03	351	0.22	< 0.01
Total sugar density (g/1,000 kcal)	422	0.27	< 0.01	354	0.12	0.02	351	0.23	< 0.01
Added sugar (E%)	422	0.31	< 0.01	354	0.13	0.01	351	0.27	< 0.01
Desserts (g/d)	422	0.13	< 0.01	354	0.11	0.05	351	0.12	0.02
Sweets (g/d)	422	0.18	< 0.01	354	0.01	0.83	351	0.16	< 0.01
Toppings (servings/d)	422	0.08	0.12	354	0.02	0.68	351	0.05	0.34
SSBs (g/d)	422	0.26	< 0.01	354	0.16	< 0.01	351	0.25	< 0.01
Juice (g/d)	422	0.05	0.33	354	0.15	< 0.01	351	0.09	0.11
Fruits (g/d)	422	−0.04	0.37	354	−0.12	0.03	351	−0.10	0.05

The partial correlations are adjusted for age, sex, energy intake and BMI (not adjusted for sex in sex-specific analyses). The urinary sugar variables are log_{10}-transformed. U-sucrose, urinary sucrose; U-fructose, urinary fructose; U-sugars, sum of urinary sucrose and fructose; SSB, Sugar-sweetened beverages; BMI, body mass index.

anthropometric measurements. The examination of spot urinary sucrose (not morning urine) in relation to obesity measures in the EPIC Norfolk cohort revealed a positive association between the risk of being overweight and higher spot urinary sucrose, whereas a negative association between risk of being overweight and higher self-reported sugar intake (12). Hence, it can be speculated that the lack of a positive association between reported sugar intake and risk of being overweight might be partly explained by a measurement error bias in the dietary assessment, which is not an issue with the objective measurement of sucrose in spot urine samples. Similar patterns were observed in our study for systolic BP, diastolic BP and fasting glucose in women; these parameters were positively associated with U-sugars but not with reported added sugar intake. However, such a pattern was not observed for the other cardiometabolic risk factors. In fact, both U-sugars and added sugar intake were positively associated with BMI and waist circumference and negatively associated with HDL cholesterol in women, indicating quite credible associations, and the combination of the two measurements strengthened the associations for these cardiometabolic risk factors. In men, however, U-sugars were negatively associated with BMI and waist circumference, while no association was found with added sugar intake. Nevertheless, cross-sectional examination of BMI and waist circumference in relation to dietary intake is difficult because large body measurements might affect one's dietary awareness more than the "nonvisual" cardiometabolic risk factors. Hence, the direction of the

TABLE 4 | Linear regression of U-sugars, added sugar intake and their composite measure (PC) on cardiometabolic risk factors in the Malmö Offspring Study.

	All				Women			Men		
	n	ß	95% CI	*P*-int sex	*n*	ß	95% CI	*n*	ß	95% CI
BMI (kg/m^2)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	763	0.15	−0.45, 0.77		412	1.03	0.14, 1.92	351	−1.00	−1.84, −0.16
Model 2	677	0.08	−0.58, 0.74	<0.01	381	1.05	0.12, 1.97	296	−1.45	−2.40, −0.51
Added sugar (E%)										
Model 1	991	0.08	0.02, 0.13		533	0.14	0.06, 0.22	458	0.01	−0.05, 0.08
Model 2	889	0.03	−0.03, 0.09	0.03	493	0.10	0.01, 0.19	396	−0.03	−0.10, 0.05
Composite measure										
Model 1	763	0.35	0.15, 0.55		412	0.55	0.28, 0.82	351	−0.04	−0.35, 0.27
Model 2	677	0.26	0.04, 0.48	<0.01	381	0.50	0.22, 0.79	296	−0.24	−0.59, 0.11
Waist circumference (cm)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	763	−0.09	−1.68, 1.50		412	2.00	−0.21, 4.21	351	−2.94	−5.18, −0.70
Model 2	677	−0.20	−1.84, 1.45	<0.01	381	2.02	−0.23, 4.28	296	−3.79	−6.19, −1.39
Added sugar (E%)										
Model 1	991	0.24	0.11, 0.37		533	0.34	0.14, 0.53	458	0.13	−0.05, 0.30
Model 2	889	0.15	0.01, 0.29	0.05	493	0.25	0.03, 0.46	396	0.05	−0.14, 0.24
Composite measure										
Model 1	763	1.00	0.49, 1.51		412	1.34	0.68, 2.00	351	0.23	−0.60, 1.06
Model 2	677	0.76	0.22, 1.30	<0.01	381	1.19	0.51, 1.88	296	0.19	−1.09, 0.70
Total cholesterol (mmol/L)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	763	−0.08	−0.22, 0.05		412	−0.06	−0.23, 0.11	351	−0.11	−0.33, 0.11
Model 2	677	−0.14	−0.28, 0.01	0.91	381	−0.12	−0.30, 0.06	296	−0.15	−0.40, 0.10
Added sugar (E%)										
Model 1	990	−0.006	−0.02, 0.01		532	−0.009	−0.02, 0.01	458	−0.002	−0.02, 0.02
Model 2	888	−0.009	−0.02, 0.004	0.41	492	−0.02	−0.03, 0.0003	396	−0.001	−0.02, 0.02
Composite measure										
Model 1	763	−0.02	−0.06, 0.03		412	−0.02	−0.07, 0.04	351	−0.01	−0.09, 0.07
Model 2	677	−0.03	−0.07, 0.02	0.69	381	−0.03	−0.08, 0.03	296	−0.02	−0.11, 0.08
Triglycerides (mmol/L)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	757	0.04	−0.05, 0.13		409	0.02	−0.07, 0.11	348	0.06	−0.11, 0.23
Model 2	671	0.02	−0.08, 0.11	0.96	378	0.007	−0.09, 0.11	293	−0.01	−0.20, 0.18
Added sugar (E%)										
Model 1	980	0.007	−0.001, 0.01		527	−0.00001	−0.01, 0.01	453	0.01	−0.001, 0.02
Model 2	878	0.003	−0.01, 0.01	0.49	487	−0.004	−0.01, 0.01	391	0.006	−0.01, 0.02
Composite measure										
Model 1	757	0.03	−0.00001, 0.06		409	0.01	−0.02, 0.04	348	0.06	−0.01, 0.12
Model 2	671	0.02	−0.01, 0.05	0.49	378	0.003	−0.03, 0.03	293	0.03	−0.03, 0.10
HDL cholesterol (mmol/L)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	763	−0.06	−0.13, −0.003		412	−0.10	−0.19, −0.01	351	−0.02	−0.10, 0.06
Model 2	677	−0.04	−0.11, 0.02	0.16	381	−0.07	−0.17, 0.02	296	0.01	−0.07, 0.09
Added sugar (E%)										
Model 1	990	−0.02	−0.02, −0.01		532	−0.02	−0.03, −0.01	458	−0.01	−0.02, −0.005
Model 2	888	−0.01	−0.02, −0.01	0.04	492	−0.02	−0.02, −0.01	396	−0.009	−0.02, −0.002
Composite measure										
Model 1	763	−0.04	−0.06, −0.02		412	−0.05	−0.08, −0.02	351	−0.03	−0.06, −0.01
Model 2	677	−0.03	−0.05, −0.01	0.32	381	−0.03	−0.06, −0.001	296	−0.03	−0.06, 0.002

(Continued)

TABLE 4 | Continued

	All			*P*-int sex	Women			Men	
	n	**ß**	**95% CI**		***n***	**ß**	**95% CI**	***n***	**ß**	**95% CI**
LDL cholesterol (mmol/L)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	763	−0.06	−0.18, 0.06		412	−0.02	−0.17, 0.13	351	−0.11	−0.31, 0.09
Model 2	677	−0.13	−0.26, 0.01	0.66	381	−0.09	−0.26, 0.07	296	−0.16	−0.39, 0.07
Added sugar (E%)										
Model 1	989	0.006	−0.004, 0.02		531	0.006	−0.01, 0.02	458	0.007	−0.01, 0.02
Model 2	887	0.002	−0.01, 0.01	0.68	491	−0.003	−0.02, 0.01	396	0.008	−0.01, 0.03
Composite measure										
Model 1	763	0.01	−0.03, 0.05		412	0.02	−0.03, 0.06	351	0.006	−0.07, 0.08
Model 2	677	−0.006	−0.05, 0.04	0.90	381	−0.006	−0.06, 0.04	296	0.008	−0.07, 0.09
Systolic BP (mmHg)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	761	2.92	1.13, −4.70		410	4.22	1.74, 6.71	351	1.54	−0.98, 4.05
Model 2	675	2.95	0.99, 4.92	0.22	379	4.63	1.96, 7.30	296	1.30	−1.55, 4.16
Added sugar (E%)										
Model 1	988	−0.09	−0.24, 0.05		530	−0.02	−0.23, 0.20	458	−0.12	−0.31, 0.07
Model 2	886	−0.09	−0.26, 0.08	0.87	490	−0.02	−0.27, 0.22	396	−0.09	−0.31, 0.13
Composite measure										
Model 1	761	0.48	−0.11, 1.06		410	0.89	0.13, 1.66	351	0.13	−0.79, 1.06
Model 2	675	0.46	−0.19, 1.12	0.55	379	1.01	0.17, 1.85	296	−0.09	−1.13, 0.96
Diastolic BP (mmHg)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	761	1.10	−0.13, 2.33		410	1.80	0.16, 3.43	351	0.17	−1.71, 2.04
Model 2	675	1.30	−0.01, 2.62	0.43	379	1.81	0.07, 3.55	296	0.58	−1.48, 2.64
Added sugar (E%)										
Model 1	988	0.03	−0.07, 0.13		530	0.008	−0.13, 0.15	458	0.05	−0.09, 0.20
Model 2	886	−0.01	−0.12, 0.10	0.98	490	−0.02	−0.18, 0.14	396	0.003	−0.15, 0.16
Composite measure										
Model 1	761	0.54	0.14, 0.94		410	0.49	−0.01, 0.99	351	0.60	−0.08, 1.28
Model 2	675	0.45	0.02, 0.88	0.86	379	0.48	−0.06, 1.02	296	0.41	−0.34, 1.16
Fasting glucose (mmol/L)										
U-sugars (μmol·L^{-1})/(mOsm·kg^{-1})										
Model 1	762	0.09	−0.003, 0.19		412	0.14	0.04, 0.25	350	0.01	−0.15, 0.18
Model 2	677	0.10	0.0003, 0.20	0.22	381	0.16	0.05, 0.26	296	0.03	−0.17, 0.22
Added sugar (E%)										
Model 1	990	−0.007	−0.01, 0.001		533	−0.003	−0.01, 0.01	457	−0.01	−0.02, −0.00001
Model 2	889	−0.008	−0.02, 0.001	0.04	493	−0.002	−0.01, 0.01	396	−0.01	−0.03, 0.001
Composite measure										
Model 1	762	−0.0003	−0.03, 0.03		412	0.009	−0.02, 0.04	350	−0.03	−0.09, 0.03
Model 2	677	−0.0004	−0.03, 0.03	0.11	381	0.01	−0.02, 0.05	296	−0.03	−0.10, 0.04

Total cholesterol, triglycerides, HDL, and LDL cholesterol and fasting glucose are measured in plasma. U-sugars are log_{10}-transformed. The composite measure is the first PC of the two variables U-sugars and added sugars.

Model 1 is adjusted for age and sex (and energy intake for added sugar and the composite measure).

Model 2 is additionally adjusted for educational level, LTPA, smoking status, alcohol habits, and fiber density. Regressions with total cholesterol, triglycerides, HDL, and LDL cholesterol are additionally adjusted for usage of lipid lowering drugs and regressions with systolic and diastolic BP are additionally adjusted for usage of antihypertensive drugs.

U-sugars, sum of urinary sucrose and fructose; PC, principal component; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BP, blood pressure; LTPA, leisure-time physical activity.

observed associations is uncertain. Furthermore, there may exist sex differences in the effects on weight gain from a high sugar diet as it has been observed that the inhibition of lipolysis by insulin is more profound in women than men (34). Our observed sex differences in the associations with cardiometabolic risk factors were unexpected findings outside the scope of our study objective, which futures studies are encouraged to elucidate.

TABLE 5 | Partial correlation coefficients between U-sugars (not adjusted for U-osm) and its potential predictors in women and men of the Malmö Offspring Study.

	Women ($n = 373$)				Men ($n = 295$)			
	Separate models[a]		Multivariate model[a, b]		Separate models[a]		Multivariate model[a, b]	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Added sugar (E%)	0.23	< 0.01			0.27	< 0.01	0.31	< 0.01
Desserts (g/d)	0.09	0.08	0.10	0.04	0.14	< 0.01		
Sweets (g/d)	0.22	< 0.01	0.21	< 0.01	0.16	< 0.01		
Toppings (servings/d)	−0.02	0.72			0.05	0.35		
SSBs (g/d)	0.07	0.15			0.25	< 0.01		
Fruits (g/d)	−0.10	0.05			−0.14	0.01		
Juice (g/d)	0.02	0.68			0.10	0.07		
Education level	−0.12	0.03			−0.12	0.05	−0.13	0.02
Smoking status	0.05	0.35			0.06	0.28		
Alcohol habits	−0.09	0.09			−0.005	0.93		
LTPA	−0.05	0.34			−0.03	0.56		
BMI (kg/m^2)	0.11	0.02			−0.08	0.12		
Waist circumference (cm)	0.09	0.08			−0.08	0.12	−0.18	< 0.01
Systolic BP (mmHg)	0.18	< 0.01	0.17	< 0.01	0.08	0.13		
Fasting glucose (mmol/L)	0.13	< 0.01	0.12	0.01	0.01	0.78		
U-osm (mOsm/kg)	0.41	< 0.01	0.41	< 0.01	0.39	< 0.01	0.40	< 0.01
e-GFR (ml/min/1.73 m^2)	−0.05	0.33			−0.08	0.16		

[a] *All partial correlations are adjusted for age and energy intake.*

[b] *The multivariate partial correlation model was determined through stepwise backward linear regression. All covariates were added simultaneously to a linear regression model and the covariate with the highest P-value was excluded in a stepwise manner from the model until all covariates were deemed significant.*

U-sugars are log_{10}-transformed. Fasting glucose is measured in plasma.

U-sugars, sum of urinary sucrose and fructose; U-osm, urine osmolality; SSB, sugar-sweetened beverages; LTPA, leisure-time physical activity; BMI, body mass index; BP, blood pressure; e-GFR, estimated glomerular filtration rate.

Previous studies have discussed whether the amount of urinary sucrose and fructose might differ between obese and lean participants (11, 35) due to the potentially higher gut permeability of obese individuals (36, 37). Therefore, the associations between U-sugars and measures of obesity might be due to other underlying causes in addition to the notion that a high sugar intake would lead to weight gain. Nevertheless, no difference in the 24-h urinary levels of either sucrose or fructose in obese compared with normal weight subjects was observed in a randomized controlled trial (35). In women, we observed positive correlations between U-sugar and systolic BP and fasting glucose and these parameters also fell out as predictors of U-sugars, but one could discuss the putative causal direction of these associations. In addition to the theory that a high sugar intake would lead to an impaired metabolic status, both systolic BP and fasting glucose are major risk factors for renal insufficiency and it is possible that this could influence urinary excretion of sugars (38). However, to our knowledge, no previous study has shown that the amounts of sucrose and fructose excreted in the urine is affected by insulin resistance. Because only glucose is regulated by insulin in the circulation, the same principle as for urinary excretion of glucose cannot be applied to sucrose and fructose.

The limitations of this study are the lack of longitudinal data for the cardiometabolic risk factors and that urinary sugar data is generated from overnight urine samples instead of 24-h samples. We were therefore also bound to use other methods than regression calibration for combining the biomarker with reported intake (10). To date, the overnight urine biomarker has only been compared to self-reported sugar intake data, which cannot be used for validating a nutritional biomarker (39), and no earlier study has ever reported the correlation between the sugar concentrations in overnight urine samples and 24-h urine samples. However, the benefit of using overnight samples over any time spot samples is that they are less affected by recent past meals (40). Furthermore, residual confounding can almost be considered indisputable, and future studies are needed to identify the determinants of spot and overnight urinary sugars. Therefore, the following important question remains: Which measurement is most valid, the self-reported added sugar intake, which is likely to be biased by misreporting, or the sum of sucrose and fructose in overnight urine samples, which only reflects a point measurement and for which determinants other than sugar intake remain unknown? At this current state of knowledge, we believe that they both contribute partly to the truth and may complement each other. However, this must be validated against true sugar intake or the 24-h urinary sucrose and fructose biomarker in the future. Future studies should also investigate potential sex differences to improve the understanding of the urinary sugar biomarker, as well as considering the use of repeated overnight or spot urine samples to obtain improved precision, while

still facilitating urine collection as compared with 24-h urine sampling.

In summary, we found statistically significant correlations at levels of $r \approx 0.20–0.30$ and demonstrated the potential for using the sugar level in overnight urine samples to complement self-reported dietary data in investigations of cardiometabolic risk. The combination of U-sugars and added sugar intake indicated that a higher sugar intake in women is associated with higher BMI, waist circumference and systolic BP and lower HDL cholesterol. Considering the potential gains from collecting only overnight urine instead of 24-h urine in regard to participant burden, drop-out rates, missing data and selective participation, the overnight urinary sugar biomarker calls for further validation.

DATA AVAILABILITY STATEMENT

The dataset for this article is not publicly available because of ethical and legal restrictions. Requests to access the dataset should be directed to the Chair of the Steering Committee for the Malmö cohorts, see instructions at https://www.malmo-kohorter.lu.se/english.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethics Committee in Lund. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SR and ES designed the research. SR performed all statistical analyses and wrote the manuscript under the supervision of ES. NG performed the laboratory analyses of the urine samples under the supervision of GK. SE contributed with data on the urine osmolality. The collection of dietary data in the MOS was administered by SH, UE, LB, and SR. PN is the principal investigator for the MOS, whereas MO-M is in charge of the dietary data in the MOS. NG, SH, LB, SE, GE, PN, MO-M, UE, GK, and ES all contributed important input to the manuscript. SR and ES had primary responsibility for the final content.

FUNDING

This study was funded by the Swedish Research Council (2016-01501); the Heart and Lung Foundation (2016-0267); and the Albert Påhlsson Foundation. The MOS was supported by grants from the Swedish Research Council (521-2013-2756, 2013-210, 2014-366, 2018-02784); the Heart and Lung Foundation (2015-0427, 2013-0598, 2017-0523); the Region Skåne, Skåne University Hospital; the European Research Council (ERC-CoG-2014-649021); the EFSD Lilly Award 2014 (2015-338); the Swedish Diabetes Foundation (DIA 2018-358); and the Novo Nordisk Foundation (NNF17OC0027348, NNF18OC0034386). We also acknowledge the support provided by the Swedish Foundation for Strategic Research (IRC15-0067).

ACKNOWLEDGMENTS

We thank all the participants and research staff in the Malmö Offspring Study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.00062/full#supplementary-material

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Conflict of Interest: GK previously received an unrestricted grant from Mars for research on flavanols unrelated to the current study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 02 July 2020
doi: 10.3389/fnut.2020.00080

Future Directions for Integrative Objective Assessment of Eating Using Wearable Sensing Technology

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Established methods for nutritional assessment suffer from a number of important limitations. Diaries are burdensome to complete, food frequency questionnaires only capture average food intake, and both suffer from difficulties in self estimation of portion size and biases resulting from misreporting. Online and app versions of these methods have been developed, but issues with misreporting and portion size estimation remain. New methods utilizing passive data capture are required that address reporting bias, extend timescales for data collection, and transform what is possible for measuring habitual intakes. Digital and sensing technologies are enabling the development of innovative and transformative new methods in this area that will provide a better understanding of eating behavior and associations with health. In this article we describe how wrist-worn wearables, on-body cameras, and body-mounted biosensors can be used to capture data about when, what, and how much people eat and drink. We illustrate how these new techniques can be integrated to provide complete solutions for the passive, objective assessment of a wide range of traditional dietary factors, as well as novel measures of eating architecture, within person variation in intakes, and food/nutrient combinations within meals. We also discuss some of the challenges these new approaches will bring.

Keywords: objective, assessment, eating, wearable, technology

OPEN ACCESS

Edited by:
Natasha Tasevska, Arizona State University Downtown Phoenix Campus, United States

Reviewed by:
Adam Hoover, Clemson University, United States
José María Huerta, Instituto de Salud Carlos III, Spain

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *02 March 2020*
Accepted: *05 May 2020*
Published: *02 July 2020*

Citation:
Skinner A, Toumpakari Z, Stone C and Johnson L (2020) Future Directions for Integrative Objective Assessment of Eating Using Wearable Sensing Technology. Front. Nutr. 7:80. doi: 10.3389/fnut.2020.00080

INTRODUCTION

Non-communicable diseases now account for almost three quarters of global mortality, with cardiovascular disease (CVD) being the leading cause of death. Diet is responsible for more than half of CVD mortality worldwide (1). The proportion of diet-related deaths has remained relatively stable since 1990 suggesting interventions to improve food intakes have had limited success (1). A major issue in combatting diet-related disease is the way in which food intake and eating behavior are assessed. Accurate measurement of eating is key to monitoring the status quo and responses to individual or systems level interventions.

Recent years have seen a shift in nutritional science away from a focus on single nutrients such as saturated fats, toward a recognition that the complexity in patterns of food intake (e.g., combinations of foods and nutrients throughout the day), is more important in determining health (2–4). In addition to what we eat, we need to extend our understanding of eating architecture—the structure within which food and drinks are consumed. Factors such as the size, timing, and

frequency of eating are increasingly recognized as independent determinants of health over and above what food is being eaten (5, 6). For example, skipping breakfast is consistently associated with higher body weight and poorer health outcomes (5, 7). Breakfast tends to be a small meal eaten in the morning made up of foods higher in fiber and micronutrients and it's not clear which of these features (meal size, timing, or food type), if any, are causing the benefits to health (8).

Traditional methods of dietary assessment, such as food diaries, 24-h recalls and food frequency questionnaires (FFQs), are self-reported and prone to substantial error and bias (9–11), which may distort diet and health associations (12). Misreporting is one widely recognized limitation of self-reported dietary assessment methods, with systematic under-reporting of energy intake identified in upto 70% of adults of adults in the UK National Diet and Nutrition Surveys (13, 14). Under-reporting occurs for a range of reasons including; difficulties estimating portion sizes for ingredients of complex meals, a desire to present one's diet positively (social desirability), and poor memory (11). People tend to under-report between-meal snacks, possibly because these snacks tend to be less socially desirable or because they are more sporadic, easily forgotten events (15).

Multi-day food diaries or 24-h recalls compare best with "gold standard" dietary biomarkers (16). But diaries or recalls are labor intensive for researchers to interpret and code, and burdensome for participants, which means data capture is limited to short time periods, (typically 3–7 days) and can take years to be available after collection (17). In addition, accurate memory is essential for 24 h recalls and even with prospective methods like food diaries, reactivity is a problem, where participants report accurately but eat less than usual because their eating is being recorded (10).

FFQs, although simpler and quicker to use, only capture average food intakes. Therefore, exposures increasingly acknowledged as important like the timing of eating (6), the way that foods are combined within a meal (18) and within person variation throughout the day or day to day (17) are unmeasured. With analyses of 4-day food diaries revealing that as much as 80% of food intake variation is within-person and only 20% variation between people (19), there are many untapped avenues for research into novel mechanisms relating diet to disease and identifying opportunities for interventions.

Online versions of "traditional" dietary assessment methods have been developed, but errors and biases remain. Validation studies of a range of online 24-h recall and food diary tools have shown the same problems as their paper-based equivalents; misreporting, portion size estimation, accurately matching foods consumed to foods in composition databases, and high participant burden (16, 20, 21). With the best methods currently available, on paper or online, a maximum of 80% of true intake can be captured and there are systematic differences in the 20% of food intake missing (10, 15).

There is a clear need to enhance dietary assessment methods to reduce error and bias, increase accuracy, and provide more detail on food intake over longer periods so that truly causal associations with health can be identified. A range of reviews and surveys have provided insights into the use of technology to advance dietary assessments (22–24). Recent reviews in particular have highlighted the potential for hybrid approaches that use multiple sensors and wearable devices to improve assessments (25–27). We offer an overview of the state of the art in the use of sensor and wearable technology for dietary assessment that covers both established and emerging methods, and which has a particular focus on passive methods—those that require little or ideally no effort from participants. We illustrate how integrating data from these methods and other sources could transform diet-related health research and behaviors.

WHAT WE EAT

The most commonly used methods for objectively identifying food and portion sizes are image-based. The widespread adoption of smartphones (28) by most adults in high income countries means individuals always have a camera to hand as they go about their daily lives. Many smartphone apps exploring the use of food photography for dietary assessment have been developed and validated. Examples include the mobile food record (mFR) (29) and Remote Food Photography Method (RFPM) (30), where participants capture images of everything they eat over a defined time period by taking a photo before and after each meal. Initial problems with these methods included ensuring all meals were captured, and that photos captured all foods. There were also issues in identifying food items, both automatically and with manual coding systems. These apps were improved by adding customized reminders [drawing on ecological momentary assessment methods (31)], real-time monitoring of photos by researchers to encourage compliance, prompts to improve photo composition, and requests for supplementary information alongside photos. For example, users can confirm or correct tagged foods automatically identified in images (mFR) or add extra text or voice descriptions (RFPM).

The mFR and RFPM systems have been validated in adults using doubly labeled water (DLW) to assess the accuracy of energy intake estimated from several days of food photographs taken in free-living conditions. The mFR underestimated DLW measured energy expenditure by 19% (579 kcal/day), while the RFPM reported a mean underestimate of 3.7% (152 kcal/day), which is similar, if not slightly better, agreement than seen in self-reported methods (30). However, food photography currently has considerable researcher and participant burden because of the requirements for training, real-time monitoring, and provision of supplementary information. Crucially, participants still have to actively take photographs of everything they eat, and this may be affected by issues with memory and social desirability (32).

The introduction of wearable camera systems recording point of view images addresses some of these issues, by making the capturing of images of meals largely passive. Among the first wearable camera systems were those developed for life logging; recording images of events and activities throughout the day in order to aid recall for a variety of benefits (33, 34). Feasibility testing of one such device, SenseCam, which was worn around the neck and automatically took photographs approximately every 30 s, indicated it was promising in enhancing the accuracy of dietary assessment by identifying 41 food items across a

range of food groups that were not recorded by self-report methods (35). However, wearing the device around the neck meant variations in body shape could alter the direction of the lens, so for some individuals the device did not record images of meals.

Another passive wearable camera system, e-Button, reduced the size of the device so that it could be worn attached to the chest (36). Chest mounting improved the ability of the device to capture images of meals. However, the system was a bespoke development, and the use of bespoke solutions produced in limited numbers brings challenges, including potentially high unit costs, limited availability of devices, and issues around ongoing technical support.

Recent studies in other research domains have used mass-market wearable cameras of a similar shape and size to e-Button. For example, studies of infant interactions with environments and parents have used pin-on camera devices that are widely available online as novelty "spy badges" (37, 38). These devices have many characteristics that make them ideal for capturing images of meals; their small form and light weight mean they can be easily worn on the body, and their low cost facilitates use at scale. However, these devices typically capture individual images or video sequences initiated by the user, so they lack the passive operation of devices like eButton that capture images automatically throughout the day.

If using camera devices that capture images throughout the day, the first major challenge is to identify which images contain food and drink. A camera taking photographs every 10 s and worn for 12 h a day for a week will capture nearly 30,000 images, of which perhaps only 5–10% contain eating events (39), so identifying food-related photographs is a non-trivial first step. Automatic detection of images containing food using artificial intelligence shows promise for photos taken in ideal conditions (achieving an accuracy of 98.7%) (39). However, photos taken with a wearable camera are uncontrolled and more susceptible to poor lighting and blurring, and the accuracy of identifying images that depict food ranges from 95% for eating a meal to 50% for snacks or drinks (39).

Once meal images have been identified, the next step is to code food content and portion size. Expert analysis of photographs by nutritionists is currently the most common method but requires trained staff, is time-consuming (typically months to return a dataset), and expensive (>$10 per image). Alternatively, automated food identification and portion size assessment, using machine learning (ML) methods, is complex and computationally intensive. The latest approaches using convolutional neural networks appear promising, with accuracy ranging from 0.92 to 0.98 and recall from 0.86 to 0.93 (40) when classifying images from a food image database (41) into 16 food groups. However, identification of individual food items remains limited (42). ML methods require large databases of annotated food photos to train their algorithms, which are time-consuming to create. With more than 50,000 foods in supermarkets (21) and product innovation changing the landscape constantly, considerable challenges remain for ML approaches.

Humans, on the other hand, have life-long experience visually analyzing food, and are excellent at food recognition. Crowdsourcing approaches, in which untrained groups of people perform a short, simple (usually Internet-based) task for a small fee, might therefore offer a rapid low-cost alternative to expensive experts while ML methods develop. Platemate is one dietary assessment app that employs this approach (43). It is an end-to-end system, incorporating all stages from photographic capture of meals through to crowd-based identification of all foods and their portion sizes and nutrient content. The system is complex, however, and by involving crowds of up to 20 people per photo it results in an average processing time of 90 min and cost of $5 per image. To be feasible for use in large-scale longitudinal studies or public health interventions, crowdsourcing of food data from photographs needs to be fast and low cost. We developed and piloted a novel system, FoodFinder (44), and found that small ($n = 5$) untrained crowds could rapidly classify foods and estimate meal weight in 3 min for £3.35 per photo. Crowds underestimated measured meal weight by 15% compared with 9% overestimation by an expert. A crowd's ability to identify foods correctly was highly specific (98%—foods not present in the photo were rarely reported) but less sensitive (64%—certain foods present were missed by the crowd). With further development crowdsourcing could be an important stepping-stone to the automated coding of meal images as ML methods mature. Crowdsourcing could also play an important role in this development, by creating annotated databases of meal photographs to facilitate training of ML algorithms.

In addition to image based methods for assessing meals, more recent developments in body-worn sensor technology have aimed to passively measure the consumption of specific nutrients. Small, tooth mounted sensors in which the properties of reflected radio frequency (RF) waves are modulated by the presence of certain chemicals in saliva can detect the consumption of salt and alcohol in real time (45). Similarly, tattoo like epidermal sensors that attach to, and stretch and flex with the skin can detect a variety of metabolites in an individual's perspiration that relate directly to their diet (46). For these devices, it is important that metabolites detected are specific to food intake, and not conflated with endogenous metabolites produced by the body as a result of eating.

To date these new oral and epidermal sensors have largely been tested in laboratory settings and are some way from becoming widely available. There are clearly compelling uses for these, for example accurate measurement of salt intake in patients with high blood pressure and sugar intakes in patients with diabetes, as well as enhancing food photography methods by providing non-visual nutritional composition information (e.g. sugar in tea or salt added in cooking). However, it is worth noting that these methods alone are not able to identify the food that contained these nutrients. For some dietary interests (e.g., changing dietary behaviors), food items need to be assessed rather than the nutrients they contain, and in these cases image-based methods for assessing meals will be required.

WHEN WE EAT

To advance our understanding of the effects of diet, we require objective assessments of not just what we eat, but when we eat too. A variety of approaches for the passive detection of eating events have been proposed, including; acoustic methods using ear-mounted microphones to detect chewing (47), throat microphones to detect swallowing (48) and detection of jaw movements using different sensor types attached to the head or neck (49–52).

Although these methods are capable of detecting eating events passively, they need the individual to wear bespoke sensing devices attached around the head and neck, and when used on a daily basis this inevitably introduces a considerable level of device burden. To address this, one approach is to use sensors that are embedded in or attached to items that are already part of people's daily lives.

One method explored has been the use of sensors that are part of spectacles. Some approaches to this have used piezoelectric strain sensors on the arms of glasses that are attached to the side of the head to measure movements from the temporalis muscle when chewing (53, 54). High levels of performance have been reported with this approach, with one study reporting an area under the curve for chewing detection (in a combination of laboratory and free-living tests) of 0.97 (55). However, it does require the sensors be manually attached to the head every time the glasses are worn. Others have used electromyography, in which the electrical activity associated with temporalis muscle contraction is detected using sensors imbedded in the arms of 3D printed eyeglass frames (56). This also gives good performance, with recall and precision for chewing bout detection above 77% in free-living conditions. This approach does not need manual attachment of sensors, but it does require individually tailored glass frames to ensure sufficiently good contact of the built-in sensors with the head. More broadly, not everyone wears glasses, so there is also the issue of how these approaches would work for those who do not.

Another method is to use wrist-worn devices equipped with motion sensors to automatically detect eating events. Data from gyroscope and accelerometer motion sensors can be used to identify the signature hand gestures of certain modes of eating (57, 58). Early adopters of this approach strapped smartphones to the wrist (59). This functionality is now more conveniently available in the form of off-the-shelf activity monitors and smartwatches. These devices can be highly effective in detecting eating events, with recent reports of 90.1% precision and 88.7% recall (60). However, building recognition models that can generalize well in free-living conditions where unstructured eating activities occur alongside confounding activities can be challenging, and can result in reduced precision in detection (61).

Recent reviews concluded that smartwatches are of particular interest for eating as they represent an unobtrusive solution for both the tracking of eating behavior (62), and the delivery of targeted, context-sensitive recommendations promoting positive health outcomes (63), such as Just-in-Time interventions (64).

The latest ML techniques are enabling researchers to go beyond detection of eating events using wrist-worn wearables, to also measure within meal eating parameters such as eating speed. In a recent example, convolutional neural networks and long short-term memory ML methods were applied to data from the motion sensors in off-the-shelf smartwatches worn by 12 participants eating a variety of meal types in a restaurant (65). Sequences of bites were first detected, which were then classified into food intake cycles (starting from picking up food from the plate until wrist moves away from the mouth).

The ability to passively detect meal onset is an essential aspect of other healthcare systems too. One example is closed-loop artificial pancreas systems for the management of blood glucose in patients with type 1 diabetes. Such systems rely on detecting a rise in interstitial fluid glucose concentrations (a proxy for blood glucose) using continuous glucose monitors (CGM). Meal detection can be challenging as interstitial glucose rises well after a meal has begun, limiting the current use of CGM in real-time monitoring systems. However, meal detection models using CGM have developed from being purely computer-based simulations to now showing promise when fitted to real-world data. The mean delay in detecting the start of a meal has reduced from 45 to 25 min (66). CGM could therefore be another method for the passive, objective detection of meal timings in future, although further research, particularly in populations without diabetes, is required. Encouragingly pilot work in the US indicates that wearing a CGM for up to a week is as acceptable as wearing accelerometer-based sensors for 7 days (67). Furthermore, our own pilot work in the UK ALSPAC-G2 cohort demonstrated that using the latest CGM devices, which no longer require finger prick tests for calibration, improves uptake of 6 days of monitoring (68). This reflects a growing demand for non-invasive methods for CGM.

Photoplethysmography (PPG) is a technique that detects changes in levels of reflected light as a result of variation in properties of venous blood, and which is routinely included in off-the-shelf smartwatches and activity monitors for the measurement of heart rate. This same technique can also be used to non-invasively measure glucose levels, and the latest enhancements give measurement performance approaching that of reference blood glucose measurement devices (69). This opens the possibility that non-invasive CGM using commercially available smartwatches and activity monitors may be widely available in the near future, and theoretically devices of this kind could detect glucose patterns associated with meal start and end times. Once again though, the latency between start of meal and detection would need to be determined, and meal detection algorithms evaluated.

INTEGRATING METHODS

The methods outlined above individually provide objective measurements of when, what and how much someone is eating. Integrating these methods offers the possibility of objectively capturing more complete and detailed pictures of dietary intake, while minimizing participant burden.

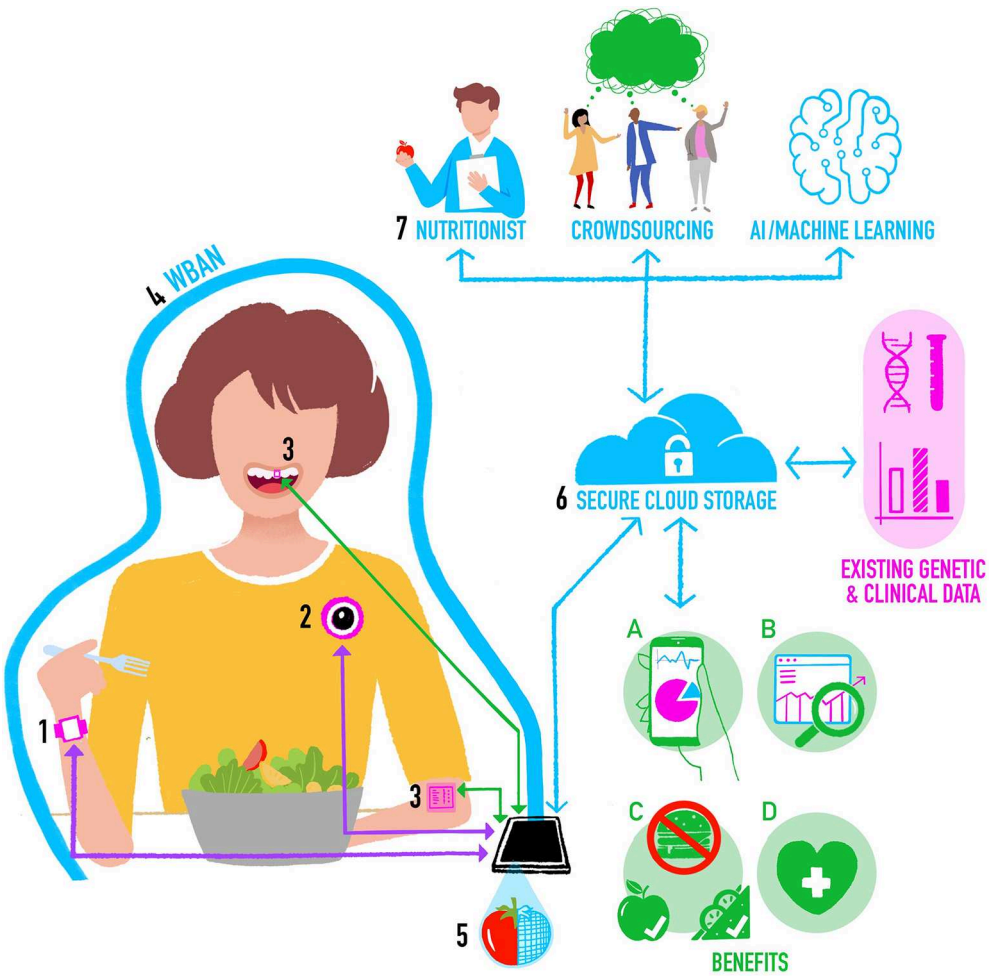

FIGURE 1 | Integrating methods for objective assessment of diet using digital and sensing technologies.

One previous proposal for an integrated system for objective dietary assessment involves combining smartwatch motion sensors with a camera built into the smartwatch (70). The motion sensors detect the start and stop of an eating event, and this triggers the camera to take an image of the meal for subsequent offline analysis. While this is a compact solution minimizing device burden, it does need the individual to direct the watch camera toward the meal to capture an image. More importantly, trends in smartwatch design have changed, and smartwatches typically no longer come equipped with built in cameras.

A more recent proposal again had a wrist-worn activity monitor to detect eating events, but this time combined with on-body sensors for detecting chewing and swallowing to capture more detailed information on bite count and bite rate within a meal (22). An interesting aspect of this system was the use of the individual's smartphone as the basis of a Wireless Body Area Network (WBAN) (71) to link up the activity monitor and different sensors. This enabled local communication between sensors via the smartphone, without the need to connect the sensors to a static wireless network or a cellular data connection.

In **Figure 1** we propose a new architecture for an integrated system for objective assessment of diet. We draw on some elements of these previous proposals, but also incorporate new and future developments in wearable sensing technology for objective dietary assessment. The operation of the system can be conceptualized as follows:

1. The individual wears a smartwatch containing accelerometer and gyroscope motion sensors. Classification algorithms applied to the motion data in real time on the watch can detect the beginning and end of an eating event, the mode of eating, and provide "within meal" metrics such as speed of eating. In the future the smartwatch may also have PPG-based CGM, which provides additional data on meal timing and size.
2. The individual is also wearing a chest-mounted camera capturing images from their viewpoint. To keep battery consumption and data storage requirements low (minimizing device size and maximizing time between charges), the camera takes still images at short intervals (e.g., every 10 s) and stores them for a brief period (e.g., for 5 min). Images are then

deleted unless the smartwatch detects the start of an eating event, in which case images before, during and after eating are stored as a complete visual record of the meal. Saving multiple photos maximizes the chances of capturing high quality images unaffected by temporary issues with lighting, camera angle, blur, etc.

3. On-body sensors including oral tooth-mounted sensors and epidermal tattoos could be added to provide more detailed nutritional assessments for monitoring of specific nutrients or calibrate estimates from other tools.
4. The individual's smartphone forms the basis of a WBAN around their body. Most devices (e.g., activity monitors, cameras) will communicate with the smartphone using a Bluetooth connection. Oral and epidermal sensing devices that do not currently have power supplies or data storage or transfer capabilities could use Near Field Communication (NFC) as a power source and to transfer data from the sensor to the smartphone.
5. Segmentation, food item recognition, and volumetric estimates of portion sizes are initially computed locally on the smartphone using data from sensors and images, and these may be used to support Just-in-Time type eating behavior change interventions.
6. The smartphone also provides a secure connection to a cloud-based central dietary profile for the individual. Data captured by sensors is processed on the smartphone and the processed data are regularly uploaded to the central profile, perhaps when the individual is at home and their smartphone connects to their home wireless network. Processed data can then, at the individual's discretion, be linked to other sources of their own health data, including omics, clinical, and imaging data. Raw data from sensors are not uploaded to reduce privacy concerns and data transfer requirements.
7. Depending on the needs of the particular scenario, and balancing speed, accuracy, and cost, data from the central profile may be sent for further analysis. For example, images of meals may be sent to a crowd-based application (44), or a dietician to refine food item identification and portion sizes (72).

The resultant cloud-based central dietary profile represents a detailed view of a person's food intake and eating behavior that will provide the following benefits:

A) Summaries of the individual's data for their personal use.
B) Dietary data that is stored and made available for future research on eating [for example prospective cohort studies like Children of the 90s (73)].
C) Information that can be automatically analyzed within computer-based personalized nutrition behavior change interventions involving monitoring progress in achieving changes in diet-related goals [e.g., see (74)].
D) Information that feeds into health professional consultations [e.g., enabling a dietician to get a better picture of an individual's overall intake and eating behavior so they can spend more time on behavior change techniques rather than having to assess diet as part of the appointment—for example see (75)].

DISCUSSION

In this article we briefly looked at how emerging digital and sensing technologies are enabling new objective assessments of dietary intake. These new methods have the potential to address many of the issues associated with current paper and online dietary assessment tools around bias, errors, misreporting, and high levels of participant or researcher burden. They do so by automating the detection and measurement of eating events, food items and portion sizes, and by providing detailed information on specific nutrients and within meal eating behaviors.

Image-based methods remain the most popular approach for objective assessment of food items and portion size. The use of on-body cameras to passively capture images of meals for subsequent processing has a number of advantages. As the individual does not have to manually initiate the capture, this helps mitigate issues such as the stigma of photographing their meals. The reliance on an individual's memory or willingness to self-report is also removed, therefore burden and bias are reduced. However, having to wear the camera device does represent a different burden, and there are issues around privacy, for example concerns from others that they may be inadvertently recorded. For nutritional assessment, image capture could be limited to eating occasions, so while concerns remain, they would hopefully be reduced.

In terms of camera devices, future developments should combine the passive operation and ease of use of a system like e-Button (36), with the low weight, size and cost, and broad availability of commercially available products. If such a device was of utility to multiple research domains (following the model of e-Button), and particularly if it had compelling mass market health or dietary use cases, demand could be sufficient for commercial production. Integration of such a device into other items already accepted for daily use (clothing, jewelery, etc.) could possibly increase acceptability further.

The emergence of sensors that attach directly to teeth or to the skin holds the promise of real time measurement of specific nutrients. These devices are at the proof of concept stage, and there are important considerations to address around durability, and how to power and read data from these devices. However, many of the mobile and wearable devices we currently use have capabilities that could possibly be adapted to work with these new sensors. For example, the near field communication wireless technology now included in most smartphones to make wireless card payments uses high frequency radio signals that could potentially be adapted to power and communicate with oral and dermal sensors (76).

In terms of detecting when people are eating, smart glasses could potentially detect the movement of, or electrical signals from the muscles used to chew, although there are the issues of sensor attachment and positioning, and how this approach

would work for people who do not normally wear glasses. Wrist-worn wearables such as smartwatches have the ability to detect the signature hand movements unique to eating. Consumer demand for these devices continues to grow, with worldwide shipments predicted to exceed 300 million by 2023 (from under 30 million in 2014) (77). Smartwatches are worn by individuals as part of their daily routine so they do not represent additional device burden. In addition, such devices have the capability to run 3rd party applications providing the opportunity for delivering just-in-time behavior change interventions based on the eating behaviors detected. However, battery life continues to be an issue, with smartwatches typically needing to be charged daily. Continuous monitoring of eating behaviors will exacerbate this. Also, the detection of eating behaviors from motion data often use computationally intense machine learning algorithms (e.g., convolutional neural networks) that cannot currently be used on wearable devices to detect eating in real time. This may change in the future as the processing power and battery life of smartwatches and other wearables improve and more sophisticated classification algorithms can run on these devices.

In the current review, we have proposed an architecture for an integrated system for the objective assessment of diet. Integrating methods will enable researchers to build a more detailed and complete picture of an individual's diet, and to link this with a wide range of related health data (e.g., omics, clinical, imaging). Storing this information in a central location will enable healthcare professionals, researchers and other collaborators the individual wishes to interact with to have controlled access to their detailed dietary data. However, this raises a number of important questions. Should cloud-based storage be used and where this would be hosted? What format to use for the stored data to maximize utility across applications? What model should be used for making the data available, given the rise of new models in which individuals can monetise their own data? (78).

Another key issue for new methods will be that they need to be financially sustainable over time. For integrated systems, architectures are required that minimize the time and cost of maintaining operation of the system when one component (e.g., a sensor) changes. For example, systems arranged with central hubs to which each sensor/device connects and communicates reduce the impact of a change in one component compared with fully connected architectures in which each sensor/device communicates to many others.

For all of these new techniques for passive measurement of dietary intake, it will be important to understand if they introduce unexpected measurement errors and biases. New methods for estimating multiple sources of error in data captured using the latest technologies could help in this respect. These are able, for example, to separate out the effects of factors such as coverage (access to the technology), non-response and measurement error (79).

Finally, whether these methods, individually or integrated, become widely adopted will rest largely with the individuals that use them. Extensive feasibility testing will be required to explore which of these new methods people are happy to use, and which ones they are not.

AUTHOR CONTRIBUTIONS

AS, ZT, CS, and LJ contributed to conceptualizing and writing the article.

FUNDING

AS was funded by a UKRI Innovation Fellowship from Health Data Research UK (MR/S003894/1). LJ, AS, and CS were funded by MR/T018984/1. LJ and AS were affiliated with, and CS was a member of a UK Medical Research Council Unit, supported by the University of Bristol and the Medical Research Council (MC_UU_00011/6). ZT was funded by the Jean Golding Institute at the University of Bristol for research unrelated to this review. Some of the work presented here was funded by a catalyst award from the Elizabeth Blackwell Institute for Health Research a Wellcome Trust Institutional Strategic Support fund.

ACKNOWLEDGMENTS

The authors would like to acknowledge Ben Kirkpatrick for the illustration.

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Conflict of Interest: AS and CS are listed as inventors on patent applications 1601342.7 (UK) and PCT/GB2017/050110 (International), Method and Device for Detecting a Smoking Gesture. LJ has received funding from Danone Baby Nutrition, the Alpro foundation and Kellogg Europe for research unrelated to this review.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

MINI REVIEW
published: 03 July 2020
doi: 10.3389/fnut.2020.00090

Traditional Self-Reported Dietary Instruments Are Prone to Inaccuracies and New Approaches Are Needed

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Background: Diet is a modifiable behavior that influences an individual's health. Because of this, diet assessment is an important component of public health surveillance, evaluating response to community health interventions, and monitoring individual compliance to medical interventions. Diet assessments are usually performed using one of three basic methods: diet recall, diet diaries, or food frequency questionnaires. Although these three assessment instruments have displayed a strong agreement between themselves, when reported intake is compared with intake measured using quantitative nutrient biomarkers, investigators have identified systematic misreporting errors for all three of these self-reported dietary instruments.

Aims: This work aims to summarize the state of knowledge regarding misreporting and why it impedes diet–health research and to introduce advances in the collection and the treatment of dietary data.

Methods: This work reviews and summarizes published data on misreporting and the recent efforts to reduce such errors.

Results: The evidence demonstrates a strong and consistent systematic underreporting of energy intake (EIn) across adults and children studies. Underreporting of EIn has been found to increase with body mass index (BMI), and the differences between macronutrient reports indicate that not all foods are underreported equally. Protein is least underreported, but which specific foods are commonly underreported are not known.

Conclusions: Because energy underreporting varies as a function of BMI, self-reported EIn should not be used for the study of energy balance in the study of obesity. The between-individual variability in the underreporting of self-reported intake of energy and other nutrients attenuates diet–disease relationships. Recent efforts to correct for underreporting have reduced misreporting of diet outcomes, but improvements have been incremental in nature and more research is needed to validate and extend these efforts.

Keywords: diet records, self-report, energy intake, underreporting, doubly labeled water method

OPEN ACCESS

Edited by:
Natasha Tasevska, Arizona State University Downtown Phoenix Campus, United States

Reviewed by:
Robin M. Tucker, Michigan State University, United States
José María Huerta, Instituto de Salud Carlos III, Spain

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *28 February 2020*
Accepted: *15 May 2020*
Published: *03 July 2020*

Citation:
Ravelli MN and Schoeller DA (2020) Traditional Self-Reported Dietary Instruments Are Prone to Inaccuracies and New Approaches Are Needed. Front. Nutr. 7:90. doi: 10.3389/fnut.2020.00090

INTRODUCTION

Investigations into the role of diet in the development of disease in humans are viewed as difficult but important because diet is one of the behaviors that individuals may employ to maintain or improve health (1). For example, human feeding studies performed or dictated by external disruptions of food supplies have clearly demonstrated that diet plays a central role in the negative impacts on health resulting from energy, vitamin, and mineral deficiencies in controlled studies conducted for at least 75 years (2). At the same time, the Minnesota Semi-starvation Study demonstrated the difficulty and the ethical issues of performing month-long feeding trials in order to investigate the quantitative nature of diet–disease relationships using only an inpatient paradigm (3).

A major source of difficulty has been documenting what individuals consume as their typical diet due to dietary measurement error (4). Because controlled and thus more accurate feeding studies are costly and difficult to perform, a far more common approach to the study of the relationship between diet and disease has been to perform studies of free-living participants. These studies usually rely on assessing diet using self-report instruments to access diet and this introduces diet measurement error into the study (5). The most common dietary assessment instruments are diet recall surveys, in which the participants report from memory each item of food consumed for the previous day, weeks, or months; food frequency questionnaires (FFQ), which have the aim of assessing the food consumption during some specified period of time or over a period such as adolescence; and diet diary methods where the subjects record dietary intake for each eating event for a period of days or weeks.

Perhaps the best documented evidence of dietary instrument measurement error is that of self-reported energy intake (EIn) being often less than that of the individual's measured energy expenditure. The aim of this short review is to summarize the development of that evidence and how misreporting impedes diet—health research. Because of the evidence, there has been a renewal of efforts to improve or develop alternatives to these instruments. These recent advances are introduced here as part of a series of reviews in this issue.

DIETARY MISREPORTING

Over the past 50 years, many traditional diet assessment instruments have undergone modification of content or structure, sometimes to the degree of being considered a new instrument, and then were validated by comparison against an older version or previously evaluated instrument. In most instances, these comparisons demonstrated a moderate or strong agreement between the basic types of dietary assessment instruments and the tool was considered to be of reasonable accuracy and precision (6). A few investigators, however, performed studies comparing self-reported dietary intake against a biomarker of dietary intake, such as urinary nitrogen, which provides an objective measure of dietary protein intake (7). Such comparisons against a biomarker often did not find these self-report instruments to be accurate. For example, Warnold et al. (8) reported that self-reported protein intake underestimated protein consumption by 47% compared to protein intake measured using urinary nitrogen outputs among women undergoing a weight loss treatment. Studies comparing self-report against biomarkers to test the accuracy of traditional diet instruments, however, were infrequent and had only a modest influence on the growth of the use of self-report dietary assessments in the study of diet–disease relationships.

The number of dietary instrument validations against a biomarker increased dramatically following the development of the doubly labeled water (DLW) method to measure total energy expenditure (TEE) in humans (9). This method, developed by Lifson, is based on the difference in the elimination kinetics of two stable isotopes in water, namely, deuterium (^{2}H) and ^{18}O (10). The difference in the elimination rate of ^{2}H and ^{18}O is proportional to carbon dioxide production (10). The latter is the end-product of oxidative phosphorylation, and TEE can be calculated using standard indirect calorimetric equations (11). The human validations that included conditions of weight stability, overfeeding, underfeeding, intravenous feeding, and heavy exercise have been summarized by Speakman et al. (12–15). These validations have displayed an average accuracy of TEE of 1 to 2% and an individual precision of 7%, which support its use as a biomarker for use in a criterion method against which one may test the accuracy and the precision of self-reported energy intake.

The development of the DLW method for the measurement of TEE created an opportunity to validate diet assessment instruments against an objective energy expenditure based on the first law of thermodynamics. The first law of thermodynamics states that energy cannot be created nor destroyed, and thus EIn equals energy expenditure plus or minus the change in body energy stores during the measurement interval. Moreover, when body energy stores are unchanged over time, the energy storage term falls to zero and then EIn equals energy expenditure. Among pregnant women, infants, and children, change in energy stores over time is expected. At 1 month of age, the average daily energy storage is about 40% of EIn, but this decreases to 3% by 1 year of age and continues to decrease to 1% at 10 years of age (16). Among pregnant women, the average increase in body energy stores is about 190 kcal/day or 8% of EIn during the 3rd trimester (17). Among most non-pregnant adults, weight gain is not recommended, but it is common. The rate of weight gain is 0.2–0.7 kg/year or about 1–2 g/day (18). Assuming that the gain is adipose tissue with 20% fat-free mass and 80% fat mass, this stores only about 8 to 16 kcal/day or about 0.3–0.6% of EIn (19) and thus energy expenditure almost equals EIn, and TEE is an excellent biomarker of EIn. There are exceptions to this essential near-equality assumption in adulthood. These include periods of voluntary weight loss, loss of appetite during illness, or periods of holiday feasts when energy intake can be quite different from expenditure and TEE will fail to be a quantitative biomarker of actual EIn. Under habitual conditions outside of these short periods, EIn roughly equals TEE. Thus, TEE is termed as a biomarker of habitual dietary energy intake rather than one of actual EIn. This is an important distinction because while TEE is generally a good measure of habitual energy intake for the study

of diet and health, it fails as a measure of actual energy intake under the above mentioned short-term conditions.

Another consideration in using TEE as a biomarker of habitual EIn is that energy intake can be expressed in one of three ways. The first is gross energy. This is the total energy available when foods are combusted to dioxide carbon, water, and nitrogen gas using bomb calorimetry (20). Not all the gross energy, however, is available to the body for metabolism. About 8% of the gross energy is not absorbed and thus lost from the body as waste products in feces (21, 22). The second expression is the absorbed energy. Finally, not all the absorbed energy is available to the body for energy production because some compounds that still contain chemical energy are lost as waste products in urine. The third expression is the portion of absorbed energy retained by the body that is available for energy production, which is the metabolizable energy. Metabolizable energy, as the name implies, is available for use in oxidative phosphorylation. This is the energy value listed in the food handbooks and tables. Metabolizable energy is thus the energy value used for calculating the dietary EIn by dietary assessment instruments.

Many of the human studies using DLW performed during the 10 years between 1982 and 1992 included an assessment of dietary EIn using traditional instruments. One of the first studies was conducted by Prentice et al. (23), in which it was observed that EIn, assessed using a 7-days food diary, was 34% ($P < 0.05$) less than TEE measured by DLW in young adult obese women (32.9 ± 4.6 kg/m^2), but there was no difference detected between EIn and TEE (2%, NS) in lean women. The authors also found that half of the EIn vs. TEE difference was due to underrating as assessed by weight loss during the dietary diary period. These findings of low self-reported EIn were confirmed in a later review (24), which included papers which found that underreporting of dietary EIn was observed in women with anorexia nervosa, who perceive that they have excess body fat, and also in individuals with measured excess body weight, who are concerned about actual excess fat. Thus, underreporting was associated with individuals likely to be concerned about excess weight and not just with actual weight status (body mass index, BMI) itself (24). Thus, as early as 1990, it was found that underreporting of dietary EIn was common among adults and linked to concerns regarding excess body weight or fat (24). Even these early studies found that the degree of underreporting was of similar magnitude regardless of whether intake was assessed using retrospective instruments such as diet recalls or histories or with instruments such as food diaries (24). Based on these observations, it was concluded that dietary assessment instruments were subject to errors that increased with the individual's concern regarding their relative weight, which would result in a correlation of increased underreporting with increased BMI (24). Because of this, it was strongly recommended that self-reported EIn should not be used as a primary assessment instrument to measure EIn in investigations into the role of EIn in weight regulation as early as 1990 (24).

Most of these early studies employing DLW as a quantitative biomarker of dietary EIn were conducted in cohorts with sample sizes categorized as small to medium and, in many cases, by investigators without extensive experience on the use of dietary assessment instruments. Based on anecdotal evidence provided by questions from the audience following oral presentations, some investigators in the audience suggested that the finding of underreporting may have been an artifact and that it might not occur if experienced investigators performed studies in large cohorts. This hypothesis, however, was not supported by the results from one study and soon thereafter by four more studies that were performed by investigators who had extensive experience on the use of dietary assessment instruments and which included cohorts with several hundreds of adult subjects each as summarized by Freedman et al. (25). The combined results of these studies confirmed that underreporting of habitual EIn in the United States was common as it was observed in each of the five studies which, when combined, involved over 2,000 participants (25). The 24-h recall (24HR) exhibited an EIn underreporting compared to the DLW-measured TEE which averaged −16% (range, −10 to −28%), and the FFQ was subject to an even larger reporting error than was 24HR (range, −26 to −32%) (25). The combined number of participants in these five studies ($n = 2{,}265$) permitted sub-analyses, and it was found that those having a BMI of >30 kg/m^2 underreported EIn by 7% more than those of a BMI in the healthy range, but there was no difference between men and women or adult age groups when centered on ages 50–59 years (25). One of these five studies (26) found that the administration of up to eight 24HRs on different days of the week did not eliminate the average reporting error, thus demonstrating that the underreporting was not simply due to day-to-day variation in actual EIn. The underreporting did decrease when two 24HRs were averaged (−11%) relative to that when only one 24HR was employed (−15%), but the percent error changed only a little when more than two 24HRs were averaged. Even when six 24HR data were collected and averaged, the bias dropped to only −9%. Thus, dietary data were more consistent when two recalls were employed in each participant, but little was gained by further replication.

The findings from the combination of the five large studies discussed above have been confirmed and extended through a systematic review conducted by Burrows et al. (27). The review identified an additional 59 studies that included 6,298 adults, including the abovementioned five studies and the 2,265 participants in the abovementioned summary by Freedman (25). The studies employed a mixture of diet instruments, including 24HR; the food diaries include weighed food records and FFQs. The degree of underreporting relative to habitual EIn as measured by DLW varied over a wide range. This included two studies that reported group averages displaying over-reporting (7 and 8%), but the vast majority identified cohort average underreporting by between 1 and 38%, and the plurality of studies found an average under-reporting between 20 and 30%. A comparison of methods indicated that the most misreporting was observed for the FFQ and the least for 24HR, but all three methods displayed underreporting errors. Studies that included advanced technology such as photography, hand-held personal digital assistants, or oral recordings did reduce the underreporting slightly compared to non-technology-assisted instruments but were still found to be subject to underreporting. Included in that review were studies conducted in countries

other than the United States, including Australia, Brazil, Canada, Finland, Germany, Japan, New Zeeland, Norway, Sweden, and the United Kingdom, demonstrating that underreporting with regard to EIn was a global problem.

Two systematic reviews concluded that underreporting of EIn was also an issue among children (28, 29). There have not been as many studies performed in children (aged 3–18 years) as have been performed in adults, but the results were similar with those in adults. Underreporting was common in children, and children with excess weight (overweight or obese) underreported more than those having a BMI in the healthy weight range. It was found that underreporting was reduced when parents assisted their children for ages <11 years. Unlike what was observed in adults, age was a significant modifier of misreporting, and underreporting was greater in adolescents than it was in younger children.

Because of the significant underreporting of EIn observed in the studies discussed above, one of the vital next steps for research directed at studying the phenomenon of EIn underreporting is to identify whether underreporting of EIn is due to a failure to accurately report specific foods or is a general underreporting of all foods. Addressing this issue is difficult because it means one has to measure something that is not reported rather than what is reported. We speculate that one means of accomplishing this would be to include multiple biomarkers in a study of self-reported dietary intake. As evidence, studies that have included DLW as a biomarker for EIn and urinary nitrogen as a biomarker for protein intake have shown that energy is underreported by a larger percentage than protein. For example, the abovementioned study combining the results from five large dietary intake studies (25) found that while energy was misreported by −16% (range, −6 to −28% using 24HR), protein was misreported by only −5% (range, −21 to +20%), indicating that protein was not as underreported as carbohydrate and/or fat. We speculate that a cluster analysis using multiple quantitative and possibly semi-quantitative biomarkers will provide vital insight into the foods that are misreported. The value of identifying what foods were being underreported as well as the difficulty of doing so without using biomarkers is illustrated by a Brazilian study performed in obese women prior to bariatric surgery (30). The study found that the under-reporters reported lower intakes of foods with high energy density but with similar intakes of calories provided by healthy foods (fruits, leafy vegetables, and vegetables) compared to those of plausible reporters. This reporting behavior influenced the determination of dietary patterns by exploratory factor analysis, in which the principal component analysis with VARIMAX rotation was applied for the selection of food groups that composed the matrix and then used for dietary pattern interpretation (30). By combining diet factor analysis with biomarker data on energy, protein, sugar, sodium, and potassium, it should be possible to infer if these differences were due to actual dietary intake differences.

Misreporting of energy and protein intake when assessing diet by self-report is well-documented and recognized by many as a major limitation to the investigation of the effects of diet on health. The problem of underreporting, particularly because of the inter-individual variation in misreporting, dramatically attenuates diet–disease relationships. Kipnis et al. (31) modeled the effects of misreporting of protein and EIn in the OPEN study and concluded that the variation in the degree of misreporting using an FFQ would severely attenuate the relative risk between true protein or EIn and disease from a true value of 2.0 to an apparent relative risk of <1.1. Even worse, it may even reverse the association between diet and disease as had occurred in an analysis of energy balance using self-reported EIn and physical activity by Kromhout et al. (32). The data from these investigators indicated that energy balance and BMI were negative and becoming more negative with increasing BMI, a result that they considered implausible and a possible artifact of underreporting EIn.

In summary, the problem of misreporting of dietary intake is limiting the ability of investigators to study diet–disease relationships (31). Investigators are, therefore, performing studies of novel approaches that may either reduce misreporting or adjust the self-reported data using *post hoc* techniques that may reduce the effect of such misreporting on study outcomes (33). These include the development of advanced technology to reduce the reporting errors themselves, adjustment of reported nutrient intake using calibration against a nutrient biomarker, statistical approaches that provide novel analyses of data from traditional self-reported dietary instruments, or direct use of dietary biomarkers to assess intake (34).

APPROACHES TO REDUCE MISREPORTING

Advanced technological tools include digital photography with on-line submission, movement monitors on the wrist or eating utensils to detect feeding, microphones to detect chewing, and scales to monitor the disappearance of food from a plate. Photographic methods provide the most detailed information about foods consumed, but they are still prone to underreporting (27) and they require a large amount of technical support (35). The other methods listed above have demonstrated the ability to detect eating events, but they provide only partial quantitative and qualitative information regarding the foods being consumed (34).

In addition, *post hoc* approaches that reduced the influence of misreporting have been presented. For example, Mozaffarian et al. (36) analyzed diet data obtained using an FFQ administered at 4-year intervals in a large longitudinal study. They used dietary change scores from the bracketing FFQs in place of raw intake scores from a single FFQ to identify foods that were associated with 4-years changes in body weight. This approach of using diet change and subsequent weight change is difficult to validate for dietary reporting accuracy, but the foods identified in this study as being associated with weight gain or loss were in general agreement with small, shorter intervention studies and thus extended the findings from the short-term interventions to the population level. Additionally, it provided high statistical power, but it did require a study design where the diet was assessed multiple times over a period of years in a large cohort and an outcome that was continuous. A second *post hoc*

data analysis approach was employed by Freedman et al. (37). They combined multiple 24HRs with an FFQ in order to use the quantitative information from 24HR along with the larger list of foods consumed from the FFQ against true intake as measured by dietary biomarkers. They reported an improvement in the correlation coefficients between reported and biomarker-measured true dietary intake of energy, protein, potassium, and sodium compared to the use of single 24HR by an average of 0.14. The highest correlation coefficient, however, was 0.64 for potassium in women, and thus the variance explained was <40%.

As an alternative to the above-discussed methods to reduce problems arising from misreporting, Prentice and Huang (38) have proposed and tested the use of a *post hoc* calibration to adjust reported intakes for misreporting identified by the use of a quantitative biomarker in the entire study cohort or a subsample of that cohort. Tasevska et al. (39) applied this approach to an analysis of self-reported sugar intake and the likelihood of developing type II diabetes or cardiovascular disease. They found that correcting reported sugar intake based on the calibration eliminated what appeared to be an implausible inverse relationship, thus avoided a false finding. The resulting positive correlation, however, was small and did not result in a significant increase in the odds ratio for disease development with increasing sugar intake during the 16-years follow-up in the Women's Health Initiative cohort of older women, thus not ending the controversy around sugar consumption and type II diabetes.

The final approach to be discussed in this review is that of Goldberg et al. (40, 41). This approach involved characterizing a self-reported intake as plausible or implausible. During a period of bodyweight stability, the ratio of $\frac{energy\ intake}{resting\ metabolic\ rate}_{reported}$ should correspond to the ratio of $\frac{total\ energy\ expenditure}{resting\ metabolic\ rate}$, which is identified as physical activity level (PAL). Considering the biological variability of the components of the equation, confidence limits (cutoffs) are calculated to classify the probable accuracy of the reported EIn, and its sensibility improves when individual PAL classification is used in the cutoff points (42). A not dissimilar approach is to calculate the ratio of reported EIn to TEE from DLW (24). As an alternative to the DLW method, it may be possible to use a predicted TEE based on weight, height, age, sex and physical activity (18). The optimal method for defining the cutoff for excluding implausible reported intakes is still under debate, but the value is recognized (43).

SUMMARY AND CONCLUSION

Dietary assessment is central to the study of diet–health relationships. The most common assessment instruments are diet recalls, diet diaries, and food frequency questionnaires, and all are dependent on self-reported data. Self-reported Ein, using all of these instruments, has been shown to yield reproducible intake results. They have also been shown to yield good to strong correlations between foods consumed when compared against one another. Comparisons against quantitative biomarkers of dietary intake, however, have clearly demonstrated that self-report is prone to misreporting errors for EIn and other nutrients and that inter-individual variability in the degree of underreporting attenuates the strength of diet–disease relationships and raises questions regarding what foods are being misreported. Recent research has identified several methods for reducing many of these reporting errors. There remains, however, a need for further research to optimize the accuracy or correct for inaccuracies in self-reported dietary data because of the importance of dietary data in the prevention and the treatment of diet-induced diseases.

AUTHOR CONTRIBUTIONS

MR and DS conceptualized the manuscript. MR and DS drafted and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 14 July 2020
doi: 10.3389/fnut.2020.00099

Reproducibility of Dietary Intake Measurement From Diet Diaries, Photographic Food Records, and a Novel Sensor Method

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Objective: No data currently exist on the reproducibility of photographic food records compared to diet diaries, two commonly used methods to measure dietary intake. Our aim was to examine the reproducibility of diet diaries, photographic food records, and a novel electronic sensor, consisting of counts of chews and swallows using wearable sensors and video analysis, for estimating energy intake.

Method: This was a retrospective analysis of data from a previous study, in which 30 participants (15 female), aged 29 ± 12 y and having a BMI of 27.9 ± 5.5, consumed three identical meals on different days. Four different methods were used to estimate total mass and energy intake on each day: (1) weighed food record; (2) photographic food record; (3) diet diary; and (4) novel mathematical model based on counts of chews and swallows (CCS models) obtained via the use of electronic sensors and video monitoring system. The study staff conducted weighed food records for all meals, took pre- and post-meal photographs, and ensured that diet diaries were completed by participants at the end of each meal. All methods were compared against the weighed food record, which was used as the reference method.

Results: Reproducibility was significantly different between the diet diary and photographic food record for total energy intake ($p = 0.004$). The photographic record had greater reproducibility vs. the diet diary for all parameters measured. For total energy intake, the novel sensor method exhibited good reproducibility (repeatability coefficient (RC) of 59.9 (45.9, 70.4), which was better than that for the diet diary [RC = 79.6 (55.5, 103.3)] but not as repeatable as the photographic method [RC = 43.4 (32.1, 53.9)].

Conclusion: Photographic food records offer superior precision to the diet diary and, therefore, would be valuable for longitudinal studies with repeated measures of dietary intake. A novel electronic sensor also shows promise for the collection of longitudinal dietary intake data.

Keywords: dietary intake, diet diary, food record, photograph, sensor, precision, reproducibility

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *03 April 2020*
Accepted: *04 June 2020*
Published: *14 July 2020*

Citation:
Fontana JM, Pan Z, Sazonov ES, McCrory MA, Thomas JG, McGrane KS, Marden T and Higgins JA (2020) Reproducibility of Dietary Intake Measurement From Diet Diaries, Photographic Food Records, and a Novel Sensor Method. Front. Nutr. 7:99. doi: 10.3389/fnut.2020.00099

INTRODUCTION

Measurement of dietary intake is a necessary but difficult undertaking in clinical and research settings. Common methods used to measure dietary intake include 24-h diet recalls, diet diaries, photographic food records, and food frequency questionnaires (1). There are advantages and disadvantages to each method in terms of cost and participant burden, but all methods share the limitations of self-report. Studies using doubly labeled water have shown that underreporting of food intake is a common problem for self-report methods (2–6). Despite the limitations of these self-report methods, they remain the only validated methods available for measuring dietary intake in free-living situations.

Proper research practice requires that methods be validated against a standard: a previously validated method and/or a biomarker, such as doubly labeled water for energy expenditure (5). Validity refers to the accuracy of any measure; that is, how close the measured value is to the actual value. An equally important, and often overlooked, feature of a method is its reproducibility or precision. Reproducibility or precision is the extent to which a measure yields the same results under similar conditions.

The reproducibility of an instrument is especially important when dietary intake will be recorded longitudinally to assess habitual intake or changes over time. A study using repeated 24-h recalls showed total energy correlation of $r = 0.59$ between measurements (7). Reproducibility research conducted with food frequency questionnaires at two time points has shown that total energy correlations between repeat administrations of questionnaires range from $r = 0.30$–0.92 (7–13). Watson et al. (9) cited under- or over-reporting as a likely contributor to the low reproducibility for the food frequency questionnaire. This concept of systematic under- or over-reporting in dietary assessment was examined by Black and Cole (3). Their review of seven studies with repeated measurements of dietary intake revealed that some persons are more likely to underreport dietary intake than others, regardless of the assessment method used. This personal reporting bias is an issue that should not be ignored when examining dietary intake data and considering the necessity of repeated measures in such research.

Although two previous studies have looked at the reliability of diet diaries, neither used a gold standard reference method, such as a weighed food record, during the same period as the diet diary was recorded, thus limiting the general applicability of the data (14, 15). To our knowledge, no previous study has rigorously examined the reproducibility of the diet diary, which is one of the most commonly used methods to measure free-living dietary intake, or the photographic food record. Both instruments have been studied for accuracy, but there exists no data on their precision. The aim of this study was to examine the reproducibility of diet diaries, photographic food records, and a novel electronic sensor from three separate, identical meals using weighed food records as the gold standard reference method.

METHODS

Participants

Thirty participants (15 females and 15 males) with a mean (±SD) age of 29 ± 12 y (range: 19–58 y) and body mass index (BMI) of 27.9 ± 5.5 kg/m^2 (range: 20.5–41.7) were recruited at the Clarkson University campus to participate in the study. The study was approved by the Institutional Review Board at Clarkson University, Potsdam, NY and all participants read and signed an informed consent form before participation. Participants with temporo-mandibular joint disease, dysphagia or other difficulties for chewing and/or swallowing were excluded from the study.

Each participant consumed three full meals at three different visits in the laboratory, ∼1–4 weeks apart and at the same clock time at each visit. At the first visit, each participant was asked to select foods according to their own preferences (content and size) from the menu offered by one of the Clarkson University food courts. Any foods or amounts could be chosen by participants, with no restrictions. The initial meal selection was documented so that the selected meal was identical for all three study visits. Participants had no limitations on the quantity of consumed foods or order in which the foods had to be consumed.

Energy Intake Measurements

Four different methods were used to estimate total mass and energy intake: (1) weighed food record; (2) photographic food record; (3) diet diary; and (4) mathematical models based on counts of chews and swallows (CCS models) obtained via the use of electronic sensors (16).

To obtain the nutritional intake data from meals, records were deidentified and sent to the Colorado Clinical and Translational Sciences Institute's (CCTSI) Nutrition Core. A single operator assessed all deidentified photographic food records and logged consumed food amounts in a standard diet dairy format. A second blinded, independent operator entered all converted photo and original participant food diaries into the nutritional analysis program Nutrient Data System for Research (NDS-R; University of Minnesota, Minneapolis, MN). None of the data entry operators at the CCTSI Nutrition Core were involved in data collection. Using a single, trained operator at each step is the current operating procedure for all CCTSI protocols and reduces variation due to inter-operator differences in data entry. All weighed food records, photographic food records, and diet diaries were de-identified before operator entered nutritional intake into NDS-R. The novel method of using models to count chews and swallows to determine total mass and energy intake was blinded so that operator processing the data was not involved in the post-ingestion annotation of chews and swallow from the original videos.

Weighed Food Records

Before and after each meal, food was weighed by a trained member of the research team to calculate the total amount consumed. Each meal was documented and logged into a chart containing detailed information of each food item such as food name and description, mass at beginning and end of the meal, and total mass consumed. For items that could be deconstructed

(e.g., a sandwich), each food item was weighed separately before and after consumption. The item was reassembled before being served to the participant. For items that could not be deconstructed (e.g., pizza or cookies) total energy intake was estimated using total weight consumed multiplied by the caloric density of the item. Weighed food records were used as the reference method for actual dietary intake. All other methods were compared to actual dietary intake measured by the weighed food records.

Photographic Records

Pre- and post-meal photographs were taken by study staff using a digital camera. The serving plate occupied the entire field of view, and photographs were taken at a 45° angle so that the depth of foods could be estimated (17). A picture of the selected meal was taken before serving and another picture was taken at the end of the eating period. A trained, validated CCTSI nutritionist used these pictures to estimate portion sizes, using the Portion Photos of Popular Foods guide (18) and entered consumed amounts into the food analysis program, NDS-R.

Diet Diary

At the beginning of the first visit, participants were trained to complete a diet diary. Oral and written instructions were given to participants for estimating portion sizes and recording foods in sufficient detail to obtain an accurate estimate of dietary intake. Examples of both fully complete and incomplete diaries were explained to demonstrate how to appropriately record intake. Participants also received a portion estimation guide that was used as a reference, but only during the first visit. All materials were supplied by the CCTSI Nutrition Core.

TABLE 1 | Repeatability coefficients (95% confidence interval) between measurement methods for percent deviation from weighed measurement.

Outcome	Assessment method	RC for percent deviation from weighed measurement[a]
Total energy (kcal)	Diary	79.6 (55.5, 103.3)
	Photo	43.3 (32.1, 53.9)*
	Sensor	59.9 (45.9, 7.4)
Carbohydrate (g)	Diary	84.1 (56.8, 109.1)
	Photo	42.2 (23.5, 59.0)
Fat (g)	Diary	96.5 (59.4, 136.4)
	Photo	80.6 (48.1, 116.4)
Protein (g)	Diary	99.3 (64.7, 131.2)
	Photo	55.0 (38.3, 70.8)
Fiber (g)	Diary	96.1 (65.9, 123.1)
	Photo	45.2 (28.8, 61.3)*
Calcium (mg)	Diary	93.2 (62.1, 125.2)
	Photo	47.0 (37.8, 55.9)*
Iron (mg)	Diary	188.6 (61.0, 300.2)
	Photo	61.1 (40.8, 79.6)
Sodium (mg)	Diary	224.9 (78.4, 363.3)
	Photo	88.8 (44.2, 134.0)

[a] *{[Weighed-Diary (or photo)]/weight} × 100.*

**Statistically significant difference in RCs from diet method at a 5% significant level.*

After each meal was finished, participants recorded the food items they just consumed in a blank food diary. Each food item was recorded on a single line indicating the type of food, preparation style, and amount consumed. Participants did not receive any help during this stage; however, the diet diary was reviewed to ensure that it was completed appropriately (i.e., all foods listed had a portion size and description assigned). Participants were not prompted to add any food items they had forgotten to record. Participants only filled out diet diaries for research meals, and no other meals consumed during the 3 days of the study.

Models Based on Counts of Chews and Swallows

Estimation of the mass and energy consumed during each meal was computed using participant-dependent models based on counts of chews and swallows. Before starting the experiments, participants were instrumented with a sensor system for monitoring ingestive behavior (19). The system consisted of: (1) a jaw motion sensor placed below the ear to capture chewing events; (2) a miniature microphone placed on the throat to capture swallowing sounds; and (3) a digital camera for video monitoring. Sensor data and video footage were used to compute the number of chews and swallows associated to each meal as previously described (16). The total mass and energy for a given meal was estimated using a counts of chews and swallows model created with the counts of chews and swallows observed in the remaining two meals consumed by the same participant (16).

Statistical Analysis

This was a retrospective data analysis of a previous study (16). The sensor method was only analyzed for total energy as this is an exploratory method, still under development and the form described in (16) was only able to estimate mass and energy intake during a meal. When the sensor method is further developed, it will be used to estimate energy, macronutrient, and micronutrient intakes.

Because the actual amount of food consumed varied between study visits, the percent difference from that assessed by the weighed food method serves as the outcome to compare reproducibility across diary, photographic, and sensor methods. The repeatability coefficient (RC) defined as $RC = 1.96 \times \sqrt{2} \times SDwithinsubject$ was used to assess the extent of reproducibility for each method. Within-participant variability (SDwithinsubject) of the outcome across three time points was assessed using the with-subject variance from a linear mixed effects model, where the fixed effect consists of intercept only and had a compound symmetry covariance structure. Five thousand Bootstrap samples were based to calculate the 95% confidence intervals for RC for each method and the difference in RC between methods as well as the *p*-values. SAS 9.4 software (SAS Institute Inc.) were used for all the analyses.

RESULTS

Comparison of the weighed intake data from the three meals indicated that there were no differences in energy or macro-

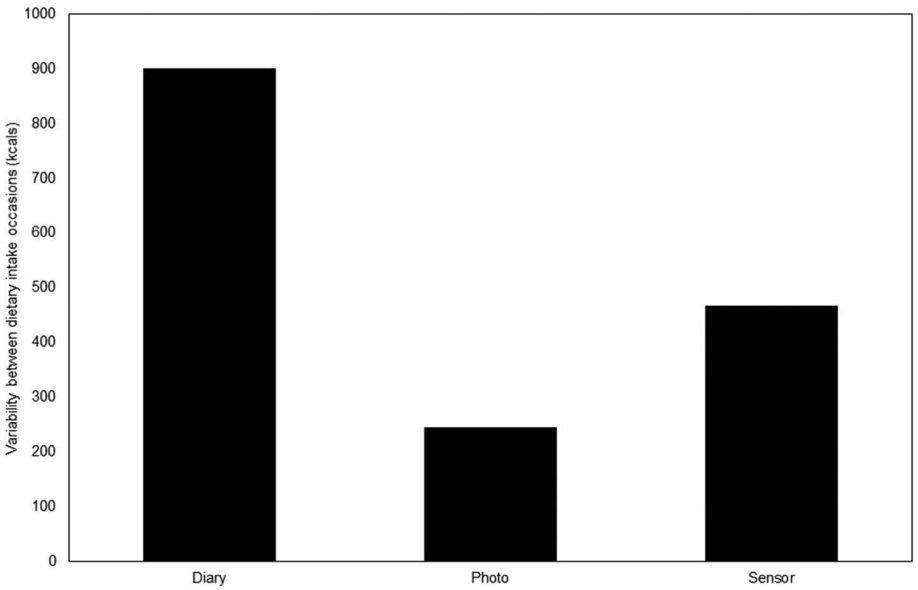

FIGURE 1 | RC and 95% CI of percent difference from weighed method for energy measurements over three time points. The photographic food record and sensor methods had greater reproducibility (lower RC values) than the diet diary for overall energy intake over three time points.

TABLE 2 | Difference in repeatability coefficient between methods over three time points.

Outcome	Comparisons	Difference in repeatability coefficient (95%) CL between two methods	p^a
Total energy (kcal)	Photo vs. Diary	−36.2 (−63.7, −10.1)	0.004*
	Sensor vs. Diary	−19.6 (−50.7, 8.0)	0.19
	Sensor vs. Photo	16.6 (−2.9, 34.2)	>0.99
Carbohydrate (g)	Photo vs. Diary	−41.9 (−74.7, −8.4)	0.01*
Fat (g)	Photo vs. Diary	−15.9 (−75.0, 39.4)	0.62
Protein (g)	Photo vs. Diary	−44.3 (−83.0, −5.0)	0.02*
Fiber (g)	Photo vs. Diary	−50.9 (−85.6, −15.3)	0.36
Calcium (mg)	Photo vs. Diary	−46.2 (−78.2, −14.3)	0.004*
Iron (mg)	Photo vs. Diary	−127.5 (−230.4, −6.9)	0.02*
Sodium (mg)	Photo vs. Diary	−136.0 (298.3, 6.3)	0.1

[a]*95% CL and 2-tailed p-values are based on 5,000 bootstrap samples. *P < 0.05.*

or micro-nutrient intake between the three meals (data not shown). The RC values for the percent difference from the weighed food records revealed that the photographic food record and sensor methods had greater reproducibility [RC = 43.4 (32.1, 53.9) and 59.9 (45.9, 70.4), respectively] than the diet diary [RC = 79.6 (55.5, 103.3)] for total energy intake over three separate meals (**Table 1** and **Figure 1**). Differences in RC values between photographic food records and diet dairies were significantly different for total energy ($p = 0.004$), carbohydrate ($p = 0.01$), protein ($p = 0.02$), calcium (0.004) and iron ($p = 0.02$) intake (**Table 2**), with photographic food records having greater reproducibility for all nutrients measured (**Table 1**).

DISCUSSION

Participants completed diet diaries immediately following each meal under supervised conditions. The method of completing the diary immediately post-meal under controlled conditions, as in this study, gives the greatest chance for this method to perform at its best. However, the diet diary method displayed the lowest reproducibility of the three methods tested for total energy intake, and it was inferior to the photographic food record for macronutrients and micronutrients examined. It should be noted that the food photographs were taken by study staff, so this method was also performed under optimal conditions that are not normally present when photographic food records are used.

Reproducibility is an important factor to consider when designing longitudinal studies in which dietary intake is to be measured repeatedly. Under these circumstances, a tool that is more reproducible will decrease the variance in the data collected over time, thereby simplifying data interpretation. Considering the cost and time spent on such studies, as well as participant burden, any instrument which is highly reproducible would add value. Indeed, previous work showed that the photographic

food record is as accurate as the diet diary in both energy intake and macronutrient composition but decreases participant burden (17). With the added benefit of higher reproducibility, the photographic food record offers increased utility over the traditional diet diary.

Although two previous studies have looked at the reliability of food records, both compared diet diaries recorded at different times, with no consistency of foods eaten during each recording period (14, 15). Therefore, any differences noted could have reflected actual differences in dietary intake rather than methodological issues. Putz et al. compared two diet diaries to a weighed food record as a reference method (15). However, the weighed food record was completed at separate time from the diet diaries so it is unclear if the dietary intake was similar across occasions and therefore, if the differences measured were due to the method used or actual differences in dietary intake on the different recording occasions. In these previous studies, for estimating total energy intake, the reproducibility of the diet diary was low to moderate [ICC of 0.49 and 0.69 for (14, 15), respectively], which compares well with our estimate of low reproducibility (RC = 43.4). Conversely, we found that the reproducibility for the sensor and photographic record methods was moderate to high, respectively.

Limitations of this study include small sample size, limited age range of participants, that energy intake was not matched between meals for each participant, and photographs in the photographic food records were taken by study staff and not participants. Whereas weighed food records are considered the gold standard and this method was used in our laboratory setting, in a free living situation doubly labeled water could be used to compare reported intake to total energy expenditure, albeit at greater expense. With regard to expense, studies have shown photographic food records to be similar in cost or less costly than self-report methods such as diet diaries and 24-h recalls (20–23). However, when compared to written diet diaries, it does take ∼20 more mins per day of intake recording to analyze photos and convert the visual information to amounts for data entry, which is likely irrelevant for smaller studies but could create higher cost overall for large studies. This study had several strengths, however, including the use of a within subject repeated measures design, the large variety of foods for participants to choose from, the use of more than two repeated measures, and that the study took place in a controlled laboratory setting where the researchers had the ability to accurately determine energy intake using weighed food records, considered the gold-standard (24–26).

An interesting finding from this study is that the photographic food record was more precise/reproducible than the sensor method, even though previous work showed that the sensor was more accurate than photographic food records (19). Accuracy may be of greater concern when working with understudied or vulnerable populations where little data currently exist, or in studies that measure dietary intake at a single point. Under these circumstances, the sensor method displays promise, particularly for vulnerable populations such as children with developmental delay or the elderly who may not be able to complete any other method for the estimation of dietary intake. As the sensor can be placed on the participant's jaw and behind the ear, unobtrusively estimating energy intake via measurement of chewing, the need for participant literacy or cognizance of food choices is abolished. In all populations, this method would significantly reduce participant burden and negate some of the pitfalls of self-report. Our future work aims to combine the strengths of the photographic and sensor methods by enabling the sensor to automatically take images of food ingested during the day.

CONCLUSION

The higher reproducibility of the photographic food record warrants its use over the diet diary in longitudinal studies which aim to measure dietary intake repeatedly. The novel sensor method for estimating energy intake also shows promise as a dietary intake assessment tool for the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Institutional Review Board at Clarkson University, Potsdam, NY and all participants read and signed an informed consent form before participation.

AUTHOR CONTRIBUTIONS

Conceptualization: JF, ZP, ES, MM, and JH. Data curation: JF and TM. Investigation: JF and ES. Project administration: ES, MM, JH, and JT. Supervision: ES, MM, and JH. Writing original draft: TM, JH, and KM. Writing—review and editing: JT, JF, ZP, KM, ES, MM, TM, and JH. All authors contributed to the article and approved the submitted version.

FUNDING

Research reported in this publication was supported by the National Institutes of Health through the National Institute of Diabetes and Digestive and Kidney Diseases award numbers R21DK085462 and R01DK122473 and National Center for Advancing Translational Sciences award number U01TR002535. The content was solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We would like to thank the Colorado Clinical and Translational Sciences Institute Nutrition Core for assistance with data analysis.

SUPPLEMENTARY MATERIAL

Photographic Records: http://dx.doi.org/10.21227/5mjw-jz09.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 21 October 2020
doi: 10.3389/fnut.2020.561010

Design and Characterisation of a Randomized Food Intervention That Mimics Exposure to a Typical UK Diet to Provide Urine Samples for Identification and Validation of Metabolite Biomarkers of Food Intake

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: 11 May 2020
Accepted: 07 September 2020
Published: 21 October 2020

Citation:
Willis ND, Lloyd AJ, Xie L, Stiegler M, Tailliart K, Garcia-Perez I, Chambers ES, Beckmann M, Draper J and Mathers JC (2020) Design and Characterisation of a Randomized Food Intervention That Mimics Exposure to a Typical UK Diet to Provide Urine Samples for Identification and Validation of Metabolite Biomarkers of Food Intake. Front. Nutr. 7:561010. doi: 10.3389/fnut.2020.561010

Poor dietary choices are major risk factors for obesity and non-communicable diseases, which places an increasing burden on healthcare systems worldwide. To monitor the effectiveness of healthy eating guidelines and strategies, there is a need for objective measures of dietary intake in community settings. Metabolites derived from specific foods present in urine samples can provide objective biomarkers of food intake (BFIs). Whilst the majority of biomarker discovery/validation studies have investigated potential biomarkers for single foods only, this study considered the whole diet by using menus that delivered a wide range of foods in meals that emulated conventional UK eating patterns. Fifty-one healthy participants (range 19–77 years; 57% female) followed a uniquely designed, randomized controlled dietary intervention, and provided spot urine samples suitable for discovery of BFIs within a real-world context. Free-living participants prepared and consumed all foods and drinks in their own homes and were asked to follow the protocols for meal consumption and home urine sample collection. This study also assessed the robustness, and impact on data quality, of a minimally invasive urine collection protocol. Overall the study design was well-accepted by participants and concluded successfully without any drop outs. Compliance for urine collection, adherence to menu plans, and observance of recommended meal timings, was shown to be very high. Metabolome analysis using mass spectrometry coupled with data mining demonstrated that the study protocol was well-suited for BFI discovery and validation. Novel, putative biomarkers for an extended range of foods were identified including legumes, curry, strongly-heated products, and artificially sweetened, low calorie beverages. In conclusion, aspects of this study design would help to overcome several current challenges in the development of

BFI technology. One specific attribute was the examination of BFI generalizability across related food groups and across different preparations and cooking methods of foods. Furthermore, the collection of urine samples at multiple time points helped to determine which spot sample was optimal for identification and validation of BFIs in free-living individuals. A further valuable design feature centered on the comprehensiveness of the menu design which allowed the testing of biomarker specificity within a biobank of urine samples.

Keywords: dietary intake, metabolomics, free-living participants, biomarkers, radomized control trial

INTRODUCTION

The amount and pattern of foods and beverages consumed influence gene expression (1) and are major determinants of multiple health outcomes (2). Despite this centrality in the atiology of health and disease, the estimation of habitual dietary intake remains difficult (3). Conventional tools based on dietary self-report are tedious and time-consuming for both study participants and researchers. Dietary misreporting is common and substantial (4) and is exacerbated in those who are overweight or obese (5). Whilst the development of digital tools to assist with dietary recording may reduce the workload for respondents and researchers (6), use of such tools does not eliminate the subjectivity and biases inherent in approaches based on self-report. To improve measurements of dietary intake, there is a need to develop strategies for the objective identification, validation and deployment of suitable biomarkers (7).

Biomarkers of food intake (BFIs) assessed in body fluids or easily-accessible tissues offer potential alternative, objective routes to estimating dietary exposure (8). Measurement of BFIs (9, 10) could overcome some of the limitations of traditional dietary assessment methodologies by providing additional objective estimates of food exposure (11). Such biomarkers are of two main types: (i) those biomarkers which attempt to estimate a major class of nutrients e.g., urinary nitrogen excretion as an index of dietary protein consumption (12) and (ii) biomarkers which attempt to estimate intake of specific foods or food constituents (13). Most foods contain large numbers of characteristic metabolites many of which are cataloged in comprehensive databases of food composition, e.g., FoodB (developed by the University of Alberta, Canada: www.foodb.ca) and of selected food components, e.g., Phenol Explorer (developed by INRA: www.phenol-explorer.eu). However, when foods are consumed, they undergo metabolic transformation during digestion and absorption by enterocytes and colonocytes, by bacteria within the gut lumen and by Phase 1 and Phase 2 enzymes within the liver. Therefore, although the patterns of metabolites present in blood or urine reflect what has been consumed, the specific metabolites in such body fluids may differ substantially from those ingested (14).

We, and others, have applied metabolomics approaches to blood, saliva and urine in human studies to discover novel BFIs (8, 15–22). We have focused on urine as the body fluid of choice because of the ease of collection and the fact that, in contrast with blood, it provides an integrated estimate of exposure over several hours and because of technical advantages in sample preparation for metabolomics assay (8). This has led to the identification of a substantial number of putative biomarkers of individual foods (23) and to potential biomarkers of the overall healthfulness of the diet (24). Consensus guidelines for the critical assessment of candidate BFIs have been proposed recently (10). Although valuable, these guidelines focused on qualifying the utility of individual BFIs to monitor exposure to specific foods/food groups. Food intervention studies to determine the impact of individual dietary components on health form a large component of nutrition research but equally important is the need for approaches to assess overall dietary exposure in epidemiological studies and clinical trials (24). Whilst these discoveries and developments are encouraging, several challenges remain, associated particularly with the deployment of BFI technology in real world settings (**Table 1**).

The MAIN (Metabolomics at Aberystwyth, Imperial and Newcastle) Study was designed to address these challenges by investigating biomarkers of food intake under conditions in which study participants consumed well-characterized foods within conventional diets with respect to meal design, cooking and eating patterns and collected urine samples at home without changing their usual behavior. This provided an opportunity to expand biomarker coverage to include a more comprehensive range of foods and beverages that are highly consumed in the UK and considered important for the UK government healthy eating policy (25). Our primary aim was to develop protocols which could be applied in large-scale epidemiological studies, clinical trials and in public health surveys. We focused on approaches that would be acceptable to the public, easy to follow and to adhere to, and which would be of modest cost. In this paper, we report the detailed design and protocol of the MAIN Study

Abbreviations: AGC, Automatic gain control; AUC, area under the Receiver Operator Characteristic curve; BFI, biomarker of food intake; BMI, Body Mass Index; CRN, Clinical Research Network; EPIC, European Prospective Investigation into Cancer and Nutrition; FFQ, food frequency questionnaire; FIE-HRMS, flow infusion electrospray ionization—high resolution mass spectrometry; FMV, first morning void; HCD, Higher-energy collision dissociation; IPAQ, International Physical Activity Questionnaire; ISRCTN, International Standard Randomized Controlled Trial Number; MAIN, Metabolomics at Aberystwyth, Imperial and Newcastle; MDS, Multi-dimensional scaling; MRC, Medical Research Council; MS, mass spectrometry; MSI, Metabolomics Standards Initiative; MS^n, Tandem mass spectrometry; NDNS, National Diet and Nutrition Survey; PA, physical activity; RF, Random Forest; ROC, Receiver Operator Characteristic; SD, Standard deviation; TMAO, Trimethylamine-N-oxide; UHPLC-HRMS, Ultra High Performance Liquid Chromatography-High Resolution MS.

TABLE 1 | Challenges associated with the design of a food intervention study to develop and assess deployment of BFI technology to monitor overall dietary exposure.

(1) Providing opportunity to expand the discovery of biomarkers to include as many commonly-consumed foods as possible
(2) Ensuring structured exposure to a sufficiently comprehensive range of foods to mimic diets typical of a specific population
(3) Validating biomarker specificity in real world settings using conventional eating patterns where a whole diet is consumed rather than focusing on single food items
(4) Evaluating the impact of food preparation/processing/formulation and cooking method on the behavior of biomarkers of specific foods
(5) Developing a urine sampling strategy that enables collection of samples with minimal burden on free-living participants and without adversely affecting the quality and comprehensiveness of biomarker measurement

conducted at Newcastle University (MAIN Study Newcastle) as well as baseline characteristics of participants recruited to the study. Elsewhere we have reported the validation of the urine sampling methodology for free-living study participants showing it was non-intrusive, imposed low participant burden, and delivered samples with high quality metabolome content assessed using metabolite fingerprinting (26, 27). Here, we include a summary of novel biomarkers discovered using samples from the MAIN Study with the aim of extending the BFI coverage to a wider range of commonly consumed foods.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

The studies involving human participants were approved by East Midlands—Nottingham 1 National Research Ethics Committee (14/EM/0040) following Proportionate Review. Caldicott approval for storage of data and data protection was granted by Newcastle-upon-Tyne Hospitals NHS Foundation Trust [6896(3109)]. The trial was adopted into the UK Clinical Research Network (CRN) Portfolio (16037) and was registered with International Standard Randomized Controlled Trial Number (ISRCTN), 88921234.

A study information sheet was given to all potential participants in advance of their first visit to the research unit. The participants provided written informed consent to participate in each study, taken by an appropriately trained researcher. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participant Recruitment

Participants were recruited using the inclusion criteria in **Supplementary Table 1** through poster and leaflet campaigns around Newcastle University campus and in local public buildings e.g., libraries. In addition, invitation letters were sent to potential participants who had registered their interest with Newcastle University in being contacted about upcoming nutrition-related studies. An advertisement for the study was also placed in a local newspaper. Some participants were recruited by word of mouth.

Based on data from our earlier studies (28), we aimed for a sample size of 15 participants for Study 1 (which incorporated experimental period 1 menu plans) and 30 participants for Study 2 (which incorporated experimental period 2 menu plans)—this allowed for a 20% drop out. To minimize the risk that current disease or medications used for their management altered metabolism and, therefore, compromised the normal behavior of food-related biomarkers detected in urine, we implemented an *a priori* exclusion list (**Supplementary Table 1**). For similar reasons, we excluded potential participants who reported that they had had a cholecystectomy or who undertook a high level of exercise such as a professional athlete or body builder. Vegetarian recruits had to be willing to eat meat and fish during the studies.

Intervention Design and Randomisation

The MAIN Study Newcastle was built around six daily menu plans, delivered in two separate, 3-day experimental periods. Menu plans 1–3 constituted experimental period 1; menu plans 4–6 constituted experimental period 2. Menu plans were designed to emulate real world conditions and to reflect the whole diet by including many commonly-eaten foods in the context of a typical UK diet. All foods and drinks were provided to free-living individuals who prepared and consumed the meals in their own homes/places of work and were responsible for collecting urine samples while carrying out their normal daily activities.

Experimental period 1 menus were based largely on foods for which a significant amount of metabolite-based biomarker research has been published. The menus permitted the provision of 4–5 target foods each day, providing opportunity for biomarker validation as described in Lloyd et al. (27). On the first experimental day, participants consumed test foods for which there were some previously published urinary metabolite biomarkers while, on the two subsequent experimental days, they consumed foods for which putative biomarkers have been proposed but for which further validation and/or discovery is necessary. This first experimental period allowed the refinement of a spot urine sampling protocol for use in real world settings. Participants collected a range of post-prandial spot urines, which were compared for chemical richness and evaluated for the presence of known and putative dietary biomarkers. The overarching aims of this part of the study were to: (i) identify the urine collection times which provided the most data-rich spot urine for accurate capture of biomarker behavior relating to recent dietary intake and (ii) develop acceptable, non-onerous and easy-to-implement urine sampling protocols for the future identification and validation of novel biomarkers of foods included within the menus [see Lloyd et al. (27) and Wilson et al. (29) for further detail].

To assess whether use of a standardized evening meal [as employed in our earlier studies (30)] was advantageous

for "normalizing" urines prior to biomarker validation and discovery, participants followed these menus in the same order on 2 separate weeks. Participants were randomized to either a standardized evening meal (chicken casserole ready meal and chocolate éclair) or a low polyphenol evening meal of their choice, to be eaten immediately before the experimental period. Participants were provided with a guide to high polyphenol foods/drinks that they should not eat/avoid and a list of low polyphenol foods/drinks which they should select from during the "Pre" day (see **Figure 1**). A cross-over design was employed so that all participants underwent both dietary interventions i.e., standardized evening meal and own choice low polyphenol evening meal.

Experimental period 2 menu plans were designed to identify novel biomarkers of food intake and to investigate whether the putative urinary biomarkers for specific foods were influenced by food preparation, processing, quality, formulation, complexity, and cooking method. Menus were created to deliver foods for which there were few, uncertain or no published biomarkers. In addition, we expanded the range of food formulations investigated in experimental period 1. For example, red and white grapes were included in the following forms: whole grapes, raisins (dried), wine (fermented), grape juice (pasteurized/heat-treated/concentrated), sparkling grape juice (carbonated drink), and a fruit smoothie (complex beverage). Whole grains were delivered across both experimental periods in the form of rye bread (with and without a sourdough starter, toasted and untoasted), wholemeal bread (toasted and untoasted), wholegrain breakfast cereal (Weetabix®), porridge oats (boiled/microwaved with milk) and wholemeal pasta (extruded). Participants consumed a range of commonly eaten foods in three different daily menu plans, which were presented in a different order in each of three experimental weeks. This design facilitated the search for novel biomarkers of specific foods, but also provided the potential to characterize the kinetics of biomarker appearance and decay and to investigate the longevity of biomarker signals in urine.

Each daily menu plan was designed to emulate conventional UK eating patterns with a breakfast, lunch, afternoon snack and dinner. All foods and drinks for the whole intervention were provided to participants in appropriate portion sizes and with cooking instructions, where necessary. Participants were encouraged to consume these items to the exclusion of any other foods or beverages, but had the freedom to eat or not the meals and to interpret the cooking instructions as they wished. During each experimental week, the allocated menu plans were followed from Tuesday to Thursday, as shown in **Figure 1**. Experimental period 1 menu plans were eaten in the same order on 2 consecutive weeks (Study 1); experimental period 2 menu plans were eaten in pre-determined orders over a 3-week period (Study 2). In each experimental week, the "Pre" day was the day before starting the experimental period and was always a Monday and included a pre-determined evening meal (Dinner). The "Post" day was the day following completion of the experimental period when the last biological samples were collected and was always a Friday. Participants visited the Clinical Aging Research Unit, Newcastle University on these 2 days only. This design minimized the number of trips the participants had to make to the University and also enabled participants to take a break from the studies at weekends, so reducing the burden of compliance.

During the "Pre" day, participants were asked to restrict, as far as possible, their polyphenol intake. In practical terms, this meant reducing their intake of brightly colored fruits and vegetables, chocolate, tea, herbal teas and coffee. Participants were asked to abstain from alcohol and stop taking any dietary supplements for the duration of the studies. In Study 1, dinner on the "Pre" day was either a standardized evening meal or a low polyphenol meal of the participant's choice whilst in Study 2 it consisted of one of three meals designed to capture biomarkers of relatively unhealthy food choices, particularly poorer-quality meats and breaded and battered foods.

Menu plans were developed using Public Health England policy advice from The Eatwell Plate which has been revised as The Eatwell Guide (25) and information on the eating habits of the British population which were collected and collated in the National Diet and Nutrition Survey (NDNS), years 1–3 (31). The process of menu plan design has been described in detail in a recent publication (27). Briefly, this involved analysis of each food grouping described in the Eatwell Plate (e.g., fruit & vegetables) and investigating the disaggregated food groups that contribute to that food grouping in the NDNS (e.g., fresh & canned fruit; fruit juice; dried fruit etc.). We then identified the most commonly eaten foods within each disaggregated food group and incorporated as many as possible of the most commonly consumed foods into the menu plans using the most commonly reported method of preparation (e.g., raw/boiled/with or without skin). To assess biomarker robustness, multiple forms of similar foods using different processing methods and formulations were delivered as discrete meal components and incorporated within complex meals, and using different cooking methods. Foods and portion sizes were compatible with normal eating behavior and were provided according to conventional UK daily meal patterns using commercially available foods. We used average (medium) portions of each food as determined by the Food Standards Agency "Food Portion Sizes" Guide (32). Exact amounts of each food provided has been described elsewhere (26, 27). An example daily menu plan from experimental period 2 is shown in **Supplementary Table 2**.

To identify the easiest to collect and most informative spot urine sample, participants were asked to collect several spot urine samples each day. These included their first morning void (FMV) before breakfast; a fasting sample, defined as a sample collected after the FMV but before breakfast, following an overnight fast of at least 12 h; a sample collected any time between finishing breakfast and eating lunch and a sample collected any time between finishing lunch and eating their afternoon snack. Each urination between dinner and the FMV the following day was also collected. To reduce variation in timing of urine sample collection, participants were encouraged to consume meals within a 2 h time slot (breakfast, lunch and dinner) or 30 min time slot (afternoon snack) as illustrated in **Figure 1**. However, the participants were free to ignore this advice and to provide a urine sample at the time most convenient for them.

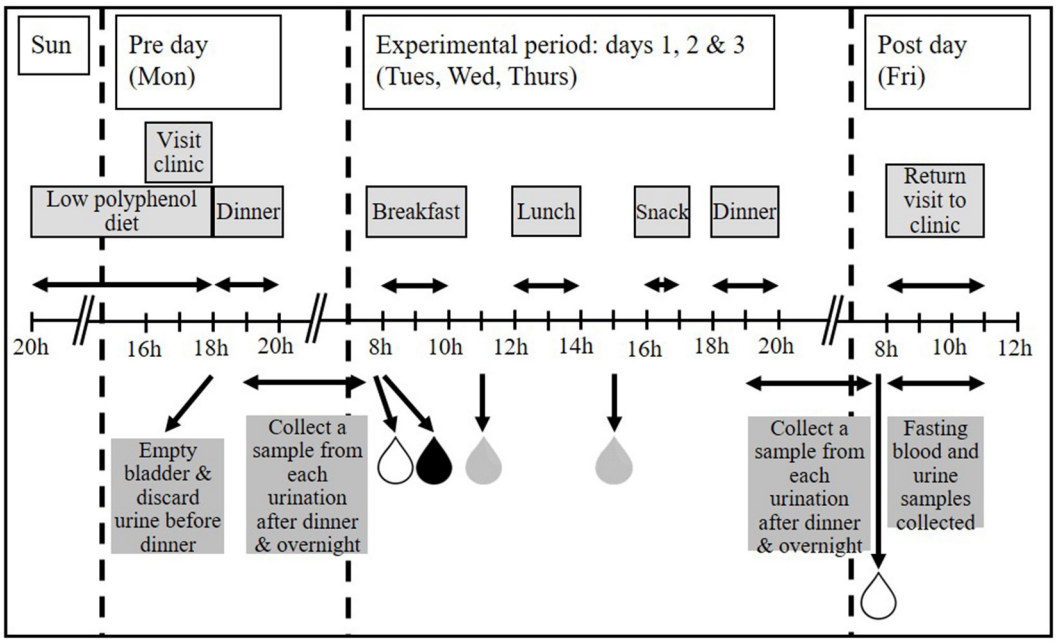

FIGURE 1 | The MAIN Study timeline. Participants were asked to follow a low polyphenol diet prior to starting the experimental period. Where applicable, dinner was provided on the "Pre" day (Monday). All foods and drinks for 3 consecutive experimental days (Tuesday, Wednesday, and Thursday) were provided. Participants collected first morning void (FMV), fasting urines and spot urines post-breakfast and post-lunch each day during the experimental period, and a FMV and fasting urine on the "Post" day. Any urination between finishing dinner and providing the FMV the following day was also collected. A fasting blood sample was taken on the "Post" day only. Figure adapted from Lloyd et al. (27).

Participants were encouraged to keep hydrated and to drink water *ad libitum*. To help maintain good hydration, participants were given eight 500 ml bottles of water per experimental week, one to be drunk with/after each dinner, one to be drunk during each experimental day and one to be drunk on the morning of the "Post" day, prior to venipuncture.

Participants in Study 1 were randomized (random.org) at enrolment to one of two dinner options on the "Pre" day. In Study 2, participants were randomized to one of 12 3 × 3 Latin squares (3 daily menu plans × 3 experimental weeks) at enrolment. The order in which these participants consumed the three less healthy dinners on the "Pre" day in each of the three experimental weeks was randomized independently.

Compliance

Dietary compliance was assessed by participant self-report. They were asked to record how much of each food/drink item they ate. If some food was uneaten, the participant recorded the amount eaten as 75, 50, 25% or 0, as appropriate. Dietary substitutions, intrusions and protocol deviations were also recorded. Participants recorded if they substituted any foods or drinks of their own choosing for those provided by the research team, ate or drank any additional items or prepared any of the meals differently from that instructed.

A 1-day food diary was used to record all foods and drinks consumed from 8pm on the evening before and throughout the "Pre" day. This was used to check participant compliance with the low polyphenol diet prior to commencement of the experimental period each week.

To monitor compliance with both the urine collection protocol and suggested meal times, participants completed a Urine sample collection record and a Meal time record—the latter asked specifically at what time they finished eating each meal. This acted as a check on the accuracy of the FMV and fasting urine samples collected. In addition, this information allowed calculation of the time interval between meal consumption and subsequent urine collections.

Study Measures

Information on participant socio-demographics (age, sex, smoking status, and alcohol consumption), medical history, current medications, and use of dietary supplements was collected at enrolment.

In Study 1, weight, height and waist circumference were measured on "Pre" and "Post" days of each experimental week. In Study 2, height and waist circumference were recorded in week 1 only, whilst weight was recorded weekly, pre- and post-the experimental period. All anthropometric measurements were made at the research unit by the same researcher.

Body weight was measured to the nearest 0.1 kg using a Tanita body composition analyzer (TBF-300 MA; Tanita Corporation, Tokyo, Japan); height was measured to the nearest 0.1 cm using a Leicester portable height measure (Chasmors Ltd, London, UK). Waist circumference (at the point equidistant between the costal margin and the iliac crest) was measured to the

nearest 0.1 cm using a non-stretch tape measure over bare skin, whenever possible. Waist circumference measurements were taken in duplicate, or repeated until two measurements agreed within 1 cm. Participants were asked to wear lightweight clothing and removed their shoes for all measurements.

Habitual dietary intake was assessed at the beginning of each study using a locally adapted version of the validated food frequency questionnaire (FFQ) used in the European Prospective Investigation into Cancer and Nutrition (EPIC) (33).

To monitor their physical activity (PA), each week participants were asked to complete the International Physical Activity Questionnaire (IPAQ) short form (34).

Blood and Urine Sample Collection

Participants collected urine samples at suggested times (**Figure 1**) in a calibrated plastic jug and recorded the date, time and total volume of collection. A 20 ml aliquot from each urination was retained and the rest discarded. If not at home, participants kept urine samples in a cool bag containing a frozen cool block, otherwise they stored them in a refrigerator before returning them to the research team at the end of each experimental week. It has been reported that no major changes in urinary metabolite fingerprints occur when samples are stored in tubes held at +4°C for up to 72 h (35). We have shown recently that the metabolome of spot urine samples collected, stored and transported as described in this manuscript is stable with negligible microbial growth at 4°C and, specifically, that inclusion of preservatives has no impact on data quality (36). For long-term storage, urine samples were divided into 10 × 1 ml aliquots [2 ml screw-cap microtubes (Starstedt, Germany)] which were free from plasticisers (in house tests; data not shown)] and 1 × 5–10 ml aliquot (25 ml Universal tubes; Starstedt) and stored at −80°C.

Whole blood, serum and plasma were archived at −80°C for future lipidomic analysis and associated metabolite fingerprinting. Venipuncture was performed on the "Post" day of each week after a minimum 12 h overnight fast. After filling, blood tubes were inverted several times, kept flat on ice and processed within 2 h. Before processing, the height of the blood in each tube was recorded to assess filling. Plasma was separated from anti-coagulated blood collected in 1 × EDTA and 2 × lithium/heparin Vacutainer® blood collection tubes (Becton Dickinson, Oxford, UK) by centrifugation at 3,100 × g for 5 min at 4°C in a Jouan CR3i centrifuge (Saint-Herblain, France). Plasma was stored at −80°C in 1 ml aliquots in plasticiser-free microtubes as described above. Serum was separated from coagulated blood collected in 2 × gel Vacutainer® tubes at least 30 min after collection, aliquoted and stored as for plasma. One tube of unprocessed whole blood, collected in an EDTA-coated tube, was also stored at −80°C.

A summary of the time points at which key data, measurements and biological samples were collected is given in **Table 2** (Study 2) and **Supplementary Table 3** (Study 1).

Metabolomic Analysis

The metabolomics methods implemented for biomarker discovery have been published elsewhere (26, 27). All urine samples were normalized by refractive index prior to analysis to ensure all MS measurements were made within a similar dynamic range. Essentially, urine samples were analysed by non-targeted metabolite fingerprinting using high resolution (HR) flow infusion electrospray (FIE) ionization mass spectrometry (MS), acquired on an Exactive Orbitrap (ThermoFinnigan, San Jose, CA) mass spectrometer coupled to an Accela (ThermoFinnigan) ultra-performance liquid chromatography system. Supervised Random Forest (RF) classification was implemented to investigate the presence of distinctive urine composition changes following consumption of specific meals. A combination of accuracy, margins of classification and area under the ROC (Receiver Operator Characteristic) curve (AUC) were used to evaluate the performance of classification models (37). To reveal potential explanatory signals responsible for discriminating between baseline and post-prandial urine samples, a combination of RF, AUC and Student's *t*-test was employed for feature selection (37).

The methodology used for biomarker identification has been described in detail elsewhere (26, 27). For metabolite signal annotation, accurate *m/z* values were extracted for high-ranked explanatory signals and queried using MZedDB, an interactive accurate mass annotation tool (38). Ultra High Performance Liquid Chromatography-High Resolution MS (UHPLC-HRMS) and Tandem mass spectrometry (MS^n) allowed further structural identification of putative biomarkers as previously described (39). Refractive index adjusted urine samples were diluted with 100% MeOH (1:1, v:v) and centrifuged at 1,400 × *g* for 5 mins at 4°C. Samples were analysed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, Waltham, MA) coupled to a Dionex Ultimate 3000 Ultra High Performance Liquid Chromatography (UHPLC) system (Thermo Scientific). Chromatographic separation was performed on a reverse phase (RP) Hypersil Gold 1.9 μm, 2.1 × 150 mm column (Thermo Scientific) using 0.1% formic acid in H_2O (mobile phase A) and 0.1% formic acid in MeOH (mobile phase B) at a flow rate of 0.6 ml/min and column oven temperature at 60°C. Each sample (5 μl) was analysed by following a gradient after 0.5 min isocratic A to 40 % B in 3.5 min and subsequently to 100% in 5 min. The column was washed with 100% B for 2.5 min and re-equilibrated for 2.5 min. Data were acquired in two runs using respective positive and negative ESI mode. Each experiment consisted of a full scan [110–1,100 *m/z* at 120,000 resolution and MS^2 scans (*ddMS2 OT HCD* event, stepped Higher-energy collision energies of 45, 60, 75%) and 15,000 Orbitrap mass resolution] within a 1 s cycle time using selected targeted mass properties for either positive or negative ionization mode between 1 and 12 min runtime. The maximum injection time was 22 ms and the Automatic Gain Control (AGC) target of 1×10^4 was set to be exceeded if there is parallelisable time. The spray voltage was 3.5 kV for positive and 2.5 kV for negative ionization modes. The temperatures of the ion transfer capillary and vaporiser were, respectively, 342° and 258°C with sheath and auxiliary gas set at 45 and 13 arbitrary units, respectively. The data were acquired using Thermo Scientific Xcalibur version 4.2.28.14.

Metabolites were annotated to Metabolomics Standards Initiative (MSI) level 1 (40) by matching masses, MS^n and retention times with authentic standards or with the respective

TABLE 2 | Summary of data and biological samples collected during Study 2.

Data collected/measures	Study time point									
		Experimental week 1			Experimental week 2			Experimental week 3		
	Screening	Pre day visit	Between visits	Post day visit	Pre day visit	Between visits	Post day visit	Pre day visit	Between visits	Post day visit
Demographics (age, sex) & self-reported anthropometrics	X									
Eligibility criteria (medical history, medications, supplements, diet & lifestyle)	X									
Written consent		X								
Randomisation		X								
One day food diary		X			X			X		
Height & waist circumference		X								
Weight		X		X	X		X	X		X
Food frequency questionnaire		X								
IPAQ Physical activity questionnaire		X			X			X		
Dietary compliance record			X			X			X	
Meal time record			X			X			X	
Urine samples			X	X		X	X		X	X
Urine sample collection record			X	X		X	X		X	X
Blood sample (plasma, serum & whole blood)				X			X			X

IPAQ, International Physical Activity Questionnaire.

aglycone (if the biotransformation product was unavailable). MSI level 2 structural assignment was achieved by putatively matching signal behavior with that of authentic standards reported in the literature (based upon physicochemical properties, retention times and spectral similarity) or fragmentation pattern alignment with data in public/commercial spectral libraries [Lipid Maps, HMDB, Metlin, and Massbank (41–44)]. MSI level 3 structural identification indicated a putatively characterized compound class.

RESULTS

Recruitment

As shown in **Figure 2**, 150 people expressed an interest, or were invited to participate, in the studies over a 10 month period: 40 people for entry into Study 1 and 116 people for entry into Study 2 (six people took part in both studies). Personal details, self-reported anthropometrics, medical history and lifestyle information (smoking, alcohol consumption, dietary supplementation) were provided by 70% people ($n = 105$). More than 70% of those invited to participate in Study 1 had taken part in earlier nutrition research studies at Newcastle University and six were subsequently recruited. A further nine participants learned about the study through word of mouth. The majority (85%) of those who expressed an interest in Study 2 and were screened for eligibility (69%) had learned about it through local community and internal university advertising and contributed 66% of enrolled participants. The remaining participants had either taken part in Study 1 (17%) or heard about the study through word of mouth (17%). The desired sample size ($n = 15$) was met in Study 1 and the target recruitment ($n = 30$) was exceeded by 20% in Study 2.

Baseline Characteristics of Participants

Participants aged 19–77 years (mean 46 years), of whom 57% were female, took part in the studies (**Table 3**). The participants were generally healthy: arthritis, previous cancer and hypertension were the most commonly reported medical history. Smokers were excluded from the study and while 78% participants were alcohol consumers, weekly consumption (median 4.5 units) was well below the recommended maximum levels. Mean BMI (24.1) was within the normal range but almost 20% of participants had central obesity. Twenty-five percent of participants reported taking dietary supplements with fish oils/cod liver oil being the most commonly consumed supplement. Self-reported physical activity levels were relatively high−59% of participants were categorized as highly physically active according to the IPAQ guidelines (45). Self-reported sitting time, measured by the IPAQ, was also high with participants reporting, on average, 6 h sitting

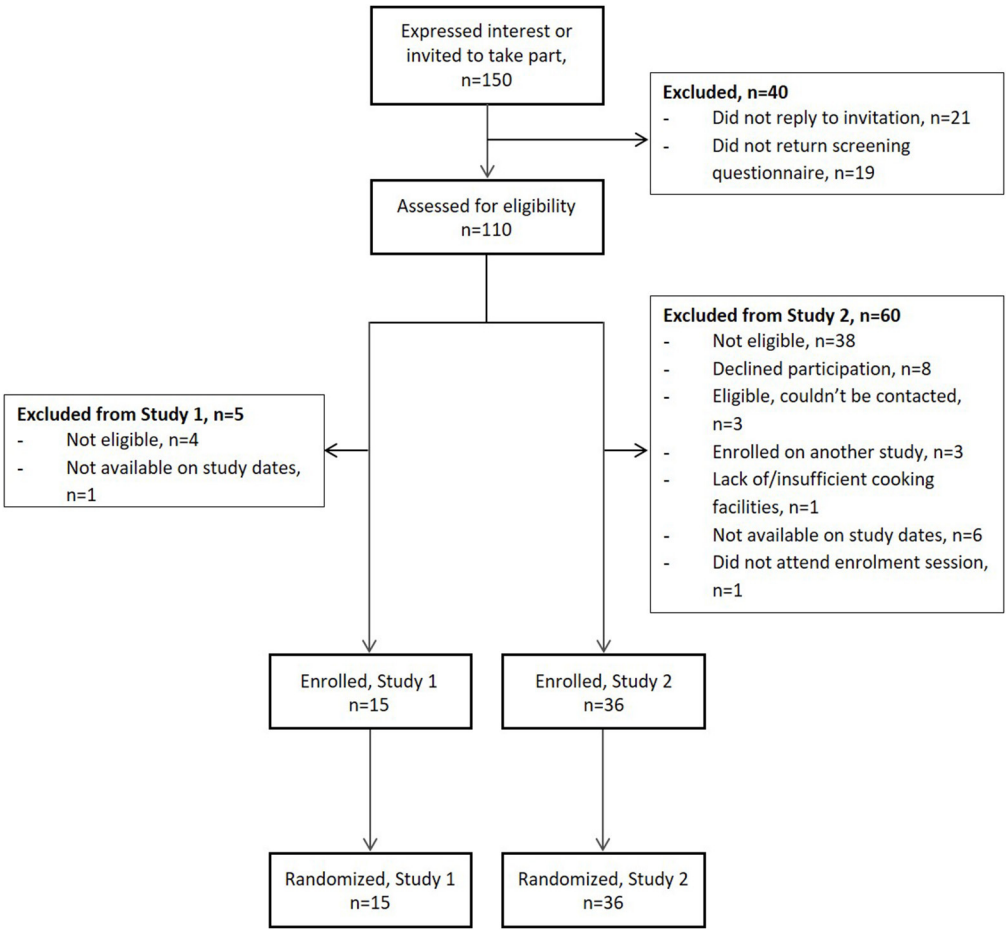

FIGURE 2 | Flow of participants during recruitment to the MAIN Study. Study 1 preceded Study 2; six participants took part in both studies.

time/day. This exceeds the 4 h sitting time/day cut-off point considered a proxy for sedentary behavior detrimental to health (46).

Participant characteristics were similar in both studies but those recruited to Study 2 were slightly older (mean 1.4 years) and had a lower BMI. They were less likely to report consuming alcohol, although weekly consumption amongst those who did was higher (based on self-reported weekly alcohol intake). Ten percent more participants were categorized as having central obesity in Study 1 than in Study 2.

Validation of Study Design for Discovery of Novel Biomarkers of Dietary Exposure

In recent publications, we have reported that dietary exposure biomarker discovery was possible within the context of the present comprehensive food intervention mimicking a typical UK diet in free-living individuals with minimal intrusion on normal daily activities (26, 27). Validation of the overall MAIN Study Newcastle design for efficient biomarker discovery was assessed initially by confirming the presence of expected BFIs in urine samples collected during and following experimental day 1 in Study 1 (26). Biomarker performance was further tested using different food formulations and processing methods with several types of meat, wholegrains, fruit and vegetables (26). Additionally it was shown that the urine sampling methodology for free-living study participants was non-intrusive and delivered samples with high quality metabolome content using metabolite fingerprinting (27).

Against this background, we now demonstrate that the MAIN Study Newcastle design, coupled with metabolomic techniques, made possible the discovery of new BFIs for use in monitoring dietary intake in free-living individuals eating conventional diets. As an example, the schematic of the dietary exposure biomarker discovery strategy using BFIs of legumes is shown in **Figure 3**. Participants were exposed to an evening meal (**Figure 3A**) containing a typically consumed legume (garden peas). Random Forest modeling of metabolite fingerprints representing urines collected at bed-time following this meal and FMV urine samples acquired earlier in the day showed that the urine

TABLE 3 | Characteristics of the MAIN Study participants at baseline.

	Study 1	Study 2	Total
Variable	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Demographics			
Total [n]	15	36	51
Sex			
Female [%]	53	58	57
Age [years]	45.3 (14.8)	46.7 (18.7)	46.3 (17.5)
Age range (min-max) [years]	22–63	19–77	19–77
Health Conditions [n]			
Arthritis	0	4	4
History of cancer	1	3	4
Hypercholesterolemia	0	1	1
Hypertension	0	4	4
Irritable bowel syndrome	1	0	1
Osteoporosis	0	1	1
Stomach/bowel problems	0	1	1
Diet [n]			
Vegetarian	0	1	1
Pescatarian	1	1	2
Food Allergies[#]	0	2	2
Did not eat pork for religious reasons	0	1	1
Supplement use	4	9	13
Sport supplement[¥]	1	0	1
Vitabiotics Osteocare[≠]	1	0	1
Guarana	1	0	1
Ginkgo Biloba	1	0	1
Aloe Vera	0	1	1
Garlic	1	1	2
Evening Primrose oil	1	1	2
Fish oils/cod liver oil	1	6	7
Glucosamine	0	2	2
Chondroitin	0	1	1
Zinc	0	1	1
Cranberry extract	0	1	1
Vitamin D	0	1	1
Vitamin C	0	1	1
Multivitamins	0	1	1
Lifestyle[a]			
Alcohol			
Consumers [%]	87	75	78
Consumption [units/wk][b]	4 (2.5–9)	6.5 (2–13.5)	4.5 (2–11)
Anthropometrics			
Weight [kg]	71.9 (13.5)	66.8 (10.4)	68.3 (11.5)
Height [cm]	169.5 (9.0)	167.6 (7.7)	168.2 (8.0)
Waist circumference [cm]	84.5 (11.3)	82.5 (9.1)	83.1 (9.7)
BMI [kg m^{-2}][c]	24.9 (3.7)	23.7 (3.1)	24.1 (3.3)
Weight Status [%]			
Normal	60.0	66.7	64.7
Overweight	33.3	27.8	29.4
Obese	6.7	5.6	5.9
Central Obesity[d]	26.7	16.7	19.6
Physical Activity[e]			
Total PA [MET-mins/week]	2,747 (1,969–5,058)	2,994 (1,866–4,878)	2,937 (1,894–4,878)
Activity Level [%]			
High	69.2	54.8	59.1
Moderate	30.8	29.0	29.5
Low	0	16.1	11.4
Sitting time [mins/day][f]	390 (285–480)	360 (240–435)	360 (255–480)

In this table data are presented as mean (standard deviation, SD) for continuous variables and as number of participants or percentage (%) for categorical variables, except for alcohol consumption, total physical activity (PA), and sitting time, which are given as median (interquartile range). MET, metabolic equivalent of task.

[a] smokers were excluded from the study.

[b] participants who reported no alcohol consumption were excluded from this analysis.

[c] body Mass Index (BMI) was calculated as [weight(kg)/height(m)2]. BMI cut-off points for determination of weight status were: normal weight 18.5–24.9 kgm−2, overweight 25.0–29.9 kgm−2, obese 29.9–39.9 kgm−2.

[d] central obesity was determined using waist circumference as a proxy, with sex-specific cut-off points (females ≥ 88 cm, males ≥ 102 cm).

[e] to classify individuals according to their self-reported PA, MET-minutes per week were calculated and participants were grouped into three activity levels (high, moderate, low) according to the cut-points defined in the International Physical Activity Questionnaire (IPAQ) guidelines (45). Outliers were excluded along with datasets of individuals containing missing values (n = 2). Reported duration of activity was truncated to 180 min, where necessary, according to the IPAQ data processing rules.

[f] sitting time is defined as a sedentary-related behavior (47) and spending 4 h or more (≥240 min) a day sitting is a proxy measure of sedentary behavior detrimental to health (46).

[#] food allergies were to shellfish (1 person) and whole egg/milk (1 person).

[¥] each serving contains 150 mg of caffeine and 1.7 g creatine monohydrate, plus specific amino acids, vitamins, fruit extracts, and black pepper extracts.

[≠] contains calcium, vitamin D, zinc, and magnesium.

samples had very distinctive compositions (**Figure 3B**). Feature selection identified several metabolite signals that were strongly explanatory of compositional differences between these two urine classes. Further analysis of urine composition after consumption of a range of legumes (beans, soy and peanuts) in other MAIN Study Newcastle menu plans revealed several explanatory metabolites in common, including *m/z* 204.98143. Detailed structural analysis indicated that this particular compound is pyrogallol sulpate (**Figure 3C**). The large relative increase in the pyrogallol sulpate signal after consumption of peas in shown in **Figure 3D**.

Following an identical rationale, a selection of potential biomarkers of an extended range of foods is summarized in **Table 4**. In addition to pyrogallol sulfate, pyrogallol glucuronide and trigonelline emerged as generic biomarkers of legume consumption (beans, peanuts, peas and soy). Eugenol glucuronide and eugenol sulfate were elevated in urine after consumption of curry and are potential biomarkers of this food group. The metabolite, 2-furoylglycine, appeared discriminatory for high temperature baked foods (e.g., pies) and toasted grain products (e.g., toasted bread). We identified furaneol (sulfate and glucuronide) and mesifurane after the consumption of strawberries and tomato products. Higher concentrations of Maillard reaction intermediates

2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (acetylformoine) in both its sulfate and glucuronide forms and norfuraneol sulfate (4-hydroxy-5-methyl-3(2H)-furanone) were observed in urine following consumption of both high temperature-baked and toasted grain products as well as strawberries, berries and tomato. After the consumption of a low-calorie beverage, urinary concentration of the sweetener acesulfame potassium was elevated for up to 12 h (**Table 4**).

DISCUSSION

Success of Free-Living Study Design

Our aim was to design and implement a prolonged dietary intervention study which would allow the collection of urine samples for biomarker studies in a home or work environment. Study recruitment achieved (Study 1) or surpassed (Study 2) original expectations with 51 healthy adults consuming specific foods and beverages, provided *in toto* by the research team. Apart from a visit to the research unit to pick up food at the beginning, and to deliver urine samples at the end, of each experimental week, participants were free-living and had the freedom to eat or not the meals and to interpret the cooking instructions as they wished. A combination of no drop outs and high compliance rates with regard to meal consumption (> 80%), suggests that this food intervention strategy was highly acceptable to participants [see Lloyd et al. (27) for details on compliance].

Participants collected urine samples within pre-specified time frames each day and fasting blood samples were collected in the morning of the final ("Post") day of each experimental week. This aspect of the study was designed to be as minimally intrusive as possible to investigate the potential for such urine collections to be incorporated into future larger-scale epidemiological studies and surveys. Data associated with urine collection has been described in a recent publication (27) and indicated that the most successful urine type collected within this cohort was the FMV and the post-dinner spot sample (both at 99% compliance). The fasting sample was the least successfully collected sample. Evidence that the urine sampling methodology imposed low burden on participants and delivered samples with high quality metabolome content has been published (27). From a metabolomics perspective, the overall experimental design was validated by confirming the presence of known BFIs in urine samples after exposure to menu plan 1 (26).

Discovery of Novel Exposure Biomarkers

For BFIs to have utility in assessing dietary intake as a whole, it is essential that the dietary exposure biomarker panel is as comprehensive as possible. To date, the focus on BFI discovery has centered largely on healthy foods of high public health significance (50, 51) rather than more unhealthy foods containing high levels of fat, sugar and salt (52–54). We used our intervention design in a free-living population to aid the discovery of novel biomarkers to help complete coverage of the UK Eatwell Guide (25) whilst aiming to monitor comprehensively both the whole diet and the range of cooking methods used in populations.

UK government policy recommends the consumption of more beans and pulses and less red and processed meat (25). However, there is very limited data on potential urinary biomarkers for non-meat protein-rich foods, such as beans, lentils, and other pulses (55, 56). Here we propose pyrogallol sulfate and glucuronide as potential markers of overall legume consumption (beans, peanuts, peas, soy). Additionally, trigonelline, despite being well-documented as a coffee (57) and pea consumption biomarker (58), and most recently, a novel candidate marker for soy (59), is, in fact, a general legume BFI. However, whilst potential BFIs for legumes, these markers are not exclusive to pulses. Pyrogallol is present in low quantities in beer (60), cocoa and coffee and is excreted as a sulfate after green tea and nut consumption (61, 62). These findings illustrate the need to utilize urine samples from a comprehensive food intervention to investigate specificity to individual foods or food groups. If the dietary source(s) of trigonelline needed to be identified further, then the relative contribution of coffee and soy consumption could be estimated using additional discriminatory biomarkers such as caffeine and daidzein sulfate, respectively (57, 63). These biomarkers could be added to a panel of BFIs to monitor protein-rich food intake, together with anserine and TMAO (Trimethylamine-N-oxide) to indicate poultry and fish intake and carnitine and carnosine to indicate red meat consumption (16, 64).

It still remains challenging to assess the overall "quality" of meat products that are consumed since processed meat products have very variable levels of striated muscle content (19, 65). However, it is possible that BFIs of meal components strongly associated with generally unhealthy diet patterns (66) such as a deep fried-potato (22) or mechanically recovered meat could be highly informative (19, 65). These issues will need to be addressed in the future to provide a comprehensive panel of biomarkers that can characterize and quantify eating habits *in toto*.

Concentrations of eugenol glucuronide and eugenol sulfate in urine increased after the consumption of curry possibly because clove (a common component of curry) is rich in eugenol (67). Having biomarkers that provide information about the cooking and processing methods used with foods would be an important addition to biomarkers that reflect the raw ingredients of the dish/meal because different cooking methods can change the quality and therefore healthiness of a food. Obtaining this level of information can be difficult using self-reported dietary assessment instruments, especially widely-used food frequency questionnaires. To date, a few such biomarkers of high-temperature cooked meats have been described (68). In the present study, the marker 2-furoylglycine appeared discriminatory for thermally treated foods including pies, grains and toasted wheat products (e.g., toasted bread). This marker has been described as an acute coffee consumption biomarker (69) which results from furan metabolites that arise through roasting of coffee beans via the Maillard reaction. Our metabolomics approach identified several other thermally treated/heated food Maillard products—furanones—including 2,4-dihydroxy-2,5-dimethyl-3-furanone sulfate and glucuronide and norfuraneol sulfate,

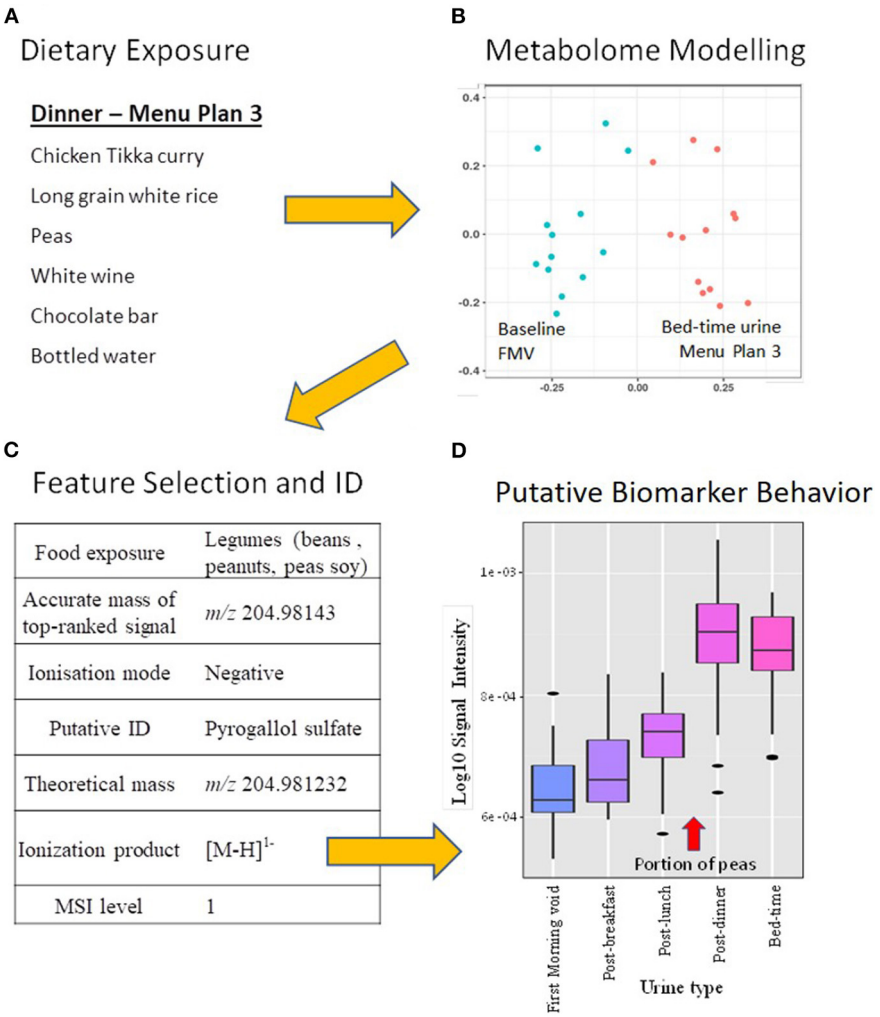

FIGURE 3 | Schematic of the dietary exposure biomarker discovery strategy within the context of a comprehensive food intervention mimicking a typical UK diet in free-living individuals. **(A)** Meal items consumed at Dinner time on Menu plan 3 [details in Lloyd et al. (27)]; **(B)** Multi-dimensional scaling (MDS) of Random Forest (RF) proximity values of the FIE-HRMS urinary fingerprint data of first morning void and bed-time urines from the same day that Menu plan 3 was consumed; **(C)** Annotation of a metabolite signal highly explanatory of legume exposure on several experimental days when legumes were included on the menu; **(D)** Box-plots showing the association between pea consumption and the relative intensity of the pyrogallol sulfate signal $[M\text{-}H]^{1-}$ in urine samples taken throughout the day that Menu plan 3 was eaten.

which have been detected as aglycones in bread crust and popcorn, respectively (70, 71), but not reported previously in urine samples. 2,4-dihydroxy-2,5-dimethyl-3-furanone sulfate and glucuronide have been reported as markers for the intake of deep-fried potatoes, but, due to their presence in other foods, these compounds are not likely to be specific (22). Although furanones are formed by the Maillard reaction during the thermal treatment of food, they can also be biosynthesized by plants, microorganisms, and insects [as reviewed by Slaughter (72)]. Other metabolites structurally identical to Maillard products, including furaneol and its methyl-ether derivative mesifurane, are well-documented natural aroma components in fruits such as pineapple, raspberry, mango, grapefruit, tomato, and strawberry (73) and the glucuronide and sulfate of these compounds have been reported in urine after strawberry consumption (48, 74). In the current study, we confirmed this observation and suggest that furaneol glucuronide and sulfate, and mesifurane sulfate, are generic biomarkers of berry and tomato consumption (**Table 4**). Assessment of the relative concentrations of some of these chemicals and strawberry/berry/tomato specific compounds {i.e., pelargonidin [strawberry], hydroxyphenylvalerolactone [procyanidin-rich food (75)] and lycopene in plasma for tomato (76)}, could provide rich information on cooking methods and could, potentially, distinguish between berry fruit and tomato consumption.

Foods high in added sugar are not needed in the diet and should only be consumed in very small amounts (25).

TABLE 4 | Discovery of novel biomarkers for foods where biomarkers have yet to be discovered in relation to UK Public health policies.

Food exposure source	Biomarker	Ionization products	MSI level
Legumes	Pyrogallol (1,2,3-Trihydroxybenzene) glucuronide	$[M-H]^{1-}$	1
	Pyrogallol (1,2,3-Trihydroxybenzene) sulfate	$[M-H]^{1-}$, $[M-H]^{1-}\ ^{13}C$, $[M-H]^{1-}\ ^{34}S$	1
	Trigonelline	$[M+H]^{1+13}C$, $[M+Na]^{1+}$, $[M+Na]^{1+}\ ^{13}C$, $[M+K]^{1+}$, $[M+K]^{1+13}C$, $[M+K]^{1+41}K$	1
Curry (clove)	Eugenol glucuronide	$[M-H]^{1-}$, $[M\text{-H-gluc}]^{1-}$, $[M\text{-H-gluc}]^{1-13}C$	1
	Eugenol sulfate	$[M-H]^{1-}$, $[M-H]^{1-34}S$	1
High temperature baked and toasted grain products	2-Furoylglycine	$[M+Na]^{1+}$, $[M+K]^{1+}$, $[M+2Na\text{-}H]^{1+}$, $[M+KNa\text{-}H]^{1+}$, $[M-H]^{1-}$	1
Strawberry, berries, and tomato	Furaneol sulfate	$[M-H]^{1-}$, $[M-H]^{1-13}C$, $[M-H]^{1-34}S$	1
	Furaneol glucuronide	$[M-H]^{1-}$	1
	Mesifurane (2,5-Dimethyl-4-methoxy-3(2H)-furanone) sulfate	$[M-H]^{1-}$	2 (48, 49)
High temperature baked and toasted grain products and strawberry, berries, and tomato	Norfuraneol sulfate (4-hydroxy-5-methyl-3(2H)-furanone)	$[M-H]^{1-}$, $[M-H]^{1-34}S$	1
	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone sulfate	$[M-H]^{1-}$	3
	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furanone glucuronide	$[M-H]^{1-}$, $[M-H]^{1-13}C$	3
Low calorie drinks	Acesulfame potassium	$[M-K]^{1-}$, $[M-K]^{1-13}C$, $[M-K]^{1-34}S$	1

MSI level 1, matching masses, MS^n and retention times with authentic standards or with the respective aglycone; MSI level 2, structural assignment achieved by putatively matching signal behavior with that of authentic standards reported in the literature or fragmentation pattern alignment with data in public/commercial spectral libraries; MSI level 3, structural identification indicated a putatively characterized compound class.

Urinary sucrose has previously been shown to be a marker of acute sugar exposure (53) and appeared discriminatory of the consumption of high sugar products, such as sweetened breakfast cereals, in the present study (26). Many food manufacturers are now substituting sugars in traditionally sugar-sweetened beverages with low-calorie sweeteners, to help combat risks associated with high sugar intake (77). The urinary concentration of the sweetener acesulfame potassium was elevated after the consumption of a low-calorie beverage and remained so for up to 12 h after consumption (**Table 4**). This low-calorie sweetener is a known component of the chosen beverage and, in addition to other commonly consumed sweeteners, is a potential biomarker of recent low-calorie beverage intake (78).

Strengths and Limitations of Study Design

The MAIN Study Newcastle is one of the largest food interventions reported to date using metabolomics approaches to discover new, and to help validate existing, biomarkers of foods frequently consumed in the UK. A key objective was to design an efficient and acceptable intervention strategy that would expose study participants to foods encountered commonly in the UK diet. We established a successful food exposure strategy using information on consumption frequencies, food groupings and eating habits from the UK NDNS data (31) together with Public Health England policy advice (25) using standard portion sizes (32). We demonstrated that it is possible to design menus that mimic major features of a typical UK diet and are suitable for short-term randomized controlled dietary interventions "experimental periods" lasting only 3 days. Importantly each experimental period contained menu plans organized to emulate conventional UK eating patterns with a breakfast, lunch, afternoon snack and dinner that differed each day. This intervention strategy may be of value for the design of future studies of BFI discovery and validation in other populations globally. This design differs from many other reported biomarker discovery studies that utilized either single meals/ingredients in isolation (15, 30, 79, 80) or repeated menus (81) or depended on supplementation of the habitual diet (82, 83). Additionally, the overall menu design ensured that participants were exposed to several formulations of individual foods. In other work within the MAIN project, we have demonstrated the value of spot urines for assessing dietary intake (29). The present protocol required all study participants to store 20 ml urine samples in their home fridges for up to 5 days before transporting them to the research unit at the end of each experimental week. We have checked that urine samples collected at home and stored at 4°C are stable and not subject to microbial degradation (36). When outside the home, small cool bags were supplied for temporary urine storage. All urine spot samples were collected with success rates exceeding 90% showing that this approach to urine sampling for biomarker studies is highly acceptable. Additionally, the collection of multiple spot urines allowed us to investigate systematically the utility of home collected spot urine samples taken at various times in the diurnal feeding and fasting cycle for BFI discovery [see Lloyd et al. (27)]. Based on these observations we suggest that the protocols used in the MAIN

study have overcome many of the design challenges summarized in **Table 1**.

From a biomarker discovery perspective, an important finding from Study 1 was that the consumption of a standardized evening meal prior to the experimental period had little impact on the ability to discover BFIs [see Lloyd et al. (26)]. This finding simplified the design of the wider food intervention in Study 2 and reduced the burden on the study participants by requiring only a limit on the consumption of polyphenol-rich foods prior to each experimental period. Furthermore, the characteristics of the MAIN study participants (**Table 3**) in respect of sex, age, adiposity, physical activity and general health indicate that BFIs discovered in this study are likely to be generalizable to the wider population. Importantly, in the MAIN Study Newcastle, all foods and beverages were prepared and consumed by free-living participants in their own homes rather than in a controlled clinical facility (24). This strategy ensured that metabolomic analyses were undertaken within the context of normal eating behaviors and in real world meal patterns, rather than following consumption of discrete items in isolation. In addition, this study design took into account the inherent variability associated with unsupervised individuals preparing meals and eating them in their own homes and collecting urine samples within flexible time ranges. Importantly, the comprehensiveness of this food intervention provides opportunity to examine the specificity of putative biomarkers in relation to exposure to a wide range of foods within the same biobank of samples.

Although the MAIN study had great value for the discovery of putative BFIs, some sources of variability were not considered. For example, the study employed relatively healthy individuals and we excluded those with current disease or disease treatment which might have altered the metabolism of ingested foods. As a consequence, future studies should explore the robustness of biomarkers of food intake in population groups with poorer health, particularly those who are taking prescribed medications that affect the P450 enzyme consortium or the gut microbiome. Our cohort of participants included one individual with irritable bowel syndrome (IBS)—contrary to the study inclusion criteria—but it seems unlikely that this would have had a major effect on the overall findings. Similarly, our cohort included three participants who were slightly obese (BMI 32.2–33.0 kg/m^2) and, again, it is unlikely that this infringement of the exclusion criteria will have affected the findings. It is possible also that participant genotype will alter the pattern of metabolites produced from a given food constituent and so for this reason further work should investigate the impact of common variants in the genes encoding Phase 1 and Phase 2 enzyme systems, an example being the P450 consortium (14), on urinary BFIs. It should be noted that several of the putative BFI reported here are biotransformation products (sulfates and glucuronides) that could be identified to the level of the aglycone only (i.e., pyrogallol, eugenol, furaneol), because chemical standards for the biotransformed products are not available commercially at a reasonable cost.

Future Work

In previous publications, we have shown that when using a combination of non-targeted metabolite profiles and targeted BFIs for assessment of dietary patterns, spot samples are suitable replacements for 24-h urine samples (29). The collection of multiple spot urine samples throughout the day using a home urine sample collection method will enable us to determine which samples (e.g., FMV or bed-time) or combinations thereof, and how many samples, are optimal for assessment of eating behavior using BFIs. It is anticipated that multiple, well-spaced spot samples collected over several weeks would be able to capture biomarker data accurately, reflecting habitual exposure to a wide range of food groups in a similar way to that achieved using multiple 24 h recall methods. The ultimate aim of our studies is to deploy a comprehensive BFI panel to aid in monitoring habitual dietary exposure in clinical trials or population surveys at a range of scales. With this aim in mind, we have developed methodology for urine collection using vacuum transfer technology which is suitable for routine use and may provide a scalable, cost-effective means to collect urine samples and to assess dietary intake in large-scale epidemiological studies and in public health surveys (36).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and a favorable ethical opinion was obtained following Proportionate Review by the East Midlands—Nottingham 1 National Research Ethics Committee (14/EM/0040). Caldicott approval for storage of data and data protection was granted by Newcastle-upon-Tyne Hospitals NHS Foundation Trust [6896(3109)]. The trial was adopted into the UK Clinical Research Network (CRN) Portfolio (16037) and was registered with International Standard Randomized Controlled Trials Number (ISRCTN), 88921234. A study information sheet was given to all potential participants in advance of their first visit to the research center. Written informed consent was obtained prior to participation from each eligible individual, for each study, by an appropriately trained researcher. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Trial registration: ISRCTN, ISRCTN88921234. Registered 3rd April 2014—retrospectively registered, http://www.isrctn.com/ISRCTN88921234. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JD, JM, and MB conceived the study. JM, JD, NW, AL, and MB designed the study and menu plans. NW and LX undertook participant recruitment, developed participant handling protocols, ran the intervention study, supervised support staff, collected all biological samples, and refined sampling methodology. IG-P and EC coordinated project input from Imperial College London. NW and MS analysed and interpreted the data with respect to study design, participant recruitment and characteristics and participant and sample handling, and produced the tables and figures. KT and MB analysed the urine samples. AL analysed and interpreted the metabolomics data and produced the tables and figures. NW, AL, JD, and JM wrote the manuscript. JM and JD coordinated the project and supervised the research teams in Newcastle and Aberystwyth Universities, respectively. All authors read and approved the final manuscript.

FUNDING

This study was funded by the Medical Research Council (MRC), Grant No. MR/J010308/1.

ACKNOWLEDGMENTS

The authors thank the study participants for their commitment and the Clinical Aging Research Unit, Newcastle University for participant support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.561010/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

METHODS
published: 22 October 2020
doi: 10.3389/fnut.2020.582999

Within- and Between-Household Variation in Food Expenditures Among Low-Income Households Using a Novel Simple Annotated Receipt Method

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition Methodology, a section of the journal Frontiers in Nutrition

Received: *13 July 2020*
Accepted: *16 September 2020*
Published: *22 October 2020*

Citation:
Valluri S, French SA, Elbel B, Oakes JM, Rydell SA and Harnack LJ (2020) Within- and Between-Household Variation in Food Expenditures Among Low-Income Households Using a Novel Simple Annotated Receipt Method. Front. Nutr. 7:582999. doi: 10.3389/fnut.2020.582999

Background: Household food purchasing behavior has gained interest as an intervention to improve nutrition and nutrition-associated outcomes. However, evaluating food expenditures is challenging in epidemiological studies. Assessment methods that are both valid and feasible for use among diverse, low-income populations are needed. We therefore developed a novel simple annotated receipt method to assess household food purchasing. First, we describe and evaluate the extent to which the method captures food purchasing information. We then evaluate within- and between-household variation in weekly food purchasing to determine sample sizes and the number of weeks of data needed to measure household food purchasing with adequate precision.

Methods: Four weeks of food purchase receipt data were collected from 260 low-income households in the Minneapolis-St. Paul metropolitan area. The proportion of receipt line items that could not be coded into one of 11 food categories (unidentified) was calculated, and a zero-inflated negative binomial regression was used to evaluate the association between unidentified receipt items and participant characteristics and store type. Within- and between-household coefficients of variation were calculated for total food expenditures and several food categories.

Results: A low proportion of receipt line items (1.6%) could not be coded into a food category and the incidence of unidentified items did not appreciably vary by participant characteristics. Weekly expenditures on foods high in added sugar had higher within- and between-household coefficients of variation than weekly fruit and vegetable expenditures. To estimate mean weekly food expenditures within 20% of the group's usual ("true") expenditures, 72 households were required. Nine weeks of data were required to achieve an $r = 0.90$ between observed and usual weekly food expenditures.

Conclusions: The simple annotated receipt method may be a feasible tool for use in assessing food expenditures of low-income, diverse populations. Within- and

between-household coefficients of variation suggest that the number of weeks of data or group sizes required to precisely estimate usual household expenditures is higher for foods high in added sugar compared to fruits and vegetables.

Keywords: nutrition methodologies, household food purchasing, food receipt method, within-household variation, between-household variation, epidemiology

BACKGROUND

Food purchasing behavior has gained interest as an intervention target to improve nutrition and nutrition-associated health outcomes in the United States (1–4). Evidence that the nutritional quality of food purchases corresponds with dietary quality (5, 6) has prompted numerous interventions targeting food purchasing behavior (3, 4). Low-income populations have generated particular attention due to socioeconomic differences in diet quality (7, 8). However, valid and feasible methods for measuring food purchasing among low-income households are limited.

Existing methods to evaluate household food purchasing behavior—including home food inventories (9–12), bar code scanners (13, 14), point of sale data (15–17), food purchase records (18–21), and food receipts (22–25)—have unique strengths, but their weaknesses present noteworthy challenges for implementation, validity, and capturing the full range of purchase information (**Table 1**) (12). Furthermore, there are important differences in food purchasing by household socioeconomic status (7, 8). Assessments methods therefore need to be evaluated among low-income households to ensure that the detail and variation in household expenditures is fully captured (12).

Food receipt methods are appealing because they can be used to assess food expenditures from a variety of retailers and for all types of foods (12, 22–25). In the annotated food receipt method, participants collect receipts and transcribe information onto forms to clarify missing details and unclear abbreviations (e.g., items described as "dairy" rather than "skim milk," or "Pillsbury white cake mix" listed as "pills white") (24, 25). However, participant burden is high and literacy is required. In contrast, participants submit receipts without transcription or annotation in the food receipt collection method (22). Although this method substantially lowers participant burden, many details that receipts generally lack may not be captured.

To capitalize on the detailed information possible using the annotated food receipt method while reducing participant burden, a simple version of the annotated food receipt method (the "simple annotated receipt method") was developed for use in a prospective trial (26). Participants are not required to transcribe all purchase information using this newly developed method; instead, they annotate items with vague or unclear descriptions directly on the receipts.

This study has two primary aims. First, we describe and evaluate the simple annotated receipt method using data from the aforementioned trial. We illustrate the food purchasing information that may be captured using this method. We also evaluate the extent to which receipt items could not be identified due to inadequate annotation and whether this varies by participant characteristic and store type. To date, this is the first study to describe and evaluate this method.

Second, we evaluate sources of variation in household food purchasing to help guide study designs using this method. Household food purchasing behavior is often evaluated for one of three research objectives: (1) to compare mean household expenditures between different groups (e.g., control vs. intervention groups), (2) to rank households by expenditures (e.g., into quartiles), (3) or to assess an individual household's expenditures (e.g., change in expenditures before and after intervention) (27, 28). Thus, this paper addresses practical and essential questions: How many households are needed in a study group to assess the group's *usual* ("true") food expenditure pattern or to rank households with reasonable precision? How many weeks of data are needed to precisely evaluate a household's usual food expenditure?

Evaluating a household's usual expenditures requires an understanding of the sources of variation in week-to-week spending. Similar to dietary intake—which varies daily and requires multiple days of assessment—household food expenditures likely vary from week to week, necessitating multiple weeks and adequate sample sizes to ascertain usual food expenditures (28, 29). Group sizes and data collection periods may also vary by food group, analogous to the differing number of dietary assessments needed to evaluate intake of specific nutrients (28).

We quantify within- and between-household variation in weekly expenditures for all foods and beverages and for two specific categories of food: fruits and vegetables, and foods high in added sugar (sugar-sweetened beverages [SSBs], candy, and sweet baked goods). Using these values, we estimate the group size needed to estimate a group's average food expenditures. We also estimate the number of weeks of data needed to rank household expenditures or estimate a household's usual food expenditures with adequate precision. Results from this paper can help researchers design efficient studies of food purchasing behavior (27–29). To our knowledge, this is the first study to provide these important metrics.

MATERIALS AND METHODS

Study Population

This paper is a secondary analysis of data from a prospective trial (26, 30, 31). Briefly, low-income households in the Minneapolis-St. Paul, Minnesota, metropolitan area were recruited between August 2013 and May 2015. Eligibility criteria included: (1) not currently enrolled in the Supplemental Nutrition Assistance Program (SNAP) or planning to enroll

TABLE 1 | Description and summary of strengths and weaknesses of existing methods to assess household food purchasing.

Method	Description	Strengths	Limitations
Home food inventories (9–12)	Collected by study staff or participants. Catalogs foods available in the home at the time inventory is completed	• Low participant burden • Relatively easy to complete	• Multiple administrations of the inventory required for accurate assessment of usual household food available • Foods purchased and consumed outside home are not ascertained • Captures types of food (e.g., soft drinks) but not quantity (e.g., fluid ounces)
Bar code scanners (12–14)	Participants scan bar codes for all foods purchased. Researchers provide codes to participants for unpackaged items.	• Does not require participant literacy • Can provide rich data on types and quantities of packaged foods	• Scanners can be expensive, susceptible to hardware malfunctioning, and rely on external database of codes to match bar codes to food items. • May not capture foods that typically lack bar codes such as bulk items, fresh produce and meats in grocery stores, and food purchased at restaurants.
Point of sale data (12, 15–17)	Uses data available from food retailers on customer food purchasing	• Minimal participant burden • Can provide rich data on types and quantities of foods	• Linking data from vendors with individual shoppers can be challenging due to proprietary nature of data, privacy concerns, and technological issues • Unable to capture comprehensive assessment of household food purchases since it is generally limited to one retailer
Food purchase record (12, 17–21)	Participants keep a written record of all foods purchased, including description of each item and quantity.	• Offers detailed and comprehensive information about types and quantities of food purchased over time	• Requires participant literacy • High participant burden
Food receipt collection (12, 22, 23)	Participants collect and mail all receipts for food purchases. Receipt purchases are coded by study staff.	• Offers details about expenditures over time • Low participant burden	• May not be able to code purchases with insufficient detail on receipt, including specific types of food (e.g., "produce" vs. "tomatoes") and quantities (e.g., fluid ounces)
Annotated food receipt (12, 24, 25)	Participants collect receipts for food purchases and transcribe receipt information onto a form to provide details not available on receipts	• Offers detailed information about all food and beverage purchases	• Requires participant literacy • High participant burden

during the study; (2) household income < 200% the federal poverty rate or participating in a government program that automatically qualifies households for SNAP (e.g., the Diversionary Work Program in Minnesota); (3) adult in the household primarily responsible for food shopping is able to read and speak English and participate in the study. Some SNAP eligibility criteria, such as citizenship status, were not applied. The University of Minnesota Institutional Review Board approved all aspects of the study (ClinicalTrials.gov: NCT02643576).

Participants were asked to annotate and submit all household food purchase receipts throughout the study using the protocol described in greater detail below. At the baseline visit, participants completed a survey to assess demographic characteristics. Household food security was evaluated using the US Household Food Security Survey Module: 6 Item Short Form (32).

Participants who completed baseline measures and submitted at least 2 weeks of receipts received a study debit card with monthly benefits for 12 weeks. Households were randomized into one of four study arms, which varied with respect to whether a financial incentive was provided for fruit and vegetable purchases and whether foods high in added sugars could be purchased with benefits. Analyses for this paper are limited to the baseline period of the trial.

Simple Annotated Receipt Method

Receipt Collection

Research staff met participants in-person to provide verbal and written instructions, and materials necessary for receipt collection. Participants were instructed to collect all food purchase receipts and to query other household members for receipts. Receipts were requested from both restaurants (retailers that serve or sell ready-to-consume food) and food retailers (retailers that primarily sell unprepared food). This paper focuses on receipts from food retailers.

Participants were instructed to annotate food retailer receipts if the item description was vague or unclear. To annotate receipts, participants were instructed to write details directly on the receipt next to the item lacking information. For example, an item described as "produce" would need annotation to specify the type of produce (e.g., "tomatoes"). Annotation was not requested for quantities of food purchased. Missing food receipt forms were requested for purchases without receipts, such as purchases made at retailers that do not provide receipts (e.g., farmer's market) or lost receipts. The missing receipt form included details such

as the store name, date of purchase, food items purchased, quantity purchased, price per item, and total price. As part of the instruction process, study staff reviewed a sample annotated receipt and missing receipt form with participants.

All receipts were to be mailed to study staff on a weekly basis using pre-addressed, postage-paid envelopes, which were pre-labeled with the participant ID number, dates comprising the week of receipt collection, and the target mailing date to facilitate tracking by staff. Participants were mailed a gift card as a reward for receipt collection every month. The reward amount was pro-rated, with $30 provided if 4 weeks of receipts were submitted, and lesser amounts for three ($15), two ($10), and one ($5) week. Research staff contacted participants to encourage submission if receipts were not received.

Food Retailer Receipt Coding

Receipts were first sorted into two categories: restaurant purchases and food retailer purchases. Restaurants were classified as full-service, limited-service, or unable to determine restaurant type. This study focuses on food retailers, which were further classified as supermarket/market (e.g., Cub, Aldi, farmers market), natural food store (e.g., co-ops), warehouse store (e.g., Costco, Sam's Club), drug store (Walgreens, CVS), convenience store/gas station (including dollar stores), superstore (e.g., Target, Walmart), or other (e.g., Home Depot, Menards) (24). Each receipt was then assigned a unique identifier to specify the participant, week, and receipt number.

Items on food retailer receipts were classified into one of 11 food categories. The choice of food categories reflects the primary aims of the original trial, which was to evaluate two food categories: fruits and vegetables, and foods high in added sugars (sugar-sweetened beverages [SSBs], sweet baked goods, and candies). Items with potential substitution effects (e.g., milk, savory snacks) were measured, while items of lesser interest to the trial (e.g., diet sodas) were categorized as "other food" purchases.

Food items that lacked sufficient detail to code into one of the 11 food categories were coded as having "insufficient detail to code" (unidentified). Before coding an item as unidentified, study staff followed a series of procedures to obtain missing information. First, an online search was conducted using the store name, item, code, and/or abbreviation. When available, the item's Universal Product Codes was searched (http://www.upcdatabase.org). Stores were contacted to verify the item for successful online searches. If these procedures failed to provide necessary details, items were coded as unidentified.

For each receipt, the total number of line items and expenditures were calculated for overall food and beverages, and for each of the food categories. Totals for each category were determined by summing across line items classified into the category. Quantities or weight of foods purchased was not considered in the tabulation. For example, a line item of "apples" would count as a frequency of one in the tally for receipt items for fruits, and total expenditure amounts are reported rather than per unit prices. The first 10 receipts coded were reviewed for accuracy by a second staff member. Errors identified were reviewed and corrected. Spot checks of coded receipts were conducted throughout for quality assurance.

Statistical Analysis

Analyses for this paper are restricted to participants who submitted at least 3 weeks of food retailer receipts during the 4-week baseline period. First, we described the food purchasing information captured by the method using total number of receipt line items and expenditures for overall food expenditures, each of the 11 food categories, and items categorized as having insufficient detail to code (unidentified). Food purchasing data was also evaluated by store, which was collapsed into four types based on previous literature and low frequency of receipts in some categories: Grocery stores, Convenience stores/Gas Stations, Drug stores, and Superstores/Mass merchandiser/Warehouse club store.

Second, we used a zero-inflated negative binomial model to evaluate whether unidentified line items on receipts were associated with participant characteristic or store type. This model was used because the distribution of unidentifed items was heavily skewed, with 94% of receipts submitted without any unidentified items. Likelihood ratio tests confirmed zero-inflation and overdispersion, supporting the model choice. The model simultaneously evaluates two processes. The logit portion of the model evaluates participant characteristics and store types associated with submitting receipts with unidentified items, yielding odds ratios (OR). The negative binomial model evaluates the incident rate ratios (IRR) of unidentified items by participant characteristics and store types among those who submitted at least one receipt with an unidentified item. Participant characteristics of interest were age, gender, race/ethnicity, marital status, household size, education level, annual household income, and food security. A dummy variable was used to indicate the store type. The model was adjusted for the total number of line items per receipt. A $p < 0.05$ was the criterion for claiming statistical significance.

Third, a mixed effects regression model with an unstructured covariance and restricted maximum likelihood estimator was used to estimate the mean, within-household variance (σ_w^2), and between-household (σ_b^2) variance for overall food expenditures, fruits and vegetables, and foods high in added sugar. We calculated within- and between-household coefficients of variation (CV_w and CV_b, respectively) as percentages using the following equations (29): $CV_w = (\sigma_w/mean)\ x\ 100$; $CV_b = (\sigma_b/mean)\ x\ 100$. The ratio of within- to between-household variation, the variance ratios, were calculated as σ_w^2/σ_b^2 (which is equivalent to $[CV_w/CV_b]^2$).

Using these values, we calculated the group size or weeks of data needed to estimate usual (or "true") food expenditures with adequate precision. The usual household food expenditures refers to the hypothetical "true" average of the study sample about which a household's expenditures vary during the period of data collection. We assume that within- and between-household variation observed in our sample is due to random variation

TABLE 2 | Baseline characteristics of households using the simple annotated receipt method as part of a trial evaluating food purchasing behavior ($n = 260$).

Characteristic	*N* (%)
AGE, YEARS	
Under 25	14 (5.4)
25–44	115 (44.2)
45–64	113 (43.5)
Over 65	18 (6.9)
GENDER	
Male	48 (18.5)
Female	212 (81.5)
RACE/ETHNICITY	
White, Non-Hispanic	77 (29.6)
Black, Non-Hispanic	131 (50.4)
Other, Hispanic	52 (20.0)
MARITAL STATUS	
Single, never married	117 (45.2)
Married or partnered	72 (27.8)
Separated/divorced/widowed	70 (27.0)
HOUSEHOLD SIZE	
1 person	58 (22.3)
2 people	58 (22.3)
3 people	61 (23.5)
4 or more people	83 (31.9)
EDUCATION LEVEL	
High school graduate or less	75 (28.9)
Some college/associates degree	138 (53.1)
College graduate or higher	47 (18.1)
ANNUAL HOUSEHOLD INCOME	
$14,999 or less	79 (33.2)
$15,000—$34,999	114 (47.9)
$35,000 or more	45 (18.9)
HOUSEHOLD FOOD SECURITY STATUS	
High or marginal	53 (19.3)
Low	96 (34.9)
Very low	126 (45.8)

about the hypothetical average, and not due to a changes in habitual spending patterns (33).

The number of households in a group (n_g) required to estimate group mean expenditure using a single week of expenditure data was calculated as follows: $n_g = Z_\alpha^2 \; x \; [(CV_b^2 + CV_w^2)/D_0^2]$, where D_0 is a specified percentage deviation of the group's usual expenditure, and Z_α is the normal deviate for the percentage of times the measured expenditure should be within a specified limit (29). For the purposes of this study, we evaluated estimates with 95% CIs (i.e., $Z_\alpha = 1.96$), with D_0 varying between 10 and 50%. The number of weeks of expenditure data (n_r) needed to obtain a given Pearson correlation coefficient, *r*, between observed and unobserved usual expenditures was also calculated (27, 33). The equation is as follows: $n_r = r^2/(1 - r^2) \; x \; (\sigma_w^2 \; / \sigma_b^2)$, where *r* varied between 0.75 and 0.95. Finally, the number of weeks of expenditure data (n_w) required to estimate mean household expenditures within the specified percentage deviation (D_0) from the household's usual ("true") expenditure was calculated as follows (29): $n_w = (Z_\alpha \; x \; CV_w/D_0)^2$. D_0 varied between 10 and 50% and Z_α was fixed at 1.96 to derive 95% CIs.

RESULTS

Of the 279 participants enrolled in the study, 260 submitted at least 3 weeks of food receipts during the baseline period and were included in the analyses. Participant characteristics are presented in **Table 2**. To summarize, most participants were female, over half were African-American, and most reported low or very low food security.

Food Purchase Information

Over a 4-week period, households included in the analyses submitted a total of 5,635 receipts. Of these, 2,094 receipts from restaurants and 11 receipts for non-food purchases were excluded from analyses. Over 98% of the receipts were submitted as original receipts; 1.5% ($n = 52$) were submitted as missing receipt forms. Over the 4-week data collection period, households submitted on average 13.6 receipts (95% CI: 12.5, 14.7). This translates to 3.4 receipts per week (95% CI: 3.1, 3.7), with an average of 8.3 (95% CI: 7.6, 9.0) line items per receipt. On average, households spent $23.30 (95% CI: $21.00, $25.51) per receipt.

Figure 1 shows the average household food expenditures over a 4-week period for selected food categories. On average, unidentified items accounted for $5.81 (95% CI: $4.25, $7.37) of household food expenditures over the 4-week period. Fruit and vegetable expenditures accounted for $15.41 (95% CI: $13.04, $17.77) and $16.34 ($13.90, $18.78), respectively. Households spent an average of $13.45 (95% CI: $11.45, $15.45) on sugar-sweetened beverages (SSBs) over the 4-week period. **Table 3** presents average household food expenditures and line items submitted over a 4-week period for all categories of food. Foods coded as "other" –food and beverages that did not fit into the 11 food categories of interest—comprised the largest share of expenditures, accounting for $180.00 (95% CI: $162.52, $197.47) of food expenditures and 58.7 (95% CI: 53.2, 64.2) receipt line items over a 4-week period.

Figure 2 shows the average household food expenditures over a four-week period by store type. Households spent the most money at grocery stores ($169.34, 95% CI: $150.37, $188.30) and superstores/mass merchandisers/warehouse club stores ($115.68, 95% CI: $98.89, $132.46). **Table 3** presents average household food expenditures and receipt line items submitted over a 4-week period by different store types. Grocery stores and superstores accounted for the greatest number of line items submitted over the 4-week period, accounting for 62.4 (95% CI: 55.5, 69.3) and 36.9 (95% CI: 31.7, 42.1) receipt line items, respectively.

Supplemental Tables 1, **2** describe the total volume of food expenditure information captured over the 4-week data collection period. The 3,530 food retailer receipts submitted by the study sample represented $70,822.21 in

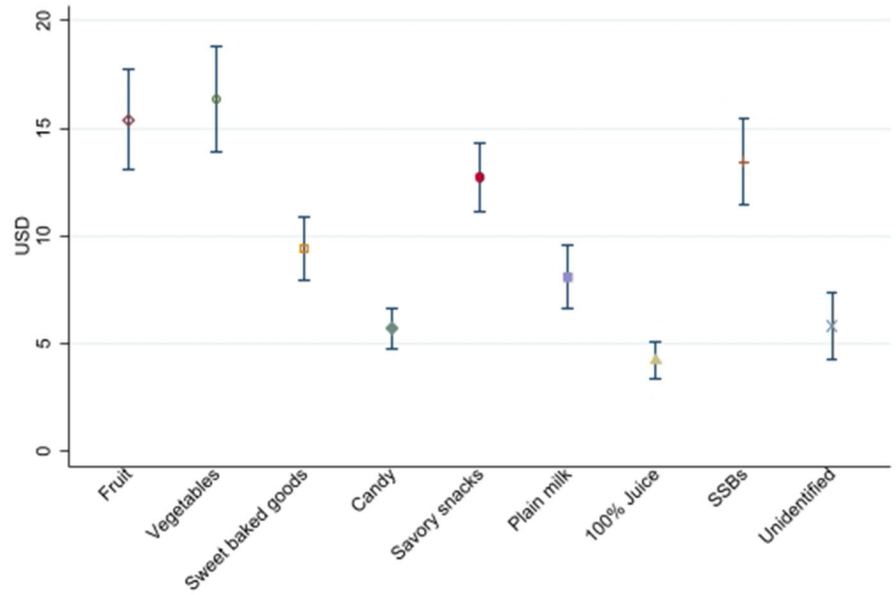

FIGURE 1 | Average household expenditures submitted over a 4-week period by low-income households using a simple annotated receipt method, for selected food categories ($n = 260$).

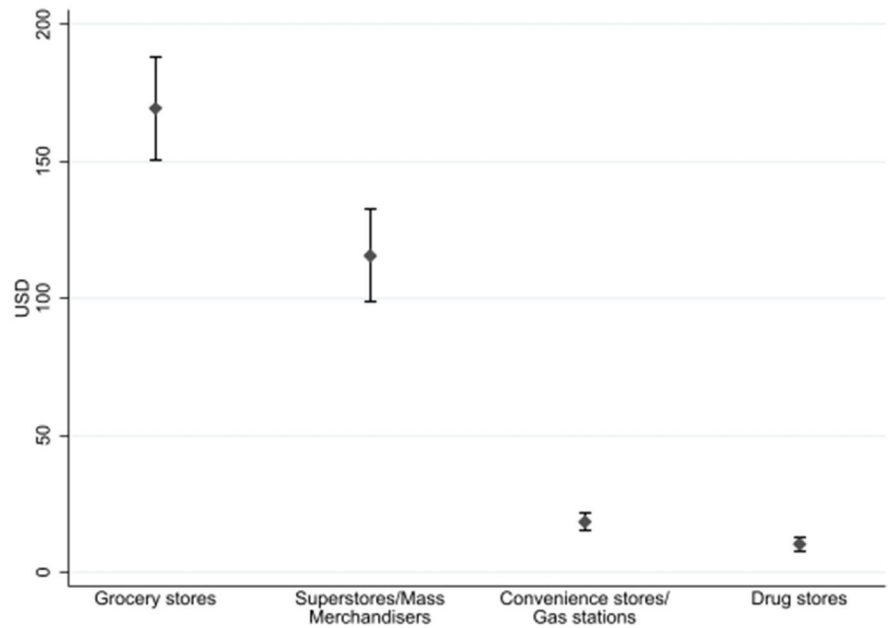

FIGURE 2 | Average household expenditures submitted over a 4-week period by low-income households using a simple annotated receipt method, by store type ($n = 260$).

total food expenditures and contained over 25,000 line items. Food purchases coded as "other" —food and beverages that do not fit into the 11 coded food categories of interest—comprised the largest share of expenditures at food retailers (66.0%), followed by vegetables (6.0%), fruits (5.7%), sugar sweetened beverages (4.9%), and savory snacks (4.7%). With respect to findings for the number of receipt line items, "other" composed the largest number of line items (59.4%) followed by vegetables (9.1%), sugar sweetened beverages (7.2%), fruits (5.7%), and savory snacks (5.5%). "Unsure fruit beverages" (fruit beverages for which it could not be determined whether the beverage was 100% fruit

TABLE 3 | Average household food expenditures and receipt line items submitted over a 4-week period by low-income households using a simple annotated receipt method, by food category and store type ($n = 260$).

	Expenditures USD (95% CI)	Receipt line items number (95% CI)
Total	*272.62 (247.13, 298.11)*	*98.8 (90.1, 107.6)*
FOOD CATEGORY		
Fruits	15.41 (13.05, 17.77)	5.6 (4.9, 6.3)
Vegetables	16.34 (13.90, 17.78)	9.0 (7.7, 10.2)
Sweet baked goods	9.39 (7.94, 10.85)	3.6 (3.1, 4.1)
Candy	5.70 (4.75, 6.65)	3.36 (2.8, 3. 9)
Savory snacks	12.72 (11.15, 14.29)	5.4 (4.8, 6.0)
Regular, unflavored milk	8.07 (6.59, 9.55)	2.5 (2.1, 2.9)
Flavored milk	0.47 (0.23, 0.71)	0.2 (0.1, 0.3)
100% Juice	4.21 (3.33, 5.10)	1.5 (1.2, 1.8)
Sugar-sweetened beverages	13.45 (11.44, 15.45)	7.1 (6.1, 8.2)
Fruit beverage, unknown type	1.04 (0.62, 1.46)	0.4 (0.3, 0.5)
Other foods	180.00 (162.52, 197.47)	58.7 (53.2, 64.2)
Unidentified	5.81 (4.25, 7.37)	1.6 (1.2, 2.0)
STORE TYPE		
Grocery stores	169.34 (150.37, 188.30)	62.4 (55.5, 69.3)
Superstores/Mass merchandisers	115.68 (98.89, 132.46)	36.9 (31.7, 42.0)
Convenience stores/Gas stations	18.30 (15.02, 24.57)	11.7 (9.7, 11.6)
Drug stores	10.38 (7.82, 12.94)	5.0 (3.9, 6.1)

juice or a fruit drink that should be classified as a sugar sweetened beverage) comprised <1 percent of both the total food spending (0.4%) and the proportion of total line items (0.4%) (**Supplemental Table 1**). Nearly 60% of total food retailer expenditures ($41,826.57) was spent in supermarkets/markets, and $24,985.98 (35.8%) was spent in superstores/mass merchandisers/warehouse club stores (**Supplemental Table 2**).

Unidentified Food Expenditures

Unidentified food expenditures comprised 2.1% of total spending and 1.6% of total line items submitted by 260 households over a 4-week period (**Supplemental Table 1**). **Table 4** presents results from the zero-inflated negative binomial model to evaluate the association between unidentified receipt items and participant characteristic and store type. Drugs stores had a lower rate of unidentified line items compared to supermarkets ($p = 0.04$). There were no significant differences in the rate of occurrence of unidentified receipt line items by the participant characteristics examined.

TABLE 4 | Adjusted* incidence rate ratios of unidentified items in receipts submitted over a 4-week baseline period by 260 low-income households using a simple annotated receipt method ($n = 3,530$ food retailer receipts).

	Unidentified receipt line items IRR (95% CI)
STORE TYPE	
Supermarket (ref)	1.00
Convenience store/Gas station	1.03 (0.58, 1.82)
Drug store	0.11 (0.01, 0.99)
Superstore/Mass merchandiser/Warehouse club store	0.92 (0.47, 1.80)
AGE, YEARS	
<25	1.40 (0.67, 2.91)
25–44 (ref)	1.00
45–64	1.56 (0.67, 3.66)
Over 65	1.04 (1.90, 5.70)
GENDER	
Female (ref)	1.00
Male	1.11 (0.70, 1.82)
RACE/ETHNICITY	
White, Non-Hispanic (ref)	1.00
Black, Non-Hispanic	0.65 (0.37, 1.13)
Other, Hispanic	1.40 (0.71, 2.62)
MARITAL STATUS	
Single, never married (ref)	1.00
Married or partnered	0.76 (0.37, 1.13)
Separated/divorced/widowed	1.37 (0.71, 2.62)
HOUSEHOLD SIZE	
1 person (ref)	0.40 (0.19, 0.84)
2 people	0.68 (0.30, 1.60)
3 people	0.68 (0.41, 1.12)
4 or more	1.00 (ref)
EDUCATION LEVEL	
High school graduate or less	1.00
Some college/associates degree (ref)	0.78 (0.50, 1.23)
College graduate or higher	1.14 (0.58, 2.23)
ANNUAL HOUSEHOLD INCOME	
$14,999 or less	1.00
$15,000–$34,999 (ref)	0.79 (0.50, 1.26)
$35,000 or more	0.87 (0.46, 1.64)
FOOD SECURITY	
Very low (ref)	1.00
Low	0.67 (0.44, 1.04)
High or marginal	0.65 (0.33, 1.27)

**Model adjusted for the total number of line items per receipt.*

Within- and Between-Household Variation

Table 5 shows the means, within-household coefficient of variation (CV_w), between-household coefficient of variation (CV_b), and ratios for weekly household expenditures for total food expenditures and selected food categories. On average, households spent $85.65 per week (standard error of the mean [SE] $5.38) on total food expenditures. Mean household expenditures on fruits and vegetables was $11.05 per week (SE $0.90), with comparable amounts spent on fruits and vegetables individually. Households spent an average of $7.95 (SE $0.78) per week on foods high in added sugar, with varying amounts spent on individual food categories. Regardless of food category, CV_w was larger than CV_b, and both values were higher when evaluating individual food categories for foods high in added sugar. The CV ratio was above 1 for all categories of food, ranging from 1.44 for fruits and vegetables to 6.44 for candy.

TABLE 5 | Mean weekly expenditures (USD), within-household coefficients of variation (CV_w), between-household coefficients of variation (CV_b), and variance ratios for food expenditures of low-income households using the simple annotated receipt method ($n = 260$).

	Mean (SE) Dollars (USD)/week	CV_w	CV_b	Variance Ratio
Total food expenditures	85.65 (5.38)	70.8	49.8	2.02
Fruits and vegetables	11.05 (0.90)	85.5	71.3	1.44
Fruits	5.67 (0.53)	100.5	75.6	1.77
Vegetables	5.38 (0.52)	104.4	81.3	1.65
Foods high in added sugar	7.95 (0.78)	105.6	79.0	1.78
Sugar-sweetened beverages	3.57 (0.45)	138.3	95.5	2.10
Sweet baked goods	2.70 (0.37)	161.9	80.3	4.07
Candy	1.70 (0.26)	184.5	72.7	6.44

CV_w, Within-household coefficient of variation; CV_b, Between-household coefficient of variation; Variance ratio, $\sigma_w^2/\sigma_b^2 = (CV_w/CV_b)^2$.

Table 6 shows the number of households in a group required to estimate the group mean weekly expenditure with 95% CIs within 10–50% deviation of the group's observed mean from the group's usual ("true") mean. To maintain precision of ±20% of the group's true total food expenditures, at least 72 households are required. Larger group sizes are required to estimate specific food categories, with the highest requirements for evaluating individual categories of food high in added sugar.

Table 7 shows the number of weeks of food expenditure data required to ensure a correlation coefficient, *r*, between observed and true expenditures. As the variance ratio decreased, fewer weeks of observation were needed to rank households by expenditure and distinguish households with low expenditures from those with high expenditures. Assuming $r = 0.90$, a minimum of 9 weeks of data are required for total food expenditures, 6 weeks of data for fruits and vegetables, and 8 weeks of data for foods high in added sugar. Compared to evaluating fruits and vegetables as individual food categories, a greater number of weeks are required for evaluating individual categories of food high in added sugar to rank households with a given *r*.

Table 8 shows the number of weeks of food expenditure data required to estimate mean weekly household expenditures with 95% CIs within 10–50% deviation from the usual ("true") household expenditure. To maintain precision of 20% within the household's true expenditures, 48 weeks are required to estimate total food expenditures with 95% CIs. Greater number of replicate weeks of data are required to estimate individual food categories, with the highest number of weeks for categories of foods high in added sugar.

TABLE 6 | Number of households in a group needed to estimate weekly expenditures with 95% CIs within 10–50% deviation of the observed group mean from the group's usual ("true") mean using a single week of expenditure data.

	Specified % of true mean				
	10%	20%	30%	40%	50%
Total food expenditures	288	72	32	18	12
Fruits and vegetables	477	119	53	30	19
Fruits	609	152	68	38	24
Vegetables	674	168	75	42	27
Foods high in added sugar	669	167	74	42	27
Sugar-sweetened beverages	1087	272	121	68	43
Sweet baked goods	1256	314	140	79	50
Candy	1514	379	168	95	61

TABLE 7 | Number of weeks of data needed to ensure a given correlation coefficient, *r*, between observed and usual ("true") weekly household expenditures.

	r-value				
	0.75	0.80	0.85	0.90	0.95
Total food expenditures	3	4	5	9	19
Fruits and vegetables	2	3	4	6	13
Fruits	2	3	5	8	16
Vegetables	2	3	4	7	15
Foods high in added sugar	2	3	5	8	17
Sugar-sweetened beverages	3	4	5	9	19
Sweet baked goods	5	7	11	17	38
Candy	8	11	17	27	60

TABLE 8 | Number of weeks of data needed to estimate mean household expenditures with 95% CIs within 10–50% of the usual ("true") household mean.

	Specified % of usual (true) mean				
	10%	20%	30%	40%	50%
Total food expenditures	192	48	21	12	8
Fruits and vegetables	281	70	31	18	11
Fruits	388	97	43	24	16
Vegetables	419	105	47	26	17
Foods high in added sugar	428	107	48	27	17
Sugar-sweetened beverages	734	184	82	46	29
Sweet baked goods	1006	252	112	63	40
Candy	1308	327	145	82	52

DISCUSSION

This paper describes and evaluates a simple annotated receipt method for assessing household food purchasing. Results show that the method can capture food purchasing information for various food categories in a variety of store types, and may be a feasible tool for use among diverse, low-income populations.

Most food items on the receipts could be coded into one of the 11 food categories of interest in the study. Only 1.6% of line items—comprising 2.1% of total spending—could not be categorized because of insufficient detail. Importantly, unidentified line items did not vary by demographic characteristics, which suggests that the tool is applicable to

diverse, low-income populations. Compared to supermarkets, drug stores had a lower rate of unidentified items. This may be because drugs stores tend to sell less produce, fresh meats, and bulk items, which often lack detail on receipts and require less annotation by the participant.

Findings also suggest that the simple annotated receipt method may be adapted for specific research questions. While the majority of food items were coded as "other," this is a result of a priori food category definitions outlined in the study protocol. The experimental trial for which this method was developed assessed policy changes to SNAP. As a result, the focus was on policy-specific food categories—specifically, fruits, vegetables, sweet baked goods, sugary sweetened beverages, and candies. The "other" category captured foods that were of lesser interest to the study aims, such as diet sodas and water. However, this category is adaptable to various study-specific questions. For example, sugar-sweetened beverages (SSBs) and fruit juices were of interest in the study. To ensure comprehensive and precise evaluation of beverage expenditures, multiple categories of beverages were specified, including a "fruit beverage, unknown type" category for fruit beverages that could not be identified as either 100% fruit juice or a sugar-sweetened fruit drink. Items labeled "fruit beverage, unknown type" comprised only 0.4% of receipt line items in comparison to 7.2% of line items for SSB and 1.5% of line items for fruit juices, suggesting that the present method can differentiate food and beverage categories as required by study-specific aims. Researchers interested in capturing different food or beverage categories can therefore adapt the method to study-specific needs using different coding protocols (e.g., "diet sodas" were included in the "other" category in the present study, but can be coded).

To our knowledge, this is the first study to apply established methods of evaluating within- and between-individual variations to a food expenditure assessment tool (27–29). The results have implications for the design of studies evaluating household food expenditures in lower-income households. CV_w and CV_b values were lowest for total food expenditures and largest for individual categories of foods high in added sugar. Larger CV_w values for foods high in added sugar values had the greatest impact on the number of replicate weeks required to assess a household's usual food expenditures. For example, candy had the highest CV_w value of the food categories evaluated, requiring 52 to 1,307 weeks to estimate the household mean weekly expenditure within 10–50% deviation of the true values. Future researchers should consider alternative or additional tools to evaluate expenditures of foods such as SSBs, candies, and sweet baked goods that are highly variably purchased week to week by households.

Our findings also suggest that the simplified annotated food receipt method is most appropriate for comparing mean expenditures of different study groups or ranking household expenditures (e.g., into quartiles). For example, a group size of at least 119 households is required to estimate the mean group expenditure on fruits and vegetables within 20% of the true mean. Similarly, at least 6 weeks of data are required to rank households by weekly fruit and vegetable expenditure level with a precision of $r = 0.90$.

Strengths and Limitations

Food purchasing behavior is strongly patterned by socioeconomic status (7, 8), but few food receipt methods have been evaluated in low-income households (12). This study addresses the need for feasible methods to evaluate food purchasing. Importantly, this novel method was evaluated in a sample of diverse, low-income households. This study also has a relatively large sample size and prolonged duration of receipt collection for evaluation of a measurement method.

There are several limitations worth noting. This study did not assess the completeness of receipt submission, the accuracy of receipt annotation, or the reliability of coding. It is possible that receipts were not submitted for some food purchases, resulting incomplete assessment of food purchasing. Future evaluations of this methodology should evaluate completeness of receipt submission and evaluate inter-rater reliability of receipt coding. Furthermore, this receipt method does not provide information on food quantities. Expenditure data may suffice if change in food purchasing is the primary outcome of interest (e.g., to evaluate whether an intervention decreases purchasing of SSBs). Previous studies also suggest that food expenditure data may be a reasonable approximation of intake (17, 19, 21). Evaluating the association of expenditure data with food quantities and dietary intake is an area for further method development. The present analyses also relies on a sample of lower-income households in one metropolitan area. The levels of variation in food expenditures may differ for other population groups and requires further research.

Finally, the present method was not directly compared to other receipt methods. A qualitative review of previously published studies shows that results are somewhat comparable. This suggests that the present method may be able to capture details similar to previous receipt methods—while potentially reducing the burden for participants (compared to the annotated receipt method) and minimizing the number of unidentified food expenditures (compared to the receipt collection method). A study using the annotated receipt collection method, which requires transcription of all receipt information, collected an average of 3.1 receipts from food retailers per household per week (24). This is comparable to an average of 3.3 receipts per household per week in the present study. The annotated receipt method also yielded an average of 25.8 line items per household per week for both food retailers and restaurant receipts (24)—compared to 24.7 line items per household per week in the present study, which included only food retailers. Results for specific beverage categories across receipt methods also suggests similarities. Sugar-sweetened beverages accounted for 9.1% of all line items using the annotated receipt method, compared to 7.2% in the present study (24). In a study using the receipt collection method—which involves neither annotation or transcription-−100% fruit juices comprised 1.6% of total grocery expenditures, similar to 1.6% of total expenditures in the present study (22). Importantly, the present method may have a lower rate of "missing/unclassified" items compared

to the receipt collection method, which was previously reported as having 7.7% "missing classified/unclassified" expenditures (22).

However, it is worth noting that the annotated receipt method and receipt collection methods discussed above were deployed in different populations and studies. The annotated receipt method followed 90 participants who were predominantly white women in Minneapolis, Minnesota, for 4 weeks. In contrast, the receipt collection method was used for a sample of 107 diverse, low-income households in Houston, Texas over a 6-weeks period. The present study is specific to ethnically and racially diverse households in the Minneapolis-St. Paul, Minnesota, metropolitan area. Future studies are needed to formally compare different methods.

CONCLUSIONS

The simple annotated food purchase receipt method is a promising approach for assessing food purchasing behavior. Our findings suggest that this method is able to capture a wide range of food purchasing information from a variety of store types. Unidentified items were limited and did not vary by participant characteristic or stores, suggesting that the present method is broadly applicable among diverse, low-income households. This paper is also the first to quantify within- and between-household variation in food expenditures using a receipt method, which is crucial information for determining sample sizes, estimating data collection periods, and interpreting findings. Research is needed to further evaluate the method and compare it to alternative receipt methods to assess food purchasing behavior.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of Minnesota Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SV performed statistical analyses. SV and LH wrote the first draft of the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The project described was supported by Award Numbers R01DK098152 and T32DK083250 from the National Institute of Diabetes and Digestive and Kidney Diseases at the Division of Epidemiology & Community Health, School of Public Health at the University of Minnesota.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.582999/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 25 November 2020
doi: 10.3389/fnut.2020.602515

Challenges Associated With the Design and Deployment of Food Intake Urine Biomarker Technology for Assessment of Habitual Diet in Free-Living Individuals and Populations—A Perspective

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OPEN ACCESS

Edited by:
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University of Bologna, Italy

Reviewed by:
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Diego A. Moreno,
Spanish National Research Council, Spain

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: 03 September 2020
Accepted: 29 October 2020
Published: 25 November 2020

Citation:
Beckmann M, Wilson T, Lloyd AJ, Torres D, Goios A, Willis ND, Lyons L, Phillips H, Mathers JC and Draper J (2020) Challenges Associated With the Design and Deployment of Food Intake Urine Biomarker Technology for Assessment of Habitual Diet in Free-Living Individuals and Populations—A Perspective. Front. Nutr. 7:602515. doi: 10.3389/fnut.2020.602515

Improvement of diet at the population level is a cornerstone of national and international strategies for reducing chronic disease burden. A critical challenge in generating robust data on habitual dietary intake is accurate exposure assessment. Self-reporting instruments (e.g., food frequency questionnaires, dietary recall) are subject to reporting bias and serving size perceptions, while weighed dietary assessments are unfeasible in large-scale studies. However, secondary metabolites derived from individual foods/food groups and present in urine provide an opportunity to develop potential biomarkers of food intake (BFIs). Habitual dietary intake assessment in population surveys using biomarkers presents several challenges, including the need to develop affordable biofluid collection methods, acceptable to participants that allow collection of informative samples. Monitoring diet comprehensively using biomarkers requires analytical methods to quantify the structurally diverse mixture of target biomarkers, at a range of concentrations within urine. The present article provides a perspective on the challenges associated with the development of urine biomarker technology for monitoring diet exposure in free-living individuals with a view to its future deployment in "real world" situations. An observational study ($n = 95$), as part of a national survey on eating habits, provided an opportunity to explore biomarker measurement in a free-living population. In a second food intervention study ($n = 15$), individuals consumed a wide range of foods as a series of menus designed specifically to achieve exposure reflecting a diversity of foods commonly consumed in the UK, emulating normal eating patterns. First Morning Void urines were shown to be suitable samples for biomarker measurement. Triple quadrupole mass spectrometry, coupled with liquid chromatography, was used to assess simultaneously the behavior of a panel of 54 potential BFIs. This panel of chemically diverse biomarkers, reporting intake of a wide range of commonly-consumed foods, can be extended successfully as new biomarker leads are discovered. Towards

validation, we demonstrate excellent discrimination of eating patterns and quantitative relationships between biomarker concentrations in urine and the intake of several foods. In conclusion, we believe that the integration of information from BFI technology and dietary self-reporting tools will expedite research on the complex interactions between dietary choices and health.

Keywords: dietary intake, metabolomics, biomarker of food intake (BFI), urinary biomarkers, habitual diet

INTRODUCTION

There is a rich history of nutrition research spanning many decades, much of which has had at its core a need for accurate information on dietary intake for investigation of the links between exposure to individual food/food groups and specific health outcomes. Food intervention projects commonly rely on participants collecting pre-prepared foods from research centres for consumption at home and then confirming compliance at a later date (1, 2). On the other hand, large-scale nutritional epidemiological projects and nutrition surveys involving free-living individuals consuming their habitual diet rely almost totally on self-reporting of dietary exposure. Long-established tools to collect self-reported quantitative dietary information include Food Frequency Questionnaires (FFQs), diet diaries, and dietary recall methodology (3). However, because of the complexity of eating patterns and the conceptual and practical difficulties in recording or recalling the types and amounts of foods and beverages consumed, errors in self-reporting of dietary intakes by cognitively-able individuals is commonplace and substantial (4, 5) and can be exacerbated in those who are overweight or obese (6, 7).

Secondary metabolites derived from individual foods or food groups present in human biofluids can provide potential biomarkers of food intake, for reviews see (8–18). The inclusion of biomarker technology in dietary assessment could help to overcome some of the limitations of traditional dietary methodologies by providing additional objective estimates of food exposure (19). Unlike blood, urine is easy to collect and it provides an integrated estimate of exposure over several hours. For a panel of dietary biomarkers to have any significant utility, it is essential that its coverage is as comprehensive as possible. Using data-driven approaches, we have shown that the potential utility of a biomarker is dependent on the type, portion size, and frequency of consumption of individual foods (20). Data concerning nationally-representative estimates of intakes of foods by the UK population are collected by the UK National Diet and Nutrition Survey (NDNS) (21) and this database can be explored to identify foods and food groups for which dietary exposure biomarker discovery might be feasible and relevant (1, 2, 22).

Over the past decade, our collaborative research projects and those of other teams (see **Supporting Data 1** for a comprehensive list) have contributed to the discovery of putative dietary intake urinary biomarkers of specific foods including poultry and red meat (23–28) citrus fruits (29, 30), crucifers (31, 32), oily fish (26, 27, 32), red berries/strawberries (2, 32–34), wholegrain/rye (35–37), sugary drinks (38, 39), artificial sweeteners (2, 40), peas/beans/legumes (2, 41, 42), grapes (41, 43–45), apples (41, 46, 47), and potatoes (48). In addition, consensus guidelines for the critical assessment of candidate BFIs has been established (49). These BFI candidate guidelines have focused generally on qualifying the utility of individual BFIs for monitoring exposure to specific foods/food groups. However, because effects on health are a consequence of the whole diet, it is equally important to develop approaches to assess overall dietary exposure in nutrition surveys, epidemiological studies, and clinical trials (45).

The ideal biomarker is highly specific for one food item or food group, is not detected in the biological sample of interest when the specific food item is not ingested, and shows a distinct dose- and time-dependent response following consumption (50). Although metabolites distinctive of dietary exposure to particular foods have been described, it is not uncommon to discover subsequently that putative biomarkers are not necessarily specific for individual foods and therefore much rigour needs to be applied during validation of their utility to monitor habitual dietary intake (51). For application in the real world, the use of multi-metabolite biomarker panels may provide more reliable estimation of dietary exposure than a single-biomarker approach [reviewed by (52)]; such panels need to have comprehensive coverage and to be extendable (53). For this reason, in the future it will be important to evaluate biomarker performance in the context of complex exposures to multiple foods, with different food formulations, cooking, and processing methods and within complex meals, in eating patterns the target study population is likely to experience (2).

Optimal sampling requirements for urine biomarker analysis will be dependent largely on the study design and objectives (**Table 1**). For example, a food intervention study with free-living

Abbreviations: AUC, area under the ROC (Receiver Operator Characteristic) curve; BFI, biomarker of food intake; CRN, Clinical Research Network; FFQ, Food Frequency Questionnaire; FMV, First Morning Void; HESI, heated electrospray ionisation; HILIC, Hydrophilic Interaction Liquid Chromatography; HPLC, high-performance liquid chromatography; IAN-AF, Portuguese National Food, Nutrition and Physical Activity Survey; ISRCTN, International Standard Randomised Controlled Trials Number; LC-QQQ-MS, liquid chromatography triple quadrupole mass spectrometry; LoD, limit of detection; logP, partition coefficients; LoQ, limit of quantification; MACCS, Molecular ACCess System; MAIN, Metabolomics at Aberystwyth, Imperial and Newcastle; MDS, multi-dimensional scaling; MRC, Medical Research Council; MRM, multiple reaction monitoring; MS, Mass Spectrometry; NDNS, National Diet and Nutrition Survey; PCA, Principal Components Analysis; QC, Quality Control; RF, Random Forest; RI, refractive index; ROC, Receiver Operator Characteristic; RP, reverse phase; RSD, relative standard deviation; SG, specific gravity; SRM, Selected Reaction Monitoring; UHPLC, Ultra High Performance Liquid Chromatography.

TABLE 1 | Study objectives and biomarker of food intake (BFI) requirements.

	Example study objectives	Typical sampling requirements		Biomarker requirements		Data requirements		Study example and reference
		Single sample only	Multiple samples	Biomarker(s) of only one food/food group	Comprehensive biomarker panel	Quantitative or semi-quantitative measurement	Exposure range assignment	
A	Confirmation of participant compliance in a food intervention study or validation of a proposed biomarker focusing on a single food/food group, short term or long term		Y	Y		Y		A validation trial: (54) A compliance trial: (55, 56)
B	Biomarker discovery and/or validation in a free-living population following a meal plan emulating normal eating patterns		Y		Y	Y		MAIN study: (1, 2, 22)
C	Investigation of individual "metabotype" in relation to interaction with specific dietary chemicals	?	?	Y		Y		Food4me study: (57)
D	Assessment of habitual (e.g., weekly, monthly, and annual) eating behavior of individuals		Y		Y	?	?	PREDIMED trial: (58, 59)
E	Observational epidemiological survey of eating habits in a large population	Y			Y	Y	?	IAN-AF: (60); EPIC: (27, 61)
F	Cohort stratification by dietary exposure levels to specific foods/food groups in a small clinical trial		Y		Y		Y	MAIN study: (45)

participants lasting several weeks investigating links between a health outcome and a specific food/food group will require appropriate samples on multiple days taken at random to assess compliance with dietary intake targets (1, 2). In contrast, assessing the general eating habits of a large population in an epidemiological survey may only require sampling of a large number of people on a single random day or multiple days (60). Any urine sampling procedure would need to be (i) acceptable for volunteers to provide samples repeatedly, (ii) require minimal researcher time and cost, and (iii) deliver samples with high quality information content. The theoretical optimal types of urine(s) to be sampled [e.g., spot, cumulative (i.e., "phase" of day) or 24 h] will also depend on study objectives (62) and, in many instances, the sampling strategy will be limited by cost constraints or the practicalities of collection. Twenty-four hour urine samples and single spot urine samples taken at random times during the day are commonly collected to monitor discrete aspects of human physiology, metabolism, or "exposome" in clinical trials and surveys (63–65). Unfortunately, such samples provide information only in relation to very recent eating behaviour and may be of limited utility in nutrition studies where the focus is on the whole diet or on the intakes of foods that are not eaten frequently. Additionally, eating behaviour and hydration levels can be very different between individuals in free-living populations and the fact that excretion half-lives of specific metabolites can vary enormously (49), means that research protocols must be in place to manage adequately these sources of variability in any biomarker discovery and validation strategy (62, 66).

Where the aim is to estimate absolute intake of specific foods, or the frequency of exposure, it will be desirable to generate quantitative or semi-quantitative data on BFI concentrations in urine. However, for other studies, it may be sufficient to be able to assign each individual into an exposure range (e.g., high-medium-low), typical of a specific reference population. Urine collection(s), sample processing, and the analytical methodology can be optimised for a target metabolite when using a single biomarker to monitor exposure to a single food/food group. In contrast, the desire to monitor habitual diet comprehensively using a panel of biomarkers requires the analytical approach to manage the complex physio-chemical attributes of the diverse range of putative biomarkers currently described, as well as coping with metabolites exhibiting differential stability during collection, transport, and storage. Other practical issues such as the commercial availability, costs, solubility, and stability of pure chemicals in mixtures as quantitation standards will also impact on the design of analytical solution likely to offer

scope for simultaneous measurement of a large number of metabolite targets.

The assessment of eating behaviour in free-living individuals is important in a wide range of types of nutrition research (**Table 1**), ranging from clinical trials investigating the mode of action of potentially beneficial "bioactive" compounds in individuals, to general surveys of national eating habits in large populations. Although considerable effort is being expended on BFI discovery and validation, there is an equally urgent need to consider the future challenges for effective deployment of dietary intake biomarker technology to assess habitual diet within populations. To summarise, these major challenges include:

- Strategies for validation of food intake biomarkers suitable for assessment of habitual dietary exposure;
- Standardised urine sampling approaches, including collection, temporary storage, transport, and long term biobanking;
- Development of biomarker analytical methodology, using a multi-panel of biomarkers, that is able to integrate new markers as they become validated;
- Algorithms to convert raw biomarker data into meaningful estimates of food intake and/or overall diet quality.

The present article aims to provide a perspective on *some* of these challenges associated with the development of urine biomarker technology to monitor recent or habitual dietary intake in free-living individuals with a view to its future deployment in "real world" situations. For example the UK government's "Better Health" campaign, a 12 week fitness and healthy eating plan announced in July 2020 to help Briton's lose weight and reduce their risk of serious complications should they contract COVID-19 (https://www.nhs.uk/better-health/). One of the key aims of the plan is to encourage people to make healthier food choices. We believe that the BFI technology we have been developing over the past 10 years, alongside the low-effort, minimally intrusive urine sampling strategies (62, 67) will soon be validated to the point that they can be used to reproducibly and objectively monitor the effectiveness of such plans at a population level. A workflow summarising the overall experimental strategy is illustrated in **Figure 1**.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

For ***Study 1***, ethical approval was obtained from the National Commission for Data Protection, the Ethical Committee of the Institute of Public Health of the University of Porto and from the Ethical Commissions of each one of the Regional Administrations of Health. All participants gave written informed consent, and the study was carried out in accordance with the Declaration of Helsinki. The MAIN (Metabolomics at Aberystwyth, Imperial, and Newcastle) food intervention trial at Newcastle (***Study 2***) was approved by the East Midlands—Nottingham 1 National Research Ethics Committee (14/EM/0040). Caldicott approval for storage of data and data protection was granted by Newcastle-upon-Tyne Hospitals NHS Foundation Trust [6896(3109)]. The MAIN food intervention trial in Newcastle was adopted into the UK Clinical Research Network (CRN) Portfolio (16037) and is registered with International Standard Randomised Controlled Trials Number (ISRCTN), 88921234.

The participants provided written informed consent to participate in each study, taken by an appropriately trained researcher. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Epidemiological Study and Urine Sampling

Study 1 involved community-living individuals consuming a freely-chosen diet. The participants ($n = 95$) were volunteers who participated in the Portuguese National Food, Nutrition, and Physical Activity Survey (IAN-AF), whose aims and methods have been described previously (60). A 24 h dietary record was collected by trained nutritionists using the "eAT24" Software (68) which facilitates the assessment of dietary data using an automated multiple-pass method (5 steps) (69). Participants were asked to collect urine samples on the day before the second 24 h dietary record. Urine samples were collected in two separate containers. The first one (a 2,700 mL container identified as container A) was used to collect all urine passed during the day before the interview, except the first void of that morning. A second one (a 500 mL container identified as container B) was used to collect just the First Morning Void (FMV) on the day of the second interview. No preservatives were added to the urine containers, and the participants were asked to keep the samples refrigerated (4°C) throughout the collection period. Participants were asked to fill in a questionnaire with the time of the beginning and the end of collections, details of any medication, and whether or not they had any problems or missed urine collections. At the laboratory, urine samples were weighed and mixed. The weights of urine from containers A and B were quantified separately and a proportionally pooled 24 h urine sample (identified as "24 h urine") was prepared by using samples A and B. From each participant, both urine samples were aliquoted: 1×45 mL (in 50 mL Falcon pre-labelled tube) + 10×1.5 mL (in 2 mL pre-labelled microtubes). These aliquots were refrigerated immediately before being moved to −80°C storage, within 24 h, for further analysis.

Food Intervention Study Design and Urine Sampling

The MAIN project at Newcastle included two controlled food intervention studies in free-living people who consumed the test foods as part of two 3day menu plans, designed to generate six distinctive "Menu Days" (1, 22). Participants were provided with all the foods and ingredients to prepare and consume meals at home, following the prescribed menus. Within this manuscript (***Study 2)*** we have used data from 15 of the individuals from the second 3-day menu plan (8 male, 7 female; non-smokers; age: 21–74). We implemented urine sampling methods based on our previous studies (23, 70) and asked participants to collect a series of urine samples including the FMV the day after each menu plan. Participants collected urine samples in a plastic jug

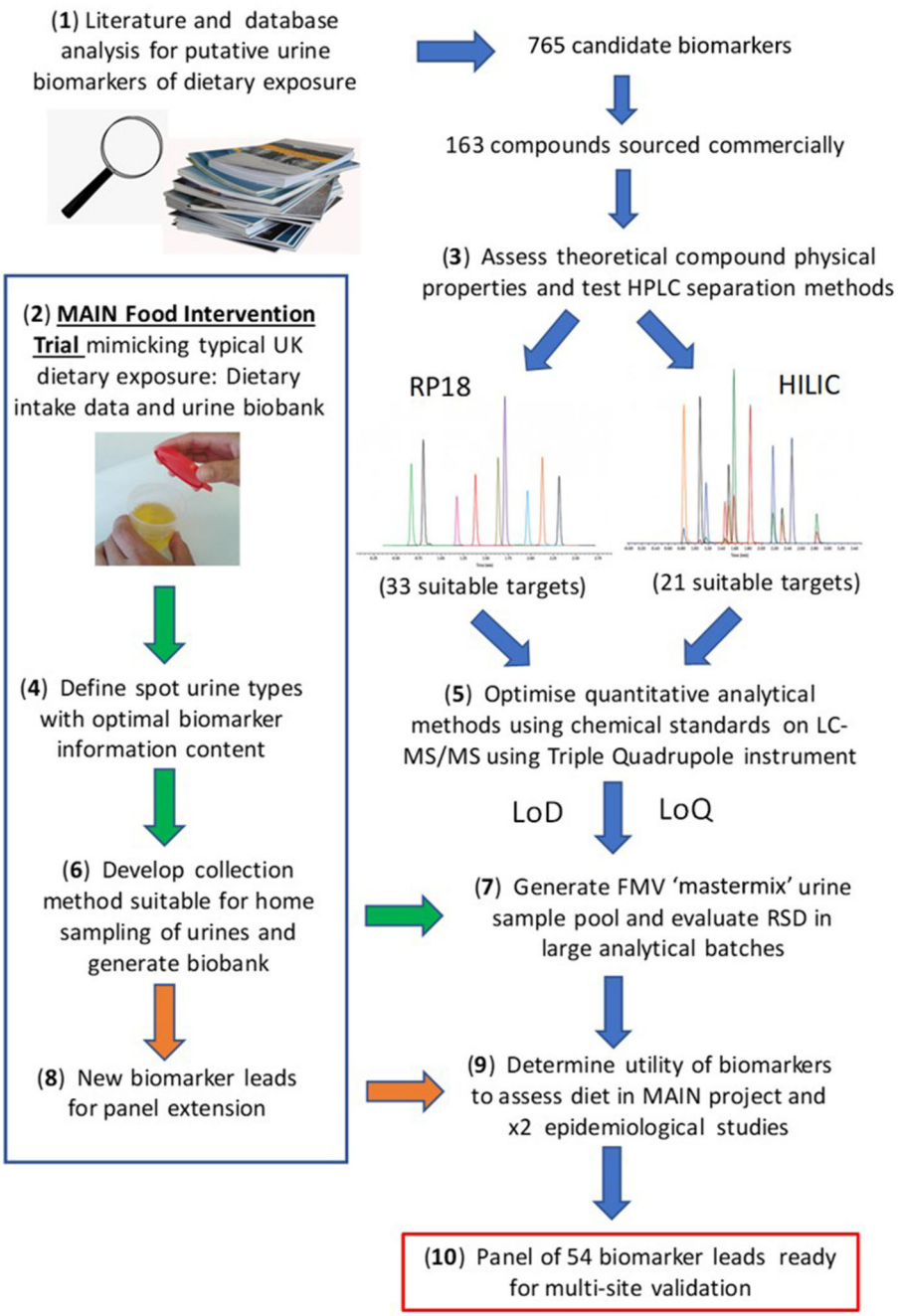

FIGURE 1 | Workflow for biomarker panel development. Where: MAIN, Metabolomics at Aberystwyth, Imperial and Newcastle; RP, reverse phase; HILIC, Hydrophilic Interaction Liquid Chromatography; LoD, limit of detection; LoQ, limit of quantification.

and transferred aliquots into labelled sterile 25 mL Universal tubes. Six of these 15 participants (2 female, non-smokers, age range 22–59) also collected FMV urine samples at home using the vacuum transfer system (67). All samples were placed in an opaque cool bag and stored at home in a fridge at 4°C for up to 4 days and then brought to the research facility in Newcastle at the end of the study week. Universal tubes were stored immediately at −80°C and the vacuum tubes remained at 4°C for a further 2 weeks before storage at −80°C. Samples were then transported to the analytical facility in Aberystwyth on dry ice for metabolite analysis.

Urine Sample Preparation

Urine samples were prepared and adjusted as reported previously (1, 22). In brief, all urine samples were normalised by refractive index (RI) prior to analysis to account for differences in fluid

intake by participants and to ensure that all Mass Spectrometry (MS) measurements were made within a similar dynamic range within the linear range of the instrument. Samples were defrosted overnight at 4°C, centrifuged (1,600 × g for 5 mins at 4°C), placed on ice and aliquots of thawed urine (1,000 μL) were transferred into labelled 2 mL Eppendorf tubes. The remaining sample were returned to a −20°C freezer. An OPTI Hand Held Refractometer (Bellingham Stanley™ Brix 54 Model) was used to record the specific gravity (SG). Using these data, aliquots of the required amounts of urine from centrifuged 2 ml Eppendorf tubes and ultra-pure (18.2 Ω) H_2O were transferred into new tubes for extraction; this ensured that all samples had the same RI.

Strategy for Selection of Candidate Dietary Exposure Biomarkers

The selection of biomarkers was initiated with a literature search to generate an initial "long list" of food-related metabolites with potential for inclusion in a panel of biomarkers that would provide comprehensive coverage of food items consumed in the MAIN Study (see **Supporting Data 2** for a summary of the foods). The search was carried out using Google Scholar and Web of Knowledge using the following search terms in a range of combinations "biomarkers + urine + food + dietary + BFI" and ended on 22/06/2020. Publications were screened and information was added to the database if they contained data relating to potential dietary exposure biomarkers measured in urine samples (see **Supporting Data 3**). Specific details on metabolite excretion profile were recorded and the availability of a commercial supply of a pure chemical standard was investigated (see **Supporting Data 3**).

Evaluation of Chemical Diversity of Biomarkers

Biomarkers were assigned to chemical class and superclass using the ClassyFire application (71). Classifications for each biomarker were retrieved using the R package classyfireR Version 0.3.3. Pairwise similarity of biomarkers was measured using the Tanimoto Distance after converting structural representations of each biomarker to its MACCS (Molecular ACCess System) fingerprint. Fingerprints were generated using the get.fingerprint function from the R package rcdk (Version 3.5) and distances computed using the fp.sim.matrix function from the R package fingerprint. The resultant matrix of fingerprint distances, was then reduced to two dimensions using the cmdscale function. Chemical descriptors (–log*P*) were calculated using the rcdk package.

Sample Analysis by Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC-QQQ-MS)

Methanol (primer trace analysis grade, Fisher Scientific, UK) was used for urine extraction and standards preparation. Acetonitrile (Optima® LC-MS grade, Fisher Scientific, UK), methanol (HPLC grade, Fisher Scientific, UK), and Ammonium acetate (Optima® LC-MS grade, Fisher Scientific, Belgium) were used for preparing the LC mobile phase. Water was ultra-pure water (18.2 Ω) drawn from an Elga Purelab® flex water purifier system (Taiwan). The suppliers of chemical standards are given in **Supporting Data 4**.

Sample analysis was performed on a TSQ Quantum Ultra EMR QQQ mass spectrometer (Thermo Scientific) equipped with a heated electrospray ionisation (HESI) source. Samples were delivered using an Accela ultra-high-performance liquid chromatography (UHPLC) system (Thermo Scientific) consisting of autosampler, column heater, and quaternary UHPLC-pump. For HILIC (Hydrophilic Interaction Liquid Chromatography) analysis, chromatographic separation was performed on a ZIC-pHILIC (polymeric 5 μm, 150 × 4.6 mm) column (Merck). The mobile phase consisted of 10 mM ammonium acetate in water: acetonitrile (95:5) (**A**) and 10 mM ammonium acetate in water: acetonitrile (5:95) (**B**). The gradient program used was as follows: 0 min, 95% B (400 μL min^{-1}); 15 min, 20% B (400 μL min^{-1}); 15.01 min, 20% B (500 μL min^{-1}); 20 min, 20 % B (500 μL min^{-1}); 20.01 min, 95 % B (500 μL min^{-1}); 25 min, 95% B (500 μL min^{-1}). The HPLC was carried out in low pressure (∼0–7,000 psi) operating mode with 0 psi and 650 psi as minimum and maximum pressures, respectively. For Reverse Phase (RP) analysis, chromatographic separation was performed on a Hypersil Gold (1.9 μm, 200 × 2.1 mm) RP-column (Thermo Scientific). The mobile phase consisted of 0.1% formic acid in H_2O (**A**) and 0.1% formic acid in MeOH (**B**). The gradient program used was as follows: 0 min, 0% B; 0.5 min, 0% B; 5 min, 60% B; 11 min, 100% B; 13 min, 100% B; 13.01 min, 0% B; 19 min, 0% B. For RP analysis, the flow rate was maintained at 400 μL/min^{-1}. The UHPLC was carried out in high pressure (∼7,000–15,000 psi) operating mode with 0 and 1,000 psi as minimum and maximum pressures, respectively. For both chromatographic analyses, column oven and autosampler tray were maintained at 60 and 14°C, respectively. To ensure consistent sample delivery, 20 μL were injected using a 20 μL loop and partial loop injection mode. After each injection, syringe, and injector were cleaned using a 10 % HPLC grade MeOH solution in ultra-pure water (1 mL flush volume; 100 μL/s^{-1} flush speed, 2 mL wash volume) to avoid sample carryover. Mass spectra were acquired in multiple reaction monitoring (MRM) mode, in positive and negative ionisation polarities simultaneously using optimised values of collision energy and tube lens for each MRM transition (**Supporting Data 4**). Spectra were collected at a scan speed of 0.010 and 0.003 s for HILIC and RP analysis, respectively. A scan width of 0.010 m/z units and peak width (Q1, Q3) of 0.7 FWHM were used for both HILIC and RP analyses.

Raw files (ThermoFisher) were converted to mzML (72) using msconvert in the ProteoWizard tool kit (73). All further processing of mzML files was performed using the R Statistical Programming Language (74). Selected Reaction Monitoring (SRM) chromatograms were extracted from mzML files using the R library, mzR and peaks areas were calculated by extracting pre-defined chromatographic windows (based on calibration standards) around each peak apex. Absolute concentrations were calculated using a nine-point calibration curve (0.006561–100 μg mL^{-1}). The limit of detection (LoD) and limit of quantification (LoQ) of all chemical standards were calculated as the lowest

concentration of each biomarker giving a signal-to-noise ratio of 3:1 and 10:1, respectively within the linear range of each calibration curve.

Quality Control (QC) Strategy for Target Biomarkers

Reproducibility of the mixture of chemical standards was determined using the relative standard deviation (RSD) of a multi component calibration standard and an external urine QC sample using a "master mix" of pooled samples. The external urine QC sample was used to determine the effect of the resultant urine matrix on the reproducibility of selected biomarkers across multiple experiments. The external QC (as distinct from an experimental QC) allowed for longitudinal monitoring of RSD without intra-experimental bias.

Data Analysis

Principal Components Analysis (PCA) was performed using the *prcomp* function in R, with variables scaled to unit variance. Supervised classification of quantitative metabolite data was performed using Random Forest (RF) classification using the randomForest package (75) in R (74). For all RF models, the number of trees (*ntree)* used was 500 and the number of variables considered at each internal node (*mtry*) was the square root of the total number of variables. Accuracy, margins of classification and area under the ROC (Receiver Operator Characteristic) curve (AUC) were all used to evaluate the performance of classification models, as described previously (76). RF classification models were plotted following multi-dimensional scaling (MDS). Proximity measures for each individual observation were extracted from RF models and scaled coordinates produced using cmdscale on *1—proximity*.

Spearman rank correlations of biomarker concentrations in 24 h vs. FMV urine were produced using the *rcorr* function from the R (Version 4.0.3) package Hmisc (Version 4.4.0). Reported *P*-values are the asymptotic *p*-values from the rank correlation. Quantile–Quantile plots were produced using the *qqnorm* function in R.

RESULTS

Selection of Target Foods and Design of a Food Intervention Study for Preliminary Survey of the Potential Utility of Urine Biomarker Technology

A major component of our strategy to develop urine biomarker technology to monitor habitual diet was the need for a biobank of urine samples from a food intervention trial that was designed to provide comprehensive exposure to foods commonly consumed in the UK. Key food groups were identified initially from *The Eatwell Guide* (77); the most commonly eaten foods were identified within each disaggregated food group using estimates of intakes of foods by the UK population from the UK National Diet and Nutrition Survey (NDNS) (21). **Supporting Data 2** describes the representative foods that were incorporated into a six-menu design as part of the MAIN food intervention trial at Newcastle [see Lloyd et al. (1) and Willis et al. (2) for full details]. The menu plans aimed to deliver foods for BFI discovery and validation, including the assessment of BFI specificity and sensitivity within the context of the whole diet. Particularly important was consideration of the impact of the likely major sources of variance on biomarker monitoring procedures including:

- The impact of exposure to targeted foods as part of complex and mixed meals, rather than foods consumed in isolation;
- The use of average portion sizes and normal eating patterns rather than exposure to huge, unrealistic portions consumed in a fasted state;
- The impact of different food formulations, processing, and cooking methods representing the range of ways in which foods are processed and eaten;
- The dynamics of putative BFI retention in the body (to inform development of biomarkers of both acute or habitual food consumption).

The selection of biomarkers was initiated with a preliminary literature search to identify putative urinary biomarkers that would provide comprehensive coverage of each specific food/food group consumed within the six menus. This database (**Supporting Data 1**) suggested that 765 urinary metabolites were potential dietary exposure biomarker candidates as summarised in **Supporting Data 2**. It is clear from these data that there is considerable choice in terms of potential biomarkers and considerable overlap of metabolites between some foods/food groups.

Evaluation of Approaches for Urine Collection and Storage for Monitoring Habitual Dietary Exposure

Using LC-MS fingerprinting methodology, we have shown previously that the metabolome of spot urine samples taken just before bed time on the study day is compositionally very similar to the corresponding 24 h urine samples (62). In the present study, RI measurements revealed that FMV spot urine samples from a national dietary intake survey (***Study 1***) also had an almost identical overall solute concentration range to that of 24 h urines (**Figure 2A**). Since creatinine concentrations are often used as a reference for normalisation in urine samples, we evaluated the relationship between creatinine concentration and RI in both FMV spot and 24 h urines. Creatinine concentrations were within the same range in FMV spot and 24 h urines and exhibited a strong linear relationship ($R^2 = 0.65$ and 0.68, respectively) with RI (**Figure 2B**), supporting the concept of sample normalisation to the same RI. The value of FMV spot urine samples for assessment of dietary exposure was explored further by examining the correspondence between the concentration of putative biomarkers in 24 h urine and spot urine samples using targeted, quantitative measurements of individual biomarkers. **Figure 2C** shows scatter plots of metabolite concentration in FMV urine vs. 24 h urine for eight example biomarkers from samples derived from ***Study 1***. Although the actual biomarker levels varied between the two urine types there was

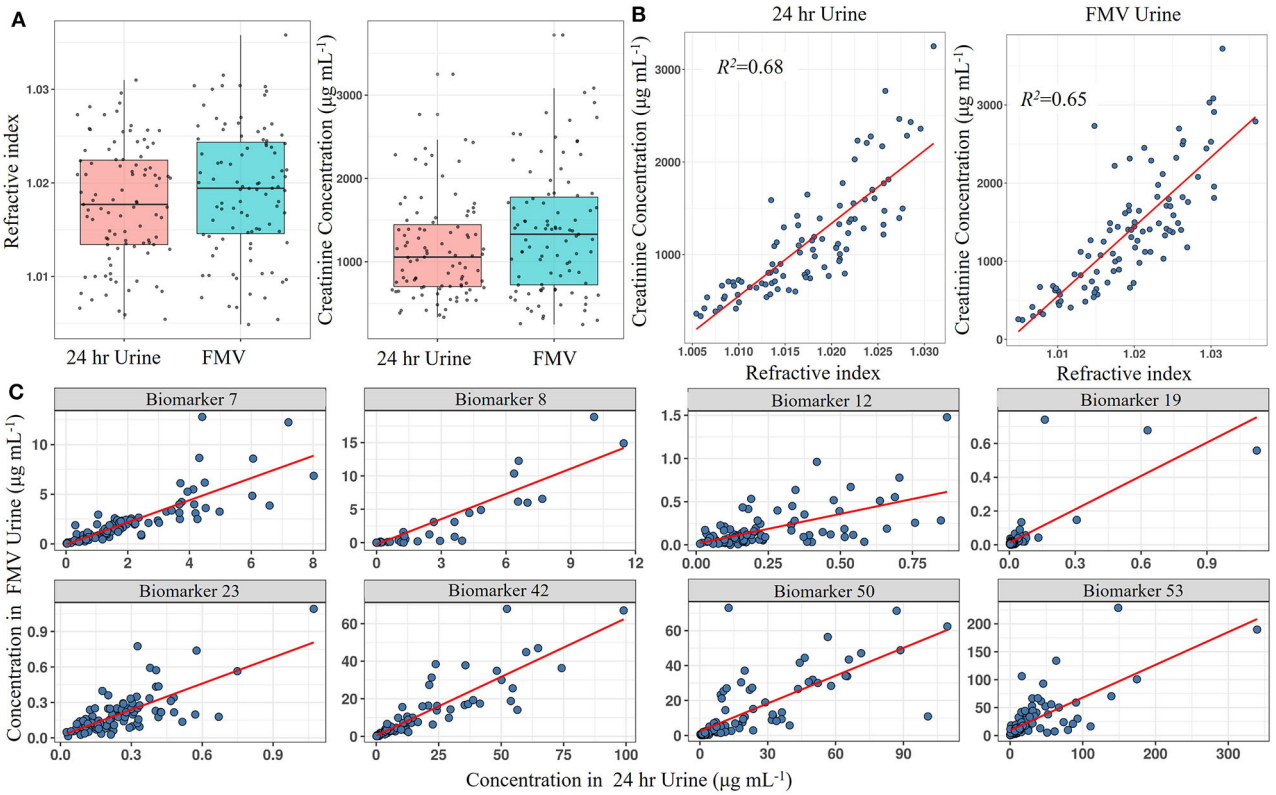

FIGURE 2 | Screening biomarkers to detect those with concentrations in spot urine that reflect levels found in 24 h urine from ***Study 1***. **(A)** Boxplot of total creatinine content and refractive index (RI) in First Morning Void (FMV) and 24 h urine samples. **(B)** Scatter plot showing the linear association between creatinine concentration and RI in FMV and 24 h urine samples. **(C)** Scatter plots showing the linear association of selected biomarker concentrations between FMV and 24 h urine samples. Where: Biomarker 7,7-Methyl xanthine; Biomarker 8, Acesulphame-K; Biomarker 12, Calystegine A3; Biomarker 19, D,L-Sulphoraphane-N-acetyl-L-cysteine; Biomarker 23, DHPPA [3-(3,5-Dihydroxyphenyl)-1-propanoic acid]; Biomarker 42, Proline betaine; Biomarker 50, Tartarate; Biomarker 53, Trimethylamine-N-oxide (Full list of number codes for biomarkers is in **Supporting Data 4**).

a strong linear relationship between concentrations in 24 h and FMV urine (**Figure 2C**). More than 50% of the biomarkers demonstrated a very strong correlation (>0.6). Further potential biomarkers exhibited a weaker correlation in concentration (**Supporting Data 4**) and it is suggested that that $r > 0.2$ (with a $p < 0.05$ from a rank correlation test) may be considered adequate. The Quantile–Quantile plot in **Supporting Data 5** shows the comparable distribution of biomarker concentrations measured in FMV and 24 h urine samples.

We have shown recently that vacuum tube technology has considerable value for spot urine sampling and that, even in the absence of preservatives, urine composition is stable for several days at 4°C (67) and under different temperature regimes. To explore further the utility of vacuum tube technology for large-scale urine sampling in community settings, we evaluated the compositional stability of FMV spot urine at 4°C for 2 weeks, to mimic longer term storage in a domestic fridge. A selection of biomarkers useful for assessment of exposure to meat, fish, wholegrain, fruit, and vegetable components of meals were targeted for analysis. Metabolite concentrations after storage in vacuum tubes at 4°C were very similar to those of the same urine samples after being frozen at −80°C (**Figure 3**).

Literature Analysis to Select Biomarker Leads for Inclusion in a Panel That Will Provide a Comprehensive Survey of Dietary Exposure

A comprehensive list of potential urinary BFIs based on a literature analysis (53) of putative dietary exposure biomarkers in various human biofluids is presented in **Supporting Data 2**. The present biomarker panel strategy aimed to assess habitual diet in individuals and populations; key to this objective was the need to use spot urine samples, specifically urine samples collected just before bedtime and FMV urines, that would be informative of overall food consumption (1, 62). A detailed examination of the dietary exposure biomarker literature was undertaken with particular emphasis on the identification of biomarker candidates present in spot urine samples > 12 h after food consumption (**Table 2** and described in further detail in **Supporting Data 3**). A shortlist of candidate biomarkers for initial biomarker panel development was generated, focusing largely on metabolites that were available from commercial providers (**Supporting Data 3**). For 28 out 54 putative biomarkers there was already evidence in the literature of their presence in FMV urine. The majority

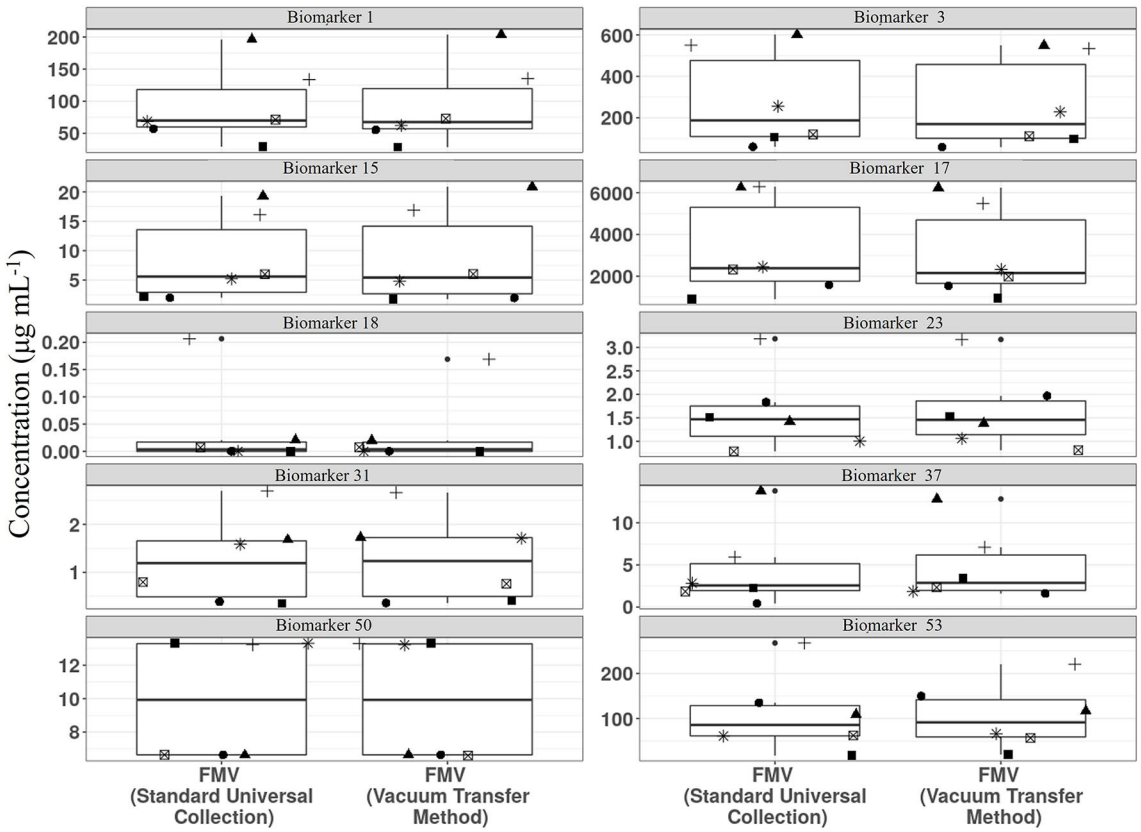

FIGURE 3 | Comparison of stability of example biomarkers in First Morning Void (FMV) urine collected in vacuum tubes stored at 4°C for 2 weeks (Vacuum transfer method) or Universal tubes (Standard Universal collection) stored at −80°C. Where: Biomarker 1, 1-Methyl histidine; Biomarker 3, 3-Methyl histidine; Biomarker 15, Carnosine; Biomarker 17, Creatinine; Biomarker 18, D,L-Sulphoraphane L-cysteine; Biomarker 23, DHPPA [3-(3,5-Dihydroxyphenyl)-1-propanoic acid]; Biomarker 31, Ferulic acid-4-O-sulphate; Biomarker 37, L-Anserine; Biomarker 50, Tartarate; Biomarker 53, Trimethylamine-N-oxide (Full list of number codes for biomarkers is in **Supporting Data 4**).

of the remaining dietary exposure biomarker leads selected had been shown to be present in 24 h urine samples and so it was reasonable to expect their presence in FMV urine samples collected the day after a specific food intervention.

Examination of Biomarker Behaviour During LC-MS and Development of a Biomarker Panel Strategy

Previous analysis of published literature revealed that the great majority of dietary exposure biomarker candidates were detected and quantified using LC-MS technology (53). Chemical classification of putative biomarkers showed great structural diversity that included metabolites from 17 Chemical Classes representative of 7 Chemical Super-Classes (**Figure 4A**). *In silico* multi-dimensional scaling of structural attribute fingerprint distances shows the large diversity in chemical structure across biomarker candidates, highlighting the necessity for employing multiple chromatography systems (**Figure 4B**). Focusing specifically on partition coefficients (log*P*) and molecular weight attributes, it is clear from the scatterplot shown in **Figure 4C** that a large percentage of biomarker candidates were quite strongly hydrophilic. Based on this distribution a decision was made to develop an analytical strategy based largely on the use of a HILIC column to measure strongly polar chemicals and a RP (C_{18}) column to quantify less polar metabolites.

LC-QQQ-MS/MS technology is used widely for measuring, with high sensitivity, the concentration of target chemicals in complex biological samples. Quantification of pre-determined fragmentation products of targeted metabolites in expected retention time windows using MRM approaches allows the investigator to obtain data on large numbers of individual metabolites in short (10–15 min) HPLC runs. Chemical mixtures designed as calibration standards for either HILIC or RP (C_{18}) chromatography were used to optimise metabolite separation and detection conditions on a Thermo QQQ instrument (see **Supporting Data 4**). Serial dilutions of the two standard chemical mixtures (30–0.00197 $\mu g\ ml^{-1}$) were used to establish LoD and LoQ and to examine analytical reproducibility over several months. The reproducibility of measurement of chemical standard mixtures was determined at nine concentration levels.

TABLE 2 | Selection of biomarkers for panel development.

Dietary component	Putative urine biomarker	Potential use as a habitual dietary exposure biomarker?	Key reference in relation to habitual dietary exposure biomarker potential
Alcohol	Ethyl-beta-D-glucuronide	**	(78)
Wine	Resveratrol	*	(59)
Coffee	Chlorogenic acid	*	(79)
Coffee	Dihydrocaffeic acid[#]	*	(80)
Coffee	Ferulic acid-4-O-sulphate	*	(80)
Coffee	Feruloylglycine	**	(1)
Coffee	m-Coumaric acid[#]	*	(46)
Cocoa	3-Methyl-xanthine	**	(81)
Cocoa	7-Methyl-xanthine	**	(81)
Cocoa/Tea	Caffeic acid[#]	*	(80)
Coffee/Cocoa	Caffeine	*	(81)
Cocoa	Vanillic acid	**	(46)
Sweetener	Acesulphame-K	**	(2)
Sugary Foods and Drinks	Sucrose	**	(39)
Fruit and Vegetables	3-Hydroxyhippuric acid	**	(82)
Fruit and Vegetables	4-Hydroxyhippuric acid	**	(82)
Fruit and Vegetables	Hippuric acid	*	(83)
Citrus	4-Hydroxyproline-betaine	**	(30)
Banana	Dopamine-3-O-sulphate[#]	**	(84)
Banana	Dopamine-4-O-sulphate[#]	**	(84)
Strawberries/red berries	Furaneol	**	(2)
Citrus (grapefruit)	Naringenin	*	(85)
Grapes/wine/red berries	p-Coumaric acid	**	(86)
Citrus	Proline betaine	**	(30)
Apple	Rhamnitol	**	(22)
Grapes	Tartarate	**	(87)
Onion and tomato	Quercetin	*	(11)
Onion and tomato	Quercetin-3-O-b-D-glucuronide	**	(88)
Cruciferous Vegetables	D,L-Sulphoraphane L-cysteine	**	(89)
Cruciferous Vegetables	D,L-Sulphoraphane-N-acetyl-L-cysteine	**	(1)
Wholegrain/Rye	BOA (1,3-Benzoxazol-2-one)	*	(35)
Wholegrain	DHBA (3,5-Dihydroxybenzoic acid)	*	(90)
Wholegrain	DHBA-3-O-sulphate	*	(91)
Wholegrain	DHPPA (3-(3,5-Dihydroxyphenyl)-1-propanoic acid)	*	(90)
Wholegrain	DHPPA-3-sulphate	*	(91)
Meat (general)	1-Methyl histidine	*	(92)
Poultry/Fish	3-Methyl histidine	*	(27)
Meat (processed)	Carnitine	*	(93)
Red meat	Carnosine	*	(24)
Meat (general)	Creatinine	*	(25)
Chicken	L-Anserine	**	(1)
Meat (general)	Taurine	*	(25)
Fish/Shellfish	Trimethylamine-N-oxide	**	(1)
Potatoes	Calystegine A_3	**	(48)
Potatoes	Calystegine B_2/B_1	**	(48)
Soy products	Daidzein	**	(94)
Legumes	Pyrogallol	**	(2)
Legumes	Trigonelline	**	(2)

(Continued)

TABLE 2 | Continued

Dietary component	Putative urine biomarker	Potential use as a habitual dietary exposure biomarker?	Key reference in relation to habitual dietary exposure biomarker potential
Strongly Heated Foods	N-(2-Furoyl)glycine	**	(2)
Polyphenol rich foods	Epicatechin(-)	*	(79)
Polyphenol rich foods	Ferulic acid	*	(95)
Polyphenol/Anthocyanin rich foods	Ferulic acid-4-O-b-D-glucuronide	*	(96)
Fruit/Grapes/Tea/wine	Gallic acid	*	(86)
Anthocyanin rich foods	Protocatechuic acid	**	(97)

[#]*Normally the conjugated forms detected; the impact/use of the selected metabolite as a potential biomarker of habitual dietary exposure where *Possible and **Likely.*

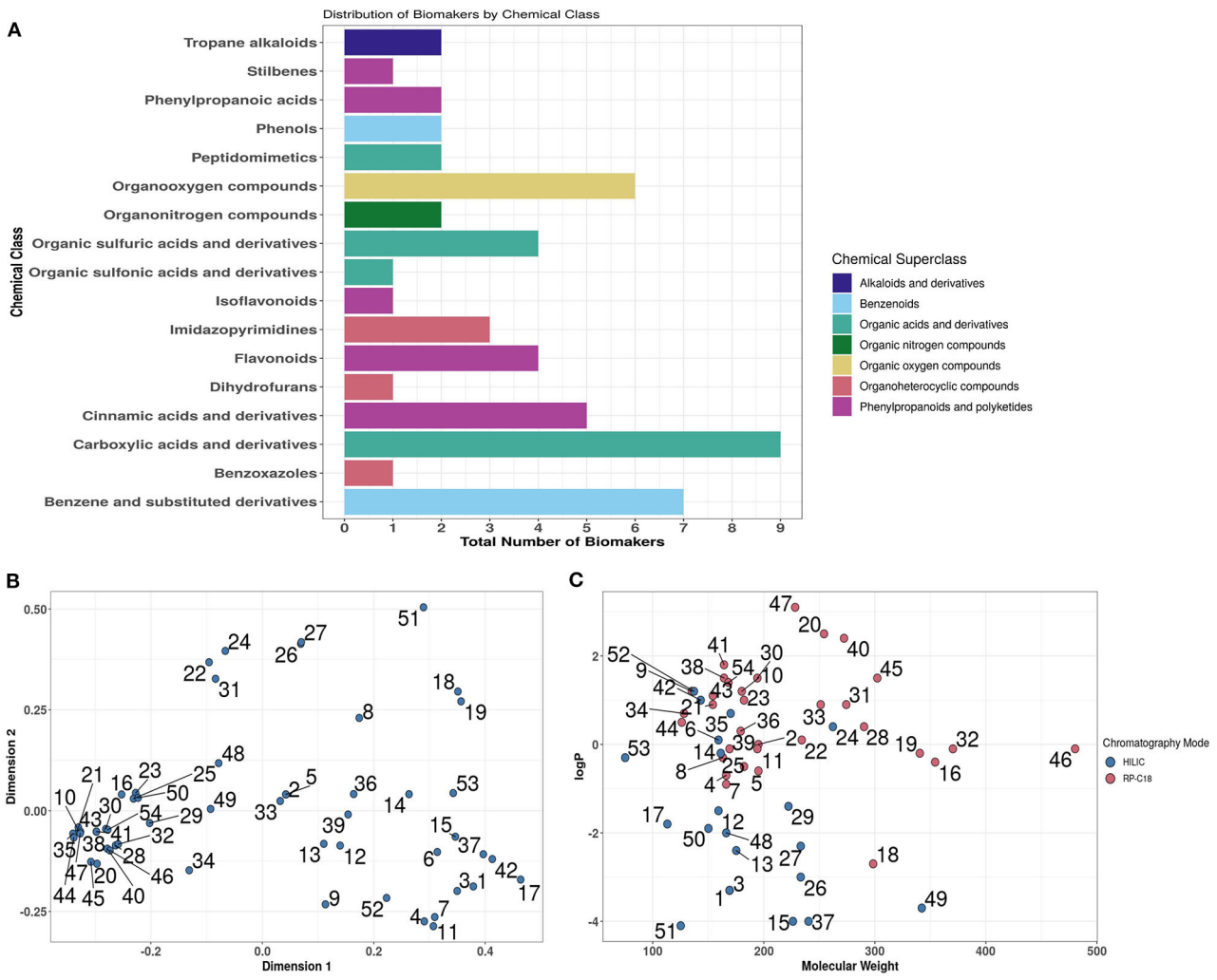

FIGURE 4 | *In silico* overview of the chemical diversity of candidate biomarkers. **(A)** Chemical class and Superclass classifications of 54 biomarkers using ClassyFire. **(B)** Multi-dimensional scaling of Tanimoto Distances between MACCS (Molecular ACCess System) fingerprints of biomarkers. **(C)**. Visualisation of biomarkers in chemical space. Where: log*P*, partition coefficients [Full list of number codes for biomarkers in panels **(B,C)** is in **Supporting Data 4**].

RSD data for biomarkers used to monitor exposure to six example foods/food groups are illustrated in **Figure 5** and in all cases, reproducibility gradually worsened as biomarker concentration dropped. Median concentrations of the same biomarkers were measured in FMV urine taken from 95 free-living participants from ***Study 1***. For each metabolite the median concentration was

substantially greater than the level at which RSD approached 20% (see dotted boxes in **Figure 5**) and usually an order of magnitude greater than the LoQ (see **Supporting Data 4**).

Demonstration of Biomarker Panel Utility to Examine Eating Behaviour in the MAIN Study

The utility of the biomarker panel to characterise eating habits within populations was explored by measuring the concentrations of 54 BFIs in FMV urine samples obtained on days following consumption of three distinctive meal plans (see text box in **Figure 6**) from 15 individuals in the Newcastle MAIN food intervention study (***Study 2***). The data were subjected to PCA which showed distinctive clustering of urine samples by Menu Day (colour coded) in relation to the zero position in PC1 and PC2 (indicated by dotted red grid lines in the scores plot shown in **Figure 6A**). Menu Day 1 and Menu Day 2 samples separated strongly in PC1, whereas Menu Day 3 samples clustered away from samples representative of the other 2 Menu Days in the PC2 dimension. Biomarkers that are strongly explanatory of differences in the composition of urines collected the morning after individual Menus Days are shown in **Figure 6B**. Examination of the 4 sectors delineated by the zero grid lines of the loadings plot revealed a strong association between specific biomarkers and particular foods consumed on each menu day. For example, TMAO (60) was strongly associated with cod fish fingers consumed on Menu Day 2, 3-Methyl histidine (3) was linked to chicken consumption on Menu Day 1 and carnosine (15) was indicative of exposure to a 100% beef burger on Menu Day 3. 1- and 3-Methyl-xanthine (4 and 7) and Epicatechin(–) (28; a marker for general polyphenol-rich foods) detected exposure to cocoa products on Menu Day 1, whilst Acesulphame-K (8) was associated with exposure to a diet soft drink on the same day. Sulphoraphane derivatives (18 and 19; D,L-Sulphoraphane L-cysteine and D,L-Sulphoraphane-N-acetyl-L-cysteine) were highly explanatory of exposure to coleslaw (containing cabbage) on Menu Day 3, whilst trigonelline (53) and N-(2-Furoyl)glycine (39; a strongly heated food marker) reflected exposure to coffee on the same day. Tartrate (51) and the calystegines (12 and 13; A_3 and B_2/B_1) contributed strongly to the clustering of urine samples from Menu Days 2 and 3 away from Menu Day 1 samples when grape products and potato products were not consumed. The example box plots (colour coded by Menu day) in **Figure 6C** demonstrate a clear increase in the concentration in urine of the selected biomarkers the day after the consumption of a specific food.

An important feature of any biomarker strategy designed to monitor habitual diet in both individuals and populations is the ability to add in new biomarkers as they are discovered and validated. RF can be used to assess the stringency of sample classification based on modelling output measures such as accuracy, AUC, and margins (76). **Figure 7** shows a MDS of proximity scores extracted from a RF classification model of the same 15 individuals consuming three unique menus, and panels of 38 dietary biomarkers used in 2018 (**Figure 7A**) and extended to 54 biomarkers in 2020 (**Figure 7B**). In both models, sample clustering by Menu Day is very similar and modelling output measures are still excellent, despite the challenge of measuring many more biomarkers in each MRM experiment in the more complex panel utilising 54 biomarkers.

DISCUSSION

More than a decade of intensive research to discover putative BFIs has yielded a wealth of information highlighting specific metabolites that appear in urine following consumption of individual foods/food groups. A large number of such metabolites have great potential as biomarker leads for particular foods/food groups when validated in isolation (49). However, their deployment in any cost-effective strategy aimed at comprehensive monitoring of habitual diet imposes a substantial number of further challenges that require both definition and investigation (1, 22). Preliminary investigation of the performance of any specific biomarker in the context of a complex biomarker panel requires urine samples from complex food intervention studies designed specifically to emulate habitual eating patterns. The MAIN Study at Newcastle was designed with this specific objective in mind (1, 2, 22). By validating biomarkers in urine samples from studies researching eating behaviour in free-living populations (62, 67) it is anticipated that BFI technology will mature rapidly over the next few years.

It is particularly important to consider carefully urine sampling approaches when deploying BFI technology to help monitor diet and to adopt a methodology that is appropriate for the study objectives (see **Table 1**). Twenty-four hour urine samples, which include the FMV after the study day, provide an ideal type of sample to assess food intake on a single day with the caveat that their collection imposes considerable burden on study participants. To assess habitual diet using BFI data would demand the collection 24 h urine samples on multiple days which can have a substantial influence on the acceptability of the study requirements and compliance by participants, as well as impacting greatly on study logistics and overall costs (1, 62, 67). Consequently, spot urine samples are becoming the urine samples of choice for studying BFIs of a single food/food group when compared with 24 h collections [e.g., (89, 98)] because their collection has little impact on normal daily activities of study participants.

Nutrikinetic studies of potential BFIs have shown that diet-derived metabolites from individual foods reach peak levels in urine at different times post-prandially (49). Thus, choosing an appropriately-timed spot urine sample is clearly problematical when considering the effective deployment of a comprehensive biomarker panel covering the whole of diet. Our recent studies have shown that spot urine samples are generally adequate substitutes for 24 h urine samples for measurement of BFIs (22, 62); particularly post-evening meal (i.e., just before bed time) and FMV urines were collected with a high degree of success (22). In the present paper, we describe a strategy to select urinary biomarkers for inclusion in a comprehensive and extendable panel to monitor habitual dietary exposure that focuses on the

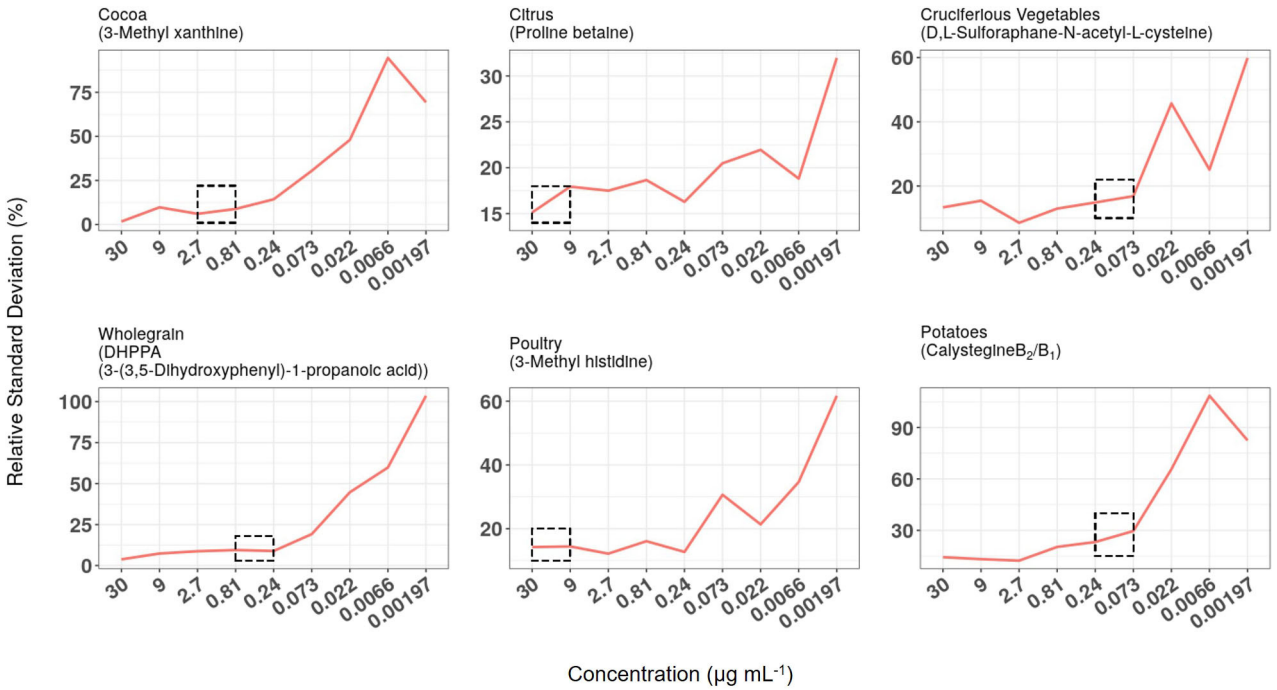

FIGURE 5 | Relationship between relative standard deviation (RSD), biomarker concentration in calibration standard mixtures, and median concentration in First Morning Void (FMV) urines from ***Study 1***. RSD data of biomarkers used to monitor exposure to six example foods/food groups when measured at nine concentration levels over a 6-month period. The concentration range within which the median concentration of the same biomarkers was measured in FMV urine taken from 95 free-living participants are highlighted by dotted boxes.

use of FMV urine samples. From an analytical perspective, sampling FMV urines after a substantial overnight sleep period allows sufficient time to elapse for any gut microbiome and liver P450 bio-transformations of targeted metabolites to be completed, thus extending the availability of characteristic biomarkers and increasing their concentrations in the collected urine. It has been shown previously that the distributions of biomarker concentrations are comparable between post-dinner spot samples, overnight cumulative samples, and 24 h urines (62). We demonstrated recently (53) that more than 50 different potential BFIs were detectable in FMV urine the day after the consumption of targeted foods. In the present study, we show that for many, but not all, BFIs there is a relatively linear correlation between concentration in 24 h and FMV urine and suggest that only those with an R^2 approaching 0.2 may be suitable for accurate quantification when using a comprehensive biomarker panel to monitor habitual diet. Although this is clearly a limitation for accurate quantification of food intake it is very likely that the presence in FMV urine of BFIs with lower correlation coefficients will still provide a useful qualitative indication of recent exposure to their target foods.

Our recent collaborations have highlighted the importance of understanding metabolic biotypes (metabotypes) in populations that may impact on nutritional status (41, 45, 99). As many dietary exposure biomarkers are derived from food chemicals that are metabolised and/or biotransformed before excretion, it is possible that chemical "signatures" reflective of common metabotype groupings in any population can be visualised using a biomarker panel. Differential metabolism of any particular BFI by metabotype sub-groups in any population would provide an additional limitation on its utility for quantitative assessment of dietary intake. The hydration levels of study participants can vary considerably and has to be adjusted for in any BFI deployment strategy. The use of 24 h urine samples for biomarker quantification demands the accurate measurement of the total volume of urine produced during any 24 h period and then the concentration and extraction of a specific aliquot before analysis in order to calculate the overall daily excretion rate.

Logistics, the analytical and computational skills required, and costs will also impact on the wider acceptance and adoption of dietary exposure biomarker technology by the nutrition research community. In the methodology we describe, urine processing is limited to a simple dilution with ultra-pure water as QQQ instruments operating in MRM mode are extremely sensitive and thus there is a need to collect only small volumes of urine for analysis (e.g., 0.5–3 ml). With this objective in mind, we have shown that spot samples can be collected in the home with high collection compliance using vacuum tube technology (67). Importantly, urine samples collected by this method are compositionally stable at room temperature for several days without preservatives (67). This feature of vacuum tube collection methodology allows transport by domestic mail without dry ice offering the opportunity to scale up dietary exposure studies in community settings. A commercial

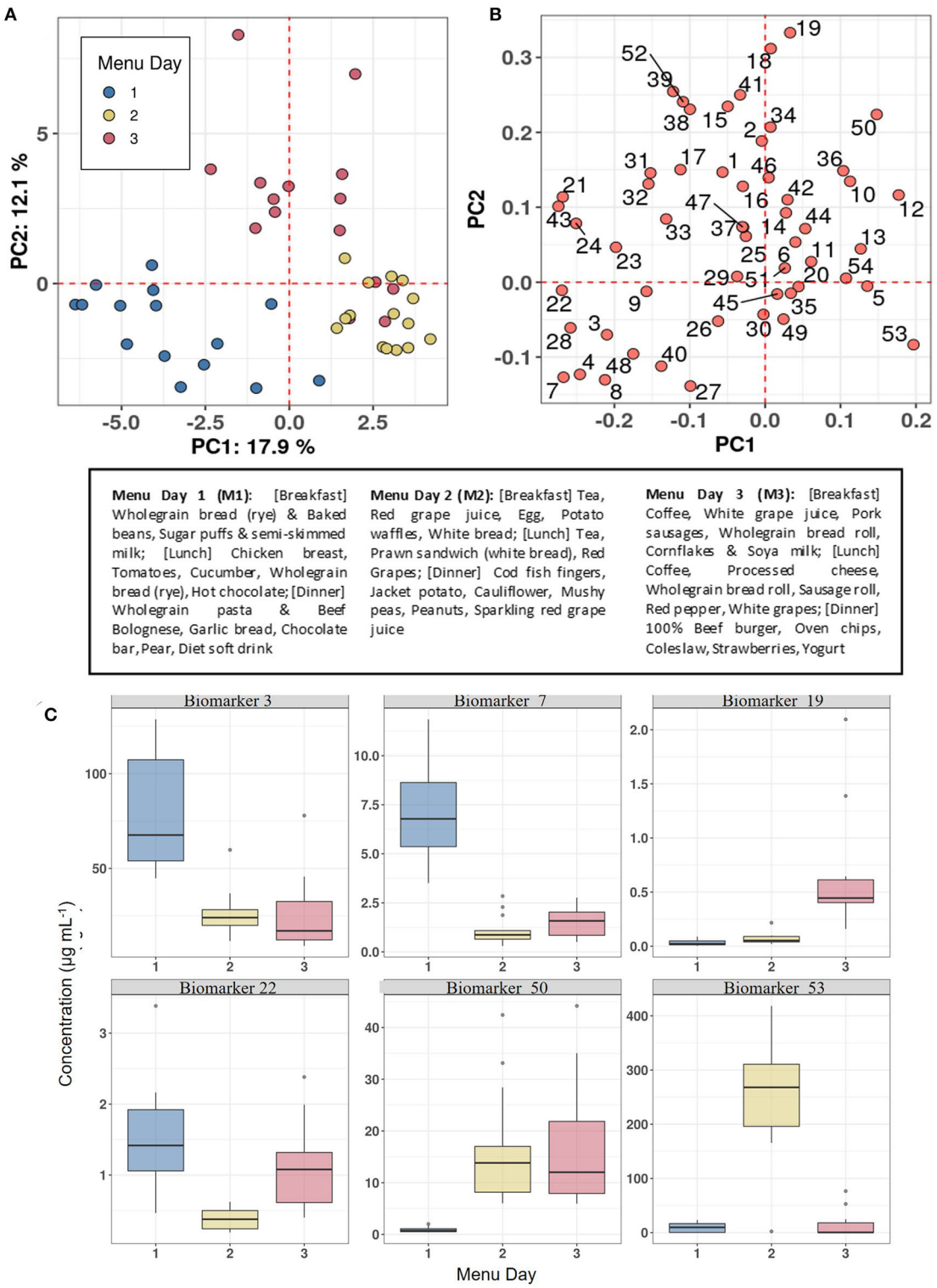

FIGURE 6 | Biomarker panel to characterise eating behaviour on individual Menu Days in ***Study 2***. **(A)** Principal Components Analysis (PCA) scores plot of biomarker panel measurements in First Morning Void (FMV) urine of 15 individuals across three Menu Days in ***Study 2***. **(B)** PCA variable loadings plot showing the variance contributions of biomarkers on each Menu Day (Full list of number codes for biomarkers in panel B is in **Supporting Data 4**). **(C)** Boxplots illustrating the concentration in FMV urine of top ranked biomarkers discriminating Menu Days following Random Forest classification. Text box provide details of meals consumed on each Menu Day. Where: Biomarker 3,3-Methyl histidine; Biomarker 7,7-Methyl xanthine; Biomarker 19, D,L-Sulphoraphane-N-acetyl-L-cysteine; Biomarker 22, DHBA-3-O-sulphate; Biomarker 50, Tartarate; Biomarker 53, Trimethylamine-N-oxide.

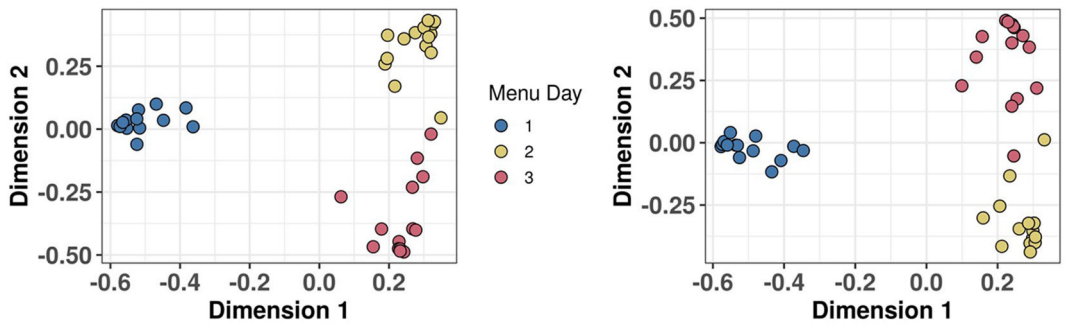

FIGURE 7 | Demonstration that biomarker panel can be extended without any loss of classification power. Multi-dimensional scaling (MDS) of Random Forest proximity extracted from a classification model of 15 individuals consuming three unique menus in ***Study 2***. **(A)** MDS using a panel of 38 biomarkers of food intake (BFIs) which was extended to 54 BFIs and **(B)** 2 years later.

product for spot urine collection (**Supporting data 6**) is now on the market (https://www.co-vertec.co.uk/) and is currently under evaluation in several clinical trials interested in monitoring vulnerable populations in community environments to study malnutrition (https://www.hra.nhs.uk/planning-and-improving-research/application-summaries/research-summaries/stream-feasibility-study/), impact of homelessness on diet (100) and evaluating the eating behaviours in pre-diabetic individuals (https://waru.org.uk/cms/waru_news/targeting-pre-diabetes-through-primary-care/).

Targeted profiling of urine using high resolution hybrid quadrupole/ion trap technology, coupled with RP C_{18} UHPLC, can capture information about the relative concentrations of a substantial number of metabolites in a sample when combined with urine concentration by solid phase extraction methodology [e.g., (101)]. However, sample processing can add significant time and cost to any analytical process and in our experience differential metabolite recovery from ion exchange cartridges can add a significant degree of uncertainty and variance in metabolite measurement. In the present study, we have described a fully quantitative approach using QQQ-MS/MS to measure biomarker abundance. This methodology uses complex mixtures of chemicals standards for quantitation and utilises two HPLC columns solutions to provide optimal resolution of a structurally diverse range of chemicals using short chromatography runs.

As outlined in **Table 1** the utility of any biomarker panel will depend on the study objectives. The biomarker panel described in the present study was optimised specifically to investigate eating behaviour in free-living populations and was targeted towards frequently consumed foods of high public health importance in the UK (2). One limitation of the present study is that only relatively small populations have been used in these initial validation studies and in future BFI technology will need to be tested rigorously in multiple larger populations. We have shown that the biomarker panel can be extended incrementally as new biomarker leads are evaluated and current evidence suggests that it should be straightforward to adapt our strategy to develop biomarker panels that provide comprehensive coverage of foods consumed frequently in other populations. Combined with existing bespoke software for data extraction, it is expected that the development of high throughput, automated biomarker measurement procedures to assess dietary intake is within scope in the near future. In addition, the routine generation of quantitative BFI data will offer further opportunities to develop novel "healthy eating indices" to summarise and "score" eating habits for use in personalised nutrition applications (26, 45). In conclusion, we believe that the integration of information from BFI technology and dietary self-reporting tools, combined with a deeper understanding of nutritional metabolic biotypes in populations, will help to provide more robust understanding of the complex interactions between dietary behaviour and human health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Study 1 involving human participants were reviewed and approved by the National Commission for Data Protection, the Ethical Committee of the Institute of Public Health of the University of Porto and from the Ethical Commissions of each one of the Regional Administrations of Health. All participants gave written informed consent, and the study was carried out in accordance with the Declaration of Helsinki. Study 2 was reviewed and approved by East Midlands—Nottingham 1 National Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MB developed quantification methods, supervised MS support staff, and wrote the manuscript. TW developed quantification methods and wrote the manuscript. AL researched literature

and wrote the manuscript. NW undertook volunteer recruitment in Newcastle, coordinated volunteer CRF visits, and supervised CRF support staff. DT and AG undertook volunteer recruitment in Portugal and coordinated volunteer visits and supervised support staff. LL and HP provided QQQ technical support and data generation. JM coordinated project and supervised research in Newcastle University. JD coordinated project, supervised research in Aberystwyth, designed Figures, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Medical Research Council (MRC), grant number MR/J010308/1.

ACKNOWLEDGMENTS

The authors thank the participants in Newcastle and Portugal for their commitment in the food intervention and dietary survey studies, respectively. The researchers acknowledge all the institutions and persons that integrated the activity of the IAN-AF (2015-2016) Consortium at all phases of the Survey.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.602515/full#supplementary-material

Supporting Data 1 | Putative urinary dietary exposure biomarkers from a literature search between the years of 2010–2020.

Supporting Data 2 | Foods consumed frequently by the UK population reported to have urine biomarkers and foods used in the MAIN study menu design.

Supporting Data 3 | Selection of biomarkers for panel development.

Supporting Data 4 | Technical detail of Triple Quadrupole targeted quantification of dietary intake biomarkers including: ionisation mode; chromatography column; retention time selected reaction monitoring (SRM) parameters of collision energy and product ion mass; quantification ion product; limitation of measurement, and concentrations in FMV urine.

Supporting Data 5 | Quantile-Quantile (QQ) plots of biomarker concentrations ($n = 52$), where **(A)** First Morning Void (FMV); **(B)** 24 h urine collection.

Supporting Data 6 | Urine collection kit characteristics and supplier.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 27 November 2020
doi: 10.3389/fnut.2020.581439

Characterizing Measurement Error in Dietary Sodium in Longitudinal Intervention Studies

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Background: Previous measurement error work that investigates the relationship between a nutritional biomarker and self-reported intake levels has typically been at a single time point, in a single treatment group, or with respect to basic patient demographics. Few studies have examined the measurement error structure in longitudinal randomized trials, and whether the error varies across time or group. This structure is crucial to understand, however, in order to correct for measurement error in self-reported outcomes and properly interpret the longitudinal effects of dietary interventions.

Methods: Using two longitudinal randomized controlled trials with internal longitudinal validation data (urinary biomarkers and self-reported values), we examine the relationship between urinary sodium and self-reported sodium and whether this relationship changes as a function of time and/or treatment condition. We do this by building a mixed effects regression model, allowing for a flexible error variance-covariance structure, and testing all possible interactions between time, treatment condition, and self-reported intake.

Results: Using a backward selection approach, we arrived at the same final model for both validation data sets. We found no evidence that measurement error changes as a function of self-reported sodium. However, we did find evidence that urinary sodium can differ by time or treatment condition even when conditioning on self-reported values.

Conclusion: In longitudinal nutritional intervention trials it is possible that measurement error differs across time and treatment groups. It is important for researchers to consider this possibility and not just assume non-differential measurement error. Future studies should consider data collection strategies to account for the potential dynamic nature of measurement error, such as collecting internal validation data across time and treatment groups when possible.

Keywords: lifestyle intervention, measurement error, nutrition, sodium intake, biomarkers, self-reporting habits

OPEN ACCESS

Edited by:
Megan A. McCrory,
Boston University, United States

Reviewed by:
Janet Tooze,
Wake Forest School of Medicine, United States
Francesco Bonomi,
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *08 July 2020*
Accepted: *05 November 2020*
Published: *27 November 2020*

Citation:
Pittman A, Stuart EA and Siddique J (2020) Characterizing Measurement Error in Dietary Sodium in Longitudinal Intervention Studies. Front. Nutr. 7:581439. doi: 10.3389/fnut.2020.581439

INTRODUCTION

Dietary interventions seek to change dietary behaviors – either to affect some clinical outcome or to change the behavior itself. These studies might use only one time point after baseline to assess participant outcomes, or they may be longitudinal, in which participant outcomes are measured several times over the course of months or years after initial group assignment.

Dietary intervention studies usually require investigators to collect nutrient intake data— such as sodium consumption in study participants—to estimate the effect of the intervention on diet. Yet properly measuring dietary intake, especially over time, with high accuracy can be difficult. Direct nutrient intake is rarely observed, and in dietary studies, researchers frequently resort to two methods to measure nutrient intake: self-report or biomarkers (1).

Self-reported measures generally rely on participants reporting their dietary intake over some period of time, such as the past 24 h or 7 days. This often takes the form of a food frequency questionnaire (FFQ), where participants fill out a survey about their eating habits or a 24-h dietary recall, where participants report everything consumed over the previous day. That is then used to extract information about the nutrients in the food reported as having been consumed. Biomarkers are biologic components from participants, such as blood, urine, or hair, which contain information about a person's nutrient levels. Biomarkers are useful because they objectively measure intake and some provide unbiased estimates of intake. Therefore, biomarkers may be closer to the "truth" than self-reported methods (but still subject to measurement error), and hence provide a better estimate of a person's nutrient intake (2, 3).

Unfortunately, biomarkers are often expensive, invasive, and/or difficult to implement in a study (4). They place potentially greater burden on study participants than self-report measures; this burden may discourage participants from taking part in a longitudinal study. Thus, there are concerns that biomarkers can contribute to poor study adherence and missing data problems (i.e., that participants will drop out of the study because of the hassle or invasiveness of the biomarker collection) (5). For these reasons, in many studies, it is often infeasible to capture biomarker data over time. Self-reported methods are more frequently implemented than biomarker measurements since they are likely easier, cheaper, and more convenient for the participant (4).

Both of these methods (biomarkers and self-report) act as "proxy" measurements of true intake, because they can be representative, but are potentially imprecise versions of the truth. They are potentially subject to two main types of error: systematic and random. Systematic error, or bias, means that a measure consistently departs from the truth in the same direction (i.e., always higher or lower), and can be hard to detect and analyze statistically (6). Systematic errors can decrease the accuracy of measurements and create potentially erroneous conclusions about the relationship between food intake or nutrients and nutrition-related diseases (7). Random error can create variability in the measurements, which may reduce precision, resulting in a loss of statistical power. However, random errors can be more easily corrected with statistical methodology (8). These errors together help create measurement error, the difference between "true" and "observed" intake.

If researchers are concerned with measurement error, they may have a slight preference for biomarker collection methods because the objective nature of biomarkers leads to less systematic error, but they are still subject to potential random errors such as daily variation in diet (3, 8–10). Self-reported measures can be more susceptible to systematic measurement errors due to the many complexities of properly reporting food intake (8, 11, 12). Even with the best due diligence, factors such as social desirability or recall problems influence final results. Examples include constant over or under-reporting (systematic error) or daily fluctuations in food consumption (random error).

Given these measurement challenges in nutrition (and many other fields), researchers have developed statistical methods such as *regression calibration* (13) and *Simulation Extrapolation* (SIMEX) (14) to deal with measurement error in settings where the variable measured with error is a covariate in an outcome model. To implement these methods, it is necessary to have information on the relationship between the variable measured with error and its true value.

The existing measurement error literature in dietary studies, and their respective correction methods, typically examine measurement error at one specific time point and/or in a single observational cohort. However, these measurement error patterns may not remain constant in longitudinal lifestyle interventions.

In addition, in randomized controlled trials (RCTs), where individuals are randomly assigned to treatment conditions and the intervention and comparison groups have different experiences, self-reporting behaviors could change over time and/or by treatment assignment. Those in the treatment group may become more cognizant of nutrition intake through intervention exposure, leading to increased reporting accuracy. Participants may also modify their self-reported values (even if not necessarily their true intake) to appear compliant with intervention recommendations, which decreases their accuracy (12).

Self-reported precision could also wane over time as participants experience fatigue with repeated reporting (15). This fatigue could lead them to be more carefree and less rigorous, introducing uncertainty into measurements. Conversely, as people repeatedly monitor sodium intake over time, they may become more accurate with increased repetitions. Thus, the structure of the measurement error could be *differential*, meaning the amount of error may differ across treatment groups and could change over time differently for each treatment group. However, to this point there has been little empirical investigation of these patterns.

As a case study, we examined sodium intake in two longitudinal intervention trials, Trials of Hypertension Prevention (TOHP) (16) and PREMIER: Lifestyle Interventions for Blood Pressure Control (17). These data sets are particularly useful for examining measurement error over time because they, unlike most dietary intervention trials, contain both self-reported sodium intake via 24-h recall and a sodium biomarker—24-h urine—for each participant at every time point. With this information, we compare the participants' self-reported values with their directly measured urinary sodium to characterize the measurement error, and assess whether the error varies across treatment group and time. Our analyses could be helpful to learn about potential measurement error in other settings, and to help researchers understand when it is important to consider differential measurement error by time or treatment condition.

MATERIALS AND METHODS

Trials of Hypertension Prevention

TOHP was a U.S. based, multicenter, randomized trial of 2,182 participants testing the efficacy of a lifestyle intervention aimed at lowering diastolic blood pressure (DBP) from the high normal range (80–89 mmHg) (16) to a lower range. Participants were assigned to one of four treatment groups: sodium reduction, weight reduction, stress management, or control. The sodium reduction group received counseling on how to reduce sodium consumption in everyday life. The weight reduction group received guidance on weight-loss techniques. The stress management group were provided coping mechanisms to handle stressful situations. The weight loss and stress management groups did not receive any counseling specifically on sodium intake. The control group did not receive any particular intervention or information; in this sense it was similar to a "usual care" condition.

Participants were considered eligible if they were healthy men and women, aged 30 through 54 years, who had high normal DBP and were not taking antihypertensive drugs for the prior 2 months (16). All participants were screened three times prior to enrollment to check eligibility requirements and then randomized to one of the four treatment groups. On the third screening, a 24-h dietary recall was conducted, and participants provided a 24-h urine sample; this served as their "baseline" measurement. All participants were contacted again—at an unannounced point in time— ∼6 and 18 months after enrollment to again provide 24-h dietary recall and 24-h urine biomarker for sodium consumption at each respective time point (**Table 1**). The 24-h recall data on individual foods was converted into nutrients using the Tufts Nutrient Data Bank based on the US Department of Agriculture Standard Reference (Release 9) in combination with extensive manufacturers' data and published nutrient data on currently consumed food products (16, 18, 19).

PREMIER: Lifestyle Interventions for Blood Pressure Control

PREMIER was also a U.S. based, multicenter randomized trial testing the effects of various lifestyle intervention on blood pressure outcomes in 810 adults with above optimal DBP (80–95 mmHg) and who were not taking antihypertensive medications (17).

Participants were randomly assigned to one of three treatment groups: Established, Established Plus Dash, or Advice Only. The Established group received guidance on improving their dietary habits (including reducing sodium consumption) and increasing physical activity. Established Plus Dash received an intervention similar to Established but also received education on the DASH diet, a diet high in fruits, vegetables and low-fat dairy products. Finally, Advice Only received general healthy behavior advice, but no specific counseling on sodium intake or physical activity.

All eligible participants attended a randomization visit, where researchers randomized them to a group and then collected baseline measurements including two 24-h dietary recalls, and a 24-h urine sample. Trial researchers contacted all participants unannounced at 6 and 18 months after enrollment, at which point individuals again provided two 24-h dietary recalls and 24-h urine samples (**Table 1**).

TABLE 1 | Study characteristics and participant demographics in TOPH and PREMIER studies.

	TOHP†	PREMIER
N	751	818
Enrollment dates	1988–1990	1999–2001
Timing of sodium assessment	Baseline	Baseline
	6 months	6 months
	18 months	18 months
Assessment method	24-h recall	Two 24-h recalls
	24-h urine	24-h urine
Treatment categories (*N* in group)	Sodium reduction* (329) Control‘ (422)	Established* (271) Established plus DASH* (272) Advice only‘ (275)
Male N (%)	534 (71)	310 (38)
Mean baseline BMI (SD)	27.3 (3.6)	33.2 (5.7)
Mean baseline age (SD)	43 (6.4)	50 (8.9)

† Some cohorts in the original TOHP study were outside the scope of our analysis and therefore were excluded from the final models. N = 751 reflects a subset of TOHP we used for this study, and subsequent BMI and age calculations are derived from the subsetted population.

**Categorized as "treatment" for our purposes.*

‘Categorized as "control" for our purposes.

Intake of nutrients and food groups was assessed from unannounced 24-h dietary recalls conducted by telephone interviewers. Two recalls (one obtained on a weekday and the other on a weekend day) were obtained at baseline, 6-, and 18-months by the Diet Assessment Center of Pennsylvania State University. The Nutrition Data System (NDS) developed and maintained by the Nutrition Coding Center of the University of Minnesota was used to generate the estimates of individual nutrient intake from the recalls (17).

We obtained the datasets for TOHP and PREMIER through an online request from the National Heart, Lung, and Blood Institute BioLINCC data repository after receiving IRB approval through Johns Hopkins Bloomberg School of Public Health and Northwestern University.

For both datasets we consolidated the original treatment and control groups into new ones for our purposes. In TOHP, only the sodium reduction group received counseling on sodium management. Hence, we discarded the stress management and weight reduction groups and only use the original control group in the control arm. For the PREMIER study we considered both behavioral intervention groups (Established, Established plus DASH) as the "treatment" condition, and used the advice only condition as the control condition. We are interested in whether participants in the sodium reduction interventions, more (or less) accurately report their actual sodium intake compared to those in the advice only group, and whether the pattern of measurement error varies over time.

The same data cleaning procedures were used for both studies prior to analysis. First, the biomarker sodium values were converted to dietary sodium values by dividing urine sodium values by 0.86, as only 86% of sodium intake appears in urine (1, 20). The dietary sodium and self-reported sodium values were both natural log-transformed to make the respective distributions approximately normal. In PREMIER, the two self-reported sodium values at each time point were averaged after log transformation. We centered log self-report (log self-report – mean log self-report at baseline) to help with the interpretability of regression coefficients.

Our model of interest is a calibration model in which a reference measure (urinary sodium) is regressed on its self-reported version (21). This relationship is used for missing data approaches (22) for handling measurement error where the variable measured without error is treated as missing data and imputation is used to fill in the unobserved data (23–26).

We began by plotting the data in order to visualize the relationship between urinary sodium and self-reported sodium and help inform our modeling efforts. We used scatterplots of urinary sodium against self-reported sodium, grouped by time, with an overlapping linear predicted regression line for each condition at each time point.

Mixed effects linear regression was used (27) to estimate the relationship between log measured urinary sodium and log self-reported sodium over time, and by treatment group, while taking into account the correlation of measures within a participant over time. To estimate these models, we used the lme4 and lmerTest packages in R version 3.5.1 (28–30).

For each trial, we started with an initial model that included main effects for follow-up time (indicators for 6- and 18-months), subjects' self-reported intake, as well as two-way interactions between self-reported intake and time, time and treatment assignment, and a three-way interaction between self-reported intake, time, and treatment. We allow each individual to have a random intercept, and the (log centered) self-reported values to have a random slope, and used an unstructured covariance matrix to model the random effects.

For each person i ($i = 1,\ldots, N$), at time j (j = baseline, 6 months, 18 months; coded categorically), in our defined treatment group (TX; 0 = control, 1 = treatment) their urine measured sodium intake is represented by U_{ij} and self-reported intake is represented by $self_{ij}$. Our model can be written as:

$$\begin{aligned} U_{ij} = {} & \beta_0 + \beta_1 * self_{ij} + \beta_2 I(time_j = 6) + \beta_3 I\left(time_j = 18\right) \\ & + \beta_4 I\left(time_j = 6\right) * TX_i + \beta_5 I\left(time_j = 18\right) \\ & * TX_i + \beta_6 * self_{ij} I\left(time_j = 6\right) + \beta_7 * self_{ij} I\left(time_j = 18\right) \\ & + \beta_8 * self_{ij} I\left(time_j = 6\right) * TX_i + \beta_9 * self_{ij} I\left(time_j = 18\right) \\ & * TX_i + b_{0i} + b_{1i} * self_{ij} + e_{ij} \end{aligned} \quad (1)$$

In Equation (1) $I()$ is an indicator function which takes on either 0 or 1. b_{0i} is the random intercept and b_{1i} is the random slope for each person's centered self-reported values, respectively. We assume correlated random effects where $b_{0i} \sim N(0, \tau_0^2)$, $b_{1i} \sim N(0, \tau_1^2)$, and residual error terms $e_{ij} \sim N(0, \sigma^2)$, independent of the random effects

We excluded a main effect for treatment (TX) from the model because the coefficient was ∼0. This is expected because we assume treatment and control groups have similar sodium levels at baseline, at least in expectation (because of randomization) and thus reduces an extra parameter.

Including the three-way (self-reported intake by time by treatment) interactions in this initial model allows the relationship between urinary sodium and self-reported sodium to vary over time and across the treatment and control groups. We include a time by treatment interaction to examine whether average levels of urinary sodium differ by time and treatment condition at a fixed level of self-report.

A backwards variable selection approach was used to obtain a final analysis model. First, the initial saturated model with the three-way interaction shown in Equation (1) was fit. We used a significance level of 0.2 to decide whether a variable should remain in the model. We first tested the two three-way interactions self-report*time*treatment. If at least one coefficient had a p-value < 0.2, we kept both interaction terms in the model (i.e., for both time points). If both coefficients had p-value > 0.2, we dropped them from the model and refit our second-stage model which omits the 3-way interaction.

In our second-stage model, we tested the significance of the self-report*time terms (β_6, β_7), which measure whether the relationship between urinary sodium and self-reported sodium changes over time, assuming any change is constant across the treatment and control groups. Once again, if both coefficients had p-values > 0.2, we dropped them from the model and fitted our final model.

Our final model allows urinary sodium levels to change across time and treatment status. In this model we test the time*treatment interactions (β_4, β_5). If both coefficients had p-values > 0.2, we dropped them from the model.

After selecting our final model we then standardized the regression coefficients. To standardize the exposure—self-reported intake—we subtracted the pooled (control and treatment) mean self-reported intake at baseline from all self-reported values and then divided that result by the standard deviation of self-reported intake at baseline. The outcome—urinary sodium—was similarly standardized, using the pooled mean and standard deviation of urinary sodium at baseline.

RESULTS

Both datasets include people who over and under report by time and treatment status (**Figures 1**, **2**). The 45-degree line in each graph represents "perfect" reporting, where measured urine biomarker equals self-reported sodium. Those who fall above the line under report, meaning their measured urine sodium levels were higher than self-reported intake. Conversely, those below the line over report, meaning their measured urine sodium levels were lower than their self-reported amounts. The wide scattering of points suggests a high degree of variability in reported sodium levels.

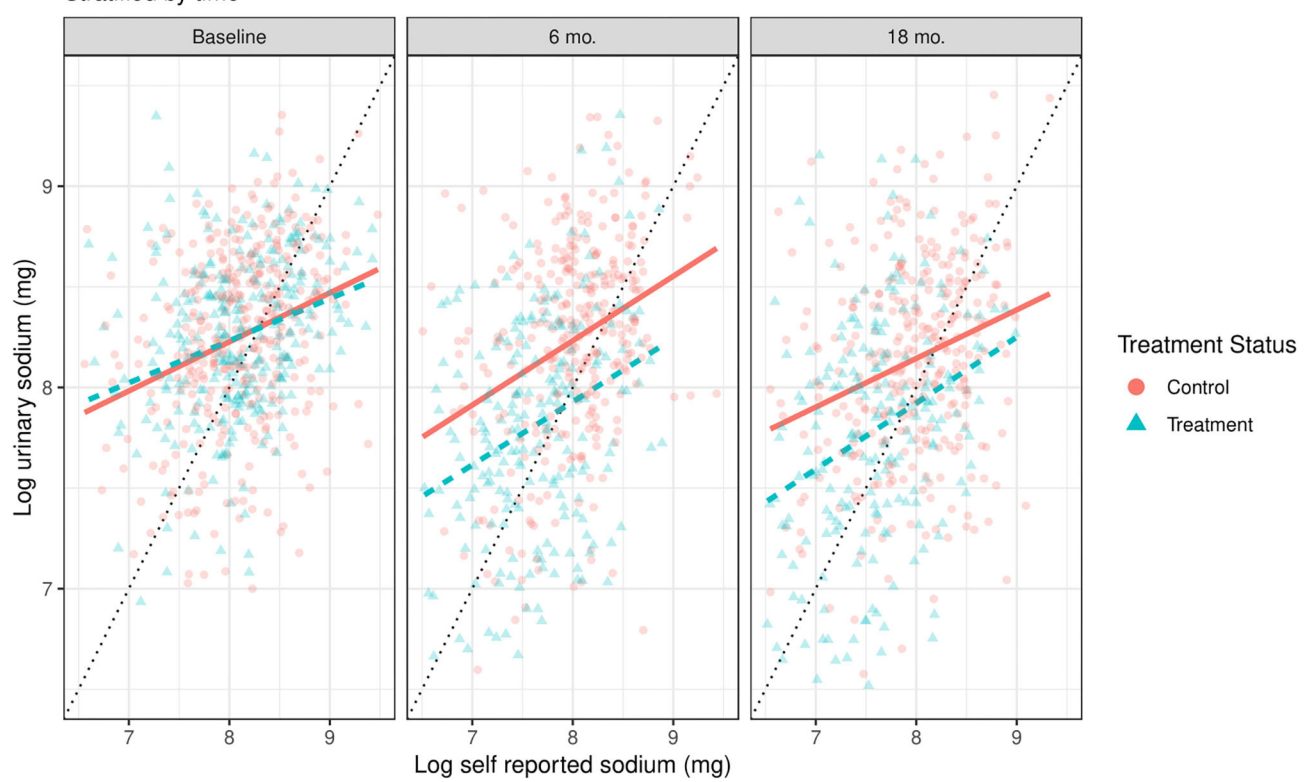

FIGURE 1 | Scatterplots of log urinary sodium vs. log self-reported sodium by time and treatment conditions in TOHP. The solid orange (control) and dashed blue lines (treatment) are linear smoothers of urinary sodium as a function of self-reported sodium in each treatment condition. 45-degree line represents where urinary sodium equals self-reported sodium. Units are on the natural log scale.

We overlapped a linear smoother on top of the scatterplot to highlight some reporting differences between the treatment and control conditions. These lines should be considered as preliminary models, as they fit the models separately by time and group, and thus do not allow formal model comparisons across time or group, but the relationships between self-reported and biomarker values appear broadly similar. In both studies at baseline, the two study conditions are approximately equal in urinary vs. dietary sodium levels, as expected from the randomization.

Regression Results

Using the stepwise procedure described above, neither the three-way interactions in model (**1**), nor the interactions between self-reported sodium levels and time in the second-stage model met the criteria for inclusion in either study. As such, the final model for both studies only includes the interaction between treatment and time. This final model is shown in Equation (2).

$$\begin{aligned} U_{ij} \;=\; & \beta_0 + \beta_1 * self_{ij} + \beta_2 I(time_j = 6) + \beta_3 I\left(time_j = 18\right) \\ & + \beta_4 I\left(time_j = 6\right) * TX_i + \; \beta_5 I\left(time_j = 18\right) * TX_i \\ & + b_{0i} + \; b_{1i} * self_{ij} \; + e_{ij} \end{aligned} \tag{2}$$

This model implies that average measured urinary sodium changes over time (β_2, β_3), and at different rates in the treatment group vs. control group (β_4, β_5) but that there is no differential change in the slope of self-reported sodium across groups over time. It is interesting to note that the final regression results in both datasets were very similar to one another.

In TOHP (**Table 2**), there was a small but significant decrease in urinary sodium between baseline and 18 months in the control group. The control group at 18 months has 0.19 SD lower urinary sodium than the control group at baseline on the log scale ($\beta_3 = -0.19$). There was, on average, a much larger significant decrease in measured urine sodium between baseline and each follow up time for the treatment group, for a given level of self-reported sodium. At 6 months, the treatment group has 0.81 SD lower urinary sodium than control group ($\beta_4 = -0.81$), and 0.65 SD lower at 18 months on the log scale ($\beta_4 = -0.81$).

In PREMIER (**Table 2**), there was a significant decrease in average measured urine sodium at 6 months compared to baseline. Both groups at 6 months had 0.24 SD lower urinary sodium at baseline on the log scale ($\beta_2 = -0.24$). However, this difference was no longer there at 18 months. There were no significant difference between treatment and control groups at any point in PREMIER.

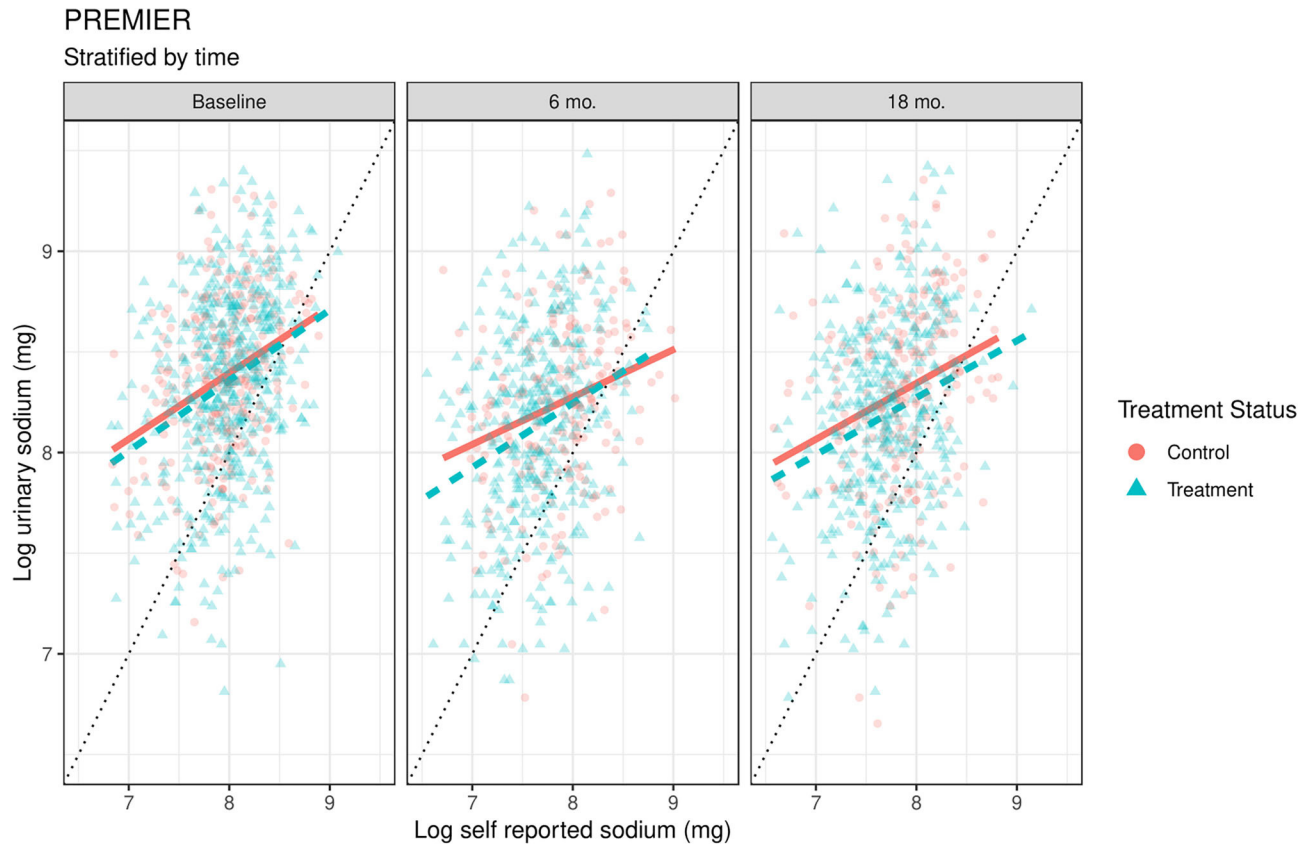

FIGURE 2 | Scatterplots of log urinary sodium vs. log self-reported sodium data time and treatment conditions in PREMIER. The solid orange (control) and dashed blue lines (treatment) are linear smoothers of urinary sodium as a function of self-reported sodium in each treatment condition. 45-degree line represents where urinary sodium equals self-reported sodium. Units are on the natural log scale.

TABLE 2 | Standardized regression output from the final regression model.

Parameter from model (2)	TOHP		PREMIER	
	Estimate (95%CI)	*p*-value	Estimate (95%CI)	*p*-value
Centered self-report β_1	0.29 (0.23, 0.34)	<0.001	0.21 (0.17, 0.26)	<0.001
Month 6 control β_2	0.03 (−0.10, 0.16)	0.68	−0.24 (−0.37, −0.10)	<0.001
Month 18 control β_3	−0.19 (−0.32, −0.06)	0.005	−0.08 (−0.21, 0.05)	0.23
Month 6 Trt. β_4	−0.81 (−1.0, −0.63)	<0.001	-0.1 (−0.26, 0.06)	0.20
Month 18 Trt. β_5	−0.65 (−0.84, −0.47)	<0.001	−0.15 (−0.30, 0.0)	0.06

β_1: Change in average log urinary sodium (in SD units) due to a 1 SD unit change in log self-reported sodium at baseline for treatment and control (assumed to be same across groups at baseline because of randomization).
β_2: Among the control group members, difference in average urinary sodium (in SD units) between baseline and 6 months on the log scale.
β_3: Among the control group members, difference in average urinary sodium (in SD units) between baseline and 18 months on the log scale.
β_4: Difference in average urinary sodium (in SD units) between treatment and control groups at 6 months on the log scale.
β_5: Difference in average urinary sodium (in SD units) between treatment and control groups at 18 months on the log scale.

If the relationship between urinary sodium and self-reported sodium did not change over time and by treatment condition, we would expect β_2, β_3, β_4, $\beta_5 = 0$. Instead, we find that β_2, β_3, β_4, $\beta_5 < 0$, an indication that the relationship between urinary sodium and self-reported sodium does in fact change over time and by treatment status. In general, for a given level of self-report, urinary sodium is *lower* at follow-up than it is at baseline.

DISCUSSION

We expand on the current nutrition literature by focusing on the differential measurement error structure of self-reported intake which may arise when the treatment group self-reports their sodium intake with increased or decreased accuracy (31). We do this by modeling the relationship of urinary sodium as a function of self-reported sodium, time, treatment condition and

all possible interactions. This information is important when designing studies where self-reported intake is a longitudinal outcome variable, and can help inform measurement error correction methods that use missing data approaches to correct for measurement error.

The final models for TOHP and PREMIER look very similar to one another, with slightly different coefficient values. The slopes of self-reported sodium did not change as a function of time or by treatment condition. The lack of significance in the three-way self-report*time*treatment interaction and the two-way self-report*time interaction indicates a lack of significant difference in systematic error in terms of the relationship between self-reported sodium and urinary sodium between the treatment arms across all three time points. However, the intercepts do change by time and/or treatment condition indicating that measurement error is affected by time and/or treatment condition. Further, our final models were much more parsimonious than our initial, fully saturated model. This result suggests that relatively simple measurement error correction models that involve only shifts in the intercept of the calibration model are sufficient to appropriately correct for measurement error.

In PREMIER, we see a decrease in measured urine sodium—conditioning on self-report—at 6 months in the control group, whereas in TOHP we see a much stronger decrease in the treatment group at 6 and 18 months. These results suggest that the relationship between biomarker and self-report can differ by treatment group and/or time, however, these differences may be study specific.

A failure to take into account differential measurement error could result in biased estimates of the treatment effect. For example, in TOHP at 6-months, for a given level of self-reported sodium, participants in the treatment condition had lower urinary sodium than did control participants. A measurement error correction model that did not take this difference into account would result in an attenuated treatment effect because this difference in reporting would not be incorporated into the difference between groups.

Discrepancies in the literature still exist about the relationship between treatment and self-reporting error. Other studies have found evidence for a relationship between treatment assignment and self-report bias, similar to the results of TOHP. In the Women's Health Eating and Living Study, a longitudinal randomized intervention trial with validation data (32), researchers found dietary intervention affected measurement error in self-reported outcomes using plasma carotenoid biomarkers. In the Women's Health Initiative Dietary Modification Trial, another dietary intervention trial (33), participants in the control group under-reported protein intake at greater amounts compared to the treatment arm. There is thus evidence that there may be differential measurement error across time and treatment group, and that this may vary depending on the dietary component being measured.

One possible solution to examine and address measurement error across time and treatment groups would be internal validation datasets with longitudinal intervention aspects. While this route is resource intensive, it may be worthwhile if it allows researchers to estimate treatment effects with less bias and greater power to detect significant effects. A cheaper or less invasive biomarker would make creating this dataset more feasible. Another option would be more measurement error correction methods, which is why it is important to study how measurement error structures change over time and by treatment status. Siddique et al. (25) performed sensitivity analyses to the assumption that measurement error structure is time invariant, treatment invariant, and time and treatment invariant. Understanding how measurement error changes over time and by treatment condition in validation datasets can help encourage the implementation of these methods and improve the accuracy of self-reported measures in longitudinal intervention trials without available biomarker data.

Limitations

One limitation of this study is the amount of missing data, with the highest being 29% at 18 months in TOHP and the lowest being 1% at baseline in both studies. The regression models were fit assuming that the missing data was "missing at random" (MAR). This means we assume participants with unobserved dietary sodium information at a given time point will have similar intake values as the observed participants at the same time after conditioning on other observed values (34). This assumption may not hold in all circumstances however, and if violated could imply differences between the observed and unobserved groups. Future work could examine how the patterns of missingness may interact with measurement error structures.

In both studies, the 24-h recalls and the 24-urine samples were not required to capture the same day of measurement. We assume that these two measures are capturing estimates of short-term intake. Even so, the limited number of measurements at each time point is likely not adequate to capture usual intake. Estimates from both the biomarker and self-reported data are therefore subject to additional variability due to day-to-day variation in diet (1).

The biomarker sodium levels—measured through urine—are also subject to additional sources of variability. Urinary sodium excretion may reflect more than 1 day of intake (35). Further, we divided urinary sodium values by 0.86 under the assumption that 86% of consumed sodium is available in urine (1, 20), this value is likely to differ by participant, introducing additional uncertainty in our estimates (36). These sources of variability in 24-h recalls and urinary sodium would have the result of attenuating the relationship between self-reported sodium and urinary sodium in our models.

Conclusion

We found that the measurement error structure in longitudinal studies can differ by time and treatment condition. When correcting for measurement error, intervention researchers need to take these differences into account, either by designing internal validation studies that are also longitudinal or by implementing measurement error correction methods that are explicitly designed to account for these changes in measurement error. Lifestyle intervention trials that fail to do this may draw erroneous conclusions of their results.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://biolincc.nhlbi.nih.gov/studies/premier/, https://biolincc.nhlbi.nih.gov/studies/tohp/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Johns Hopkins Bloomberg School of Public Health IRB Northwestern University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS originally conceived the idea for studying measurement error in longitudinal studies, and created the original model. AP performed the analyses and drafted the manuscript. ES provided assistance on analyses. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported through grant R01MH099010 (PI: Stuart) and R01HL127491 (PI: Siddique).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

SYSTEMATIC REVIEW
published: 01 December 2020
doi: 10.3389/fnut.2020.558598

Genetic Variants Shaping Inter-individual Differences in Response to Dietary Intakes—A Narrative Review of the Case of Vitamins

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: 03 May 2020
Accepted: 03 November 2020
Published: 01 December 2020

Citation:
Niforou A, Konstantinidou V and Naska A (2020) Genetic Variants Shaping Inter-individual Differences in Response to Dietary Intakes—A Narrative Review of the Case of Vitamins. Front. Nutr. 7:558598. doi: 10.3389/fnut.2020.558598

Recent advances in the field of nutrigenetics have provided evidence on how genetic variations can impact the individuals' response to dietary intakes. An objective and reliable assessment of dietary exposures should rely on combinations of methodologies including frequency questionnaires, short-term recalls or records, together with biological samples to evaluate markers of intake or status and to identify genetic susceptibilities. In an attempt to present current knowledge on how genetic fingerprints contribute to an individual's nutritional status, we present a review of current literature describing associations between genetic variants and levels of well-established biomarkers of vitamin status in free-living and generally healthy individuals. Based on the outcomes of candidate gene, genome-wide-association studies and meta-analyses thereof, we have identified several single nucleotide polymorphisms (SNPs) involved in the vitamins' metabolic pathways. Polymorphisms in genes encoding proteins involved in vitamin metabolism and transport are reported to have an impact on vitamin D status; while genetic variants of vitamin D receptor were most frequently associated with health outcomes. Genetic variations that can influence vitamin E status include SNPs involved in its uptake and transport, such as in SCAR-B1 gene, and in lipoprotein metabolism. Variants of the genes encoding the sodium-dependent vitamin C transport proteins are greatly associated with the body's status on vitamin C. Regarding the vitamins of the B-complex, special reference is made to the widely studied variant in the MTHFR gene. Methodological attributes of genetic studies that may limit the comparability and interpretability of the findings are also discussed. Our understanding of how genes affect our responses to nutritional triggers will enhance our capacity to evaluate dietary exposure and design personalized nutrition programs to sustain health and prevent disease.

Keywords: genes, diet, nutrigenetics, nutritional status, SNPs, genetic variants, vitamins

INTRODUCTION

The assessment of the population's nutritional status lies in the heart of health monitoring surveys and epidemiological studies aiming to elucidate diet-disease associations. Methods employed have traditionally relied on questionnaires, food diaries as well as markers assessed in biological samples (1). The thus accumulated evidence has enriched our understanding of the complex interplay among nutrients and other food substances; patterns of dietary intake; and, lifestyle and environmental exposures that shape an individual's nutritional status. Furthermore, advances in the field of nutrigenetics illustrate how genetic variations can impact on an individual's response to a nutritional trigger (i.e., a dietary intake) and, possibly, on the risk of nutrition-related diseases (2). It has now been generally recognized that objective dietary assessment should not solely rely on one method, but should employ combinations of methodologies including participants' self-reporting, as well as biological samples to assess established biomarkers and to identify routes of genetic susceptibilities.

In an attempt to present current knowledge on how genetic fingerprints participate in shaping the body's vitamin status, we have conducted a narrative review of studies evaluating associations between genetic variants and levels of well-established biomarkers of status of vitamin D, tocopherols and tocotrienols (vitamin E), vitamin C and vitamins of the B-complex, namely folic acid (B9) and cobalamin (B12), for which there is adequate evidence relating genetic variants and body status in free-living and generally healthy individuals.

METHODS

Studies Identification and Selection

We performed an online literature search in PubMed until March 2020. The search terms used included one term related to the vitamins under study (i.e., vitamin D; tocopherol, vitamin E; ascorbic acid, vitamin C; folic acid, folate, vitamin B9; and cobalamin, vitamin B12) followed by the term "status" and combined with either "nutrigenetics" or "polymorphisms." A study was considered if: (a) it was conducted among free-living healthy individuals and (b) was available in English. Original research papers, systematic reviews, and meta-analysis were all combined. In addition, reference lists of identified publications were further screened to identify additional literature, with the application of citation chasing techniques including reference list scanning of included studies and previous reviews, as well as backward and forward references of included studies. No limits on geographical location were applied.

Based on the search criteria and keywords used, 848 articles were identified. Among these, 817 articles were excluded after either title/abstract or full text screening since they did not address the aim of the present review, i.e., to collectively report on evidence regarding genetic variants that could impair the body's vitamin status. Finally, 31 articles were initially considered and through reference screening, we identified 30 additional publications. Overall, 60 articles have been considered in this review (**Table 1**).

VITAMIN D

Vitamin D is a fat-soluble vitamin that regulates bone growth and health through promotion of calcium absorption and maintenance of adequate calcium and phosphate concentrations. Vitamin D is further involved in the cell growth process and neuromuscular and immune activities (63, 64).

Vitamin D exists in two main forms: ergocalciferol (D2) and cholecalciferol (D3). Vitamin D3 is naturally present in animal foods (e.g., fatty fish, fish liver oils, dairy, egg yolk) and vitamin D2 is naturally present in some higher fungi. Vitamin D3 can also be synthesized endogenously in the skin from its provitamin 7-dehydrocholesterol, following exposure to UV-B radiation and subsequent thermal isomerization. The cutaneous synthesis in the human body is the main source of vitamin D3 and varies depending on the individual's exposure to sun, age, skin color, time spent outdoors, use of sunscreen, as well as the season of the year (64).

Vitamin D Metabolism

In the body, dietary or endogenous vitamin D (D2 and D3) is either converted into its biologically active metabolite, following two hydroxylations, or transferred to the storage tissues. Activation of vitamin D involves two steps. The first occurs in the liver, where vitamin D is hydroxylated to 25-hydroxyvitamin D, 25(OH)D, also known as calcidiol. The 25(OH)D attaches to the vitamin D binding protein (DBP) and is transported to the kidneys where it is hydroxylated to form the 1,25-dihydroxyvitamin D, $1,25(OH)_2D$, also known as calcitriol. A mitochondrial enzyme (CYP27A1) and several microsomal enzymes (including CYP2R1) play an important role in the process of the 25-hydroxylation of vitamin D in the liver. Vitamin D, in the form of either $1,25(OH)_2D$ or 25(OH)D, is transported in the blood primarily bound to the DBP. Upon its release, the biologically active form $1,25(OH)_2D$ reaches the main target tissues including the intestine, the kidneys, and the bone, where it binds to the intracellular vitamin D receptor (VDR). After hydroxylation of vitamin D in the liver, serum 25(OH)D is also delivered to the adipose tissue, muscle, and liver for long-term storage (65). Metabolites of both 25(OH)D and $1,25(OH)_2D$ are degraded in an oxidative pathway mediated by the CYP24A1 inactivation protein (24-hydroxylase) (64, 66).

Biomarkers of Vitamin D Status

The intake through diet and the level of exposure to sun are the most important determinants of vitamin D status. Serum concentration of 25(OH)D indicates the overall vitamin D levels derived from both cutaneous synthesis and dietary sources. It is considered a reliable marker of vitamin D status with a half-life of ∼13 to 15 days because of its strong affinity for DBP (63, 67). In populations with low exposure to UV-B radiation, serum 25(OH)D can also be used as a biomarker of intake (68). However, the individual variability observed in vitamin D status could be attributed to different analytical methods, the use of distinct reference values to assess the body status and to genetic factors (28). Thus, assessments of vitamin D status in population studies should be interpreted with caution (64).

TABLE 1 | Characteristics of the publications included in the review ($n = 60$).

References	Type of study	Reference to vitamin	Study participants (original research articles) or number of publications in meta-analyses
Ahn et al. (3)	GWAS	Vitamin D	Five cohorts of 4,501 persons of European ancestry
Andrew et al. (4)	Cross-sectional (Twin Study)	Vitamin B12	$n = 2,110$ female twins of women of North European descent (Caucasian), 18–79 y
Bahrami et al. (5)	Review	Vitamin D	
Batai et al. (6)	Cross-sectional	Vitamin D	$n = 1,057$ ($n = 652$ African Americans, $n = 405$ European Americans), US
Bendik et al. (7)	Review	Vitamin D	
Block et al. (8)	Cross-sectional	Vitamin C	$n = 383$ non-smokers Caucasians 18–85 y (participants in previous RCT)
Bhan (9)	Review	Vitamin D	
Borel et al. (10)	Cross-sectional	Vitamin E	$n = 128$, Caucasians 18–70 y (participants enrolled in the Medi-RIVAGE study)
Borel Desmarchelier (11)	Review	Vitamin E	
Bu et al. (12)	Cross-sectional (CGAS)	Vitamin D	$n = 496$, non-Hispanics
Bueno et al. (13)	Cross-sectional	Folate	$n = 726$, Spanish adults 18–75 y
Cahill et al. (14)	Cross-sectional	Vitamin C	$n = 905$ non-smoking subjects, 20–29 y (participants in the Toronto Nutrigenomics and Health Study)
Cahill and El Solemy (15)	Cross-sectional	Vitamin C	$n = 1.277$ non-smoking subjects, 20–29 y (participants in the Toronto Nutrigenomics and Health Study)
Cahill and El Solemy (16)	Cross-sectional	Vitamin C	$n = 1,046$ non-smoking subjects, 20–29 y (participants in the Toronto Nutrigenomics and Health Study)
Castro et al. (17)	Cross-sectional	Vitamin B12	$n = 122$ Portuguese individuals (mean age 46 y)
de Batlle et al. (18)	Cross-sectional	Folate	$n = 988$ French women 40–65 y (participants in French E3N-EPIC cohort)
Duan et al. (19)	Systematic Review and meta-analysis	Vitamin D	Sixteen publications with a total of 52,417 participants of Asian and Caucasian origin
Duarte et al. (20)	Cross-sectional	Vitamin C	$n = 80$ Brazilian subjects
Duell et al. (21)	Case-control (nested within EPIC Cohort)	Vitamin C	$n = 1,649$ subjects, 57–70 y
Galmés et al. (22)	Review	Vitamin E	
Grarup et al. (23)	GWAS	Vitamin B12	$n = 45,574$ Icelandic and Danish individuals
Engelman et al. (24)	Cross-sectional	Vitamin D	$n = 1,379$ participants of 40 y mean age (from IRAS Family Study), Hispanics and African Americans
Ferrucci et al. (25)	GWAS	Vitamin E	$n = 3,941$ Caucasians (participants in InCHIANTI, WHAS, and ATBC studies)
Hansen et al. (26)	Cross-sectional	Vitamin D	$n = 989$, African Americans, 70–79 y
Hazra et al. (27)	Meta-analysis	Vitamin B12	$n = 4,763$, Caucasians (from three GWA scans)
Herrmann et al. (28)	Review	Vitamin D	
Hiraoka and Kagawa (29)	Review	Folate	
Horska et al. (30)	Cross-sectional	Vitamin C	$n = 388$ Slovakian factory workers
Hustad et al. (31)	Cross-sectional	Folate	$n = 10,601$ adults 50–64 y (from the Norwegian Colorectal Cancer Prevention cohort)
Jolliffe et al. (32)	Review	Vitamin D	
Kwak et al. (33)	Cross-sectional	Vitamin D	$n = 2,264$, Korean adults >20 y
Lauridsen et al. (34)	Cross-sectional	Vitamin D	$n = 595$ Danish Caucasian postmenopausal women (participants in DOPS Study)
LeCompte et al. (35)	Cross-sectional	Vitamin E	$n = 1,614$ Caucasian adults (participants in SU.VI.MAX and HELENA studies)
Major et al. (36)	GWAS	Vitamin E	$n = 5,006$ men of European descent (in two adult cohorts)
Major et al. (37)	GWAS	Vitamin E	$n = 2,112$, Finnish male smokers, 50–69 y (participants in the ATBC trial)
McGrath et al. (38)	Review	Vitamin D	
McNulty et al. (39)	Cross-sectional	Folate	$n = 123$, Irish individuals, 19–63 y
McNulty et al. (40)	RCT	Folate	$n = 89$ Irish individuals, 18–65 y

(Continued)

TABLE 1 | Continued

References	Type of study	Reference to vitamin	Study participants (original research articles) or number of publications in meta-analyses
Michels et al. (41)	Review	Vitamin C	
Molloy (42)	Review	Folate	
Na et al. (43)	Cross-sectional	Vitamin C	$n = 110$ Chinese individuals, 18–50 y
Orton et al. (44)	Longitudinal population-based twin study	Vitamin D	$n = 178$ Canadian twins,34–78 y
Robien et al. (45)	Cross-sectional	Vitamin D	$n = 504$, Singapore Chinese men and women, 45–74 y
Schwartz (46)	Review	Vitamin C, Folate	
Shane (47)	Review	Folate	
Shane et al. (48)	GWAS analysis	Folate	$n = 2,232$ Irish subjects, 18–28 y
Signorello et al. (49)	Cross-sectional	Vitamin D	$n = 758$ ($n = 379$ African Americans, $n = 379$ Caucasians), 40–79 y
Stanislawska-Sachadyn et al. (50)	Cross-sectional	Vitamin B12	$n = 613$ Northern Irish Men (Caucasians) 30–49 y
Surendran et al. (51)	Review	Vitamin B12	
Thuesen et al. (52)	Cross-sectional	Vitamin B12 Folate	$n = 6,784$ Danish, 30–60 y
Timpson et al. (53)	Meta-analysis	Vitamin C	Five cohorts with a total of 15.087 Caucasians
Tsang et al. (54)	Systematic Review and meta-analysis	Folate	Forty publications with non-pregnant, non-lactating females 12–49 y across various population groups
Touvier et al. (55)	Cross-sectional	Vitamin D	$n = 1,828$, Caucasian adults, <65 y
Voipio et al. (56)	Cross-sectional	Vitamin D	$n = 2,204$, Finnish subjects, 30–45 y (participants in prospective Cardiovascular Risk in Young Finns Study)
von Castel-Dunwood et al. (57)	Cross-Sectional	Vitamin B12	$n = 344$ non-pregnant women, 20–30 y, non-Hispanic white (93%), non-Hispanic black (7%)
Wang et al. (58)	GWAS	Vitamin D	∼30,000 ($n = 33.869$) Individuals of European descent from 15 cohorts.
Wright et al. (59)	Case-control (nested within the ATBC Trial)	Vitamin E	$n = 1,833$ (982 cases, 851 controls), Finnish male smokers, 50–69 y
Xu et al. (60)	Cross-sectional	Vitamin D	$n = 1,873$ ($n = 945$ Uygur/$n = 928$ Kazak ethnic)
Zanon-Moreno et al. (61)	Case-control study	Vitamin C	$n = 300$ (150 cases, 150 controls) Caucasian participants, 50–80 y
Zinck et al. (62)	Cross-sectional	Vitamin B12	$n = 3,114$ Canadians (85% Caucasians, 15% non-Caucasians) 20–79 y

Vitamin D Deficiency

Taking into account risk for rickets or symptomatic osteomalacia, serum 25(OH)D levels below 25 nmol/L have been suggested as an indicator of vitamin D deficiency in Europe (64). The Institute of Medicine concluded that people with serum concentrations below 30 nmol/L and between 30 and 50 nmol/L are susceptible to vitamin D deficiency and inadequacy, respectively (63). The Endocrine Society Task Force suggests that 25(OH)D concentration below 50 nmol/L indicates vitamin D deficiency (69). In general, a threshold of less than 25–30 nmol/L characterizes vitamin D deficiency, but to date a standard definition regarding the "optimal" 25(OH)D levels is still lacking (70). Recently, a target population value of 50 nmol/L for serum 25(OH)D concentration is proposed by EFSA (64).

Genetic Variations and Vitamin D Status

Genetic variants of proteins participating in the vitamin D metabolism, its binding to receptors and transport can have an impact on vitamin D availability and status (5, 7). Several single nucleotide polymorphisms (SNPs) related with serum 25(OH)D that have been detected in candidate gene studies and genome-wide association studies (GWAS) have enhanced our understanding of vitamin D balance; the detection of individuals more vulnerable to deficiency; as well as those who could benefit more than others from supplementation (5).

The Study of Underlying Genetic Determinants of Vitamin D and Highly Related Traits (the SUNLIGHT study) is the largest GWAS published to date that identified significant genetic determinants of 25(OH)D levels. The study involved individuals of European descent from 15 cohorts ($n = 33,996$) and reported variants at three loci reaching genome-wide significance in both discovery and replication cohorts for association with 25(OH)D concentrations. The genetic variants identified in this large study belong to the genes encoding DBP (GC gene), enzyme 7-dehydrocholesterol reductase (DHCR7 gene), vitamin D 25-hydroxylase (cytochrome P450, family2, subfamily R, member 1, CYP2R1 gene), and vitamin D 24-hydroxylase (cytochrome P450, family 24, subfamily A, member 1 CYP24A1 gene) (58)

and confirm the results of previous smaller-scale studies (12). With respect to variants of the gene encoding the intracellular VDR, there are no consistent findings relating to vitamin D status. In a recent systematic review, Jolliffe et al. (32) reported that polymorphisms in DBP, CYP2R1, and DHCR7 are the ones most frequently associated with 25(OH)D blood concentrations, while variants in the VDR gene have mostly been reported as determinants of phenotypes. Genes implicated in vitamin D metabolism and signaling pathways have been schematically presented by Jolliffe et al. (32).

Vitamin D Binding Protein Related Variants (DBP)

The DBP initially known as Gc-globulin (group-specific component of serum), is a circulating alpha globulin produced primarily by the liver at relatively stable levels throughout life, except from conditions of high estrogen levels (e.g., during pregnancy, when production is increased) The DBP is a multi-functional compound that is involved in the binding of the majority (>85%) of circulating 25(OH) D, and also of extracellular actin and in the transport of fatty acids (71). The DBP has the highest affinity for 25(OH)D-lactones, followed by 25(OH)D and $1,25(OH)_2D$ (72) and binds more effectively to vitamin D3 and its metabolites than vitamin D2 and its metabolites (73). Variants of the DBP gene have been widely investigated as sources of variation in the circulating levels of vitamin D (5, 32).

The GC gene encodes the DBP and is located on chromosome 4q12-q13. The two most frequently investigated variants in the GC gene that modify the amino acid sequence of the protein are the SNPs found in exon 11. The rs4588 DBP SNP causes an amino acid change from lysine to threonine in codon 420 (substituting A for C, ACG→AAG, Thr→Lys) resulting in a Gc-2 protein. In addition, the rs7041 SNP leads to a change of aspartate to glutamate in codon 416 (substituting G for T, GAT→GAG, Asp→Glu) resulting in a Gc-1s protein (9). Although over 120 variants in the DBP gene have been recognized, three main phenotypic alleles-GC haplotypes (Gc1S, Gc1F, and Gc2) have been identified. Regarding electrophoretic migration, the slowest is Gc2, followed by Gc1S (slow) and Gc1F (fast) (74). The associated serum concentration of DBP and its affinity toward 25(OH)D differ among the phenotypic alleles (75, 76). The median concentration of 25(OH)D is the highest in Gc1s-1s (CC rs4588; GG rs7041), intermediate in Gc1-2 (Gc1F) (CC rs4588; TT rs7041), and lowest in Gc2-2 (AA rs4588; TT rs7041) (28, 34). These haplotypes further exhibit an important racial and geographic variation, since GC1F is more prevalent among dark-skinned individuals (particularly those of African descent), while Gc1S and Gc2 are more common in Caucasians (77, 78). The association between the rs4588/rs7041 SNPs with serum 25(OH)D levels has been confirmed in studies including different ethnic populations (32). Minor A allele of rs4588 is consistently associated with lower 25(OH)D levels, while minor G allele of rs7041 with higher 25(OH)D levels (32). In Caucasian adults, these associations were found to be significant after adjustment for vitamin D dietary intake, sun exposure, socio-demographic, anthropometric, and lifestyle factors ($P < 0.0001$) (55). Furthermore, in a recent study among African Americans the T allele of rs7041 was associated with lower serum 25(OH)D (b = −0.93, SE = 0.53, close to significance thresholds, $P = 0.08$) (26). Individuals with the Gc2-2 haplotype (AA rs4588 /TT rs7041) have significantly lower 25(OH)D concentrations compared to all other Gc haplotypes (p-trend < 0.001). In addition, individuals with Gc1s-1s haplotype (CC rs4588/GG rs7041) are characterized by higher 25(OH)D status (45). These findings confirm earlier observations between plasma 25(OH)D concentrations and Gc phenotypes reported by Lauridsen et al. (34).

Two GWAS meta-analyses identified another polymorphism in the GC gene (the rs2282679) which is located within intron 12 (A>C) and is in a high linkage disequilibrium with rs4588 (3, 58). In the Ahn et al. (3) meta-analysis of five cohorts of individuals with European ancestry, the (minor) risk C allele has been found to be inversely associated with circulating vitamin D levels (b = −0.36, SE: 0.05). Per copy of the minor allele of this variant, individuals have 49% higher risk of vitamin D insufficiency [<50 nmol/L OR = 1.49, 95% CI = 1.40–1.59, $P = 7.5 \times 10^{-33}$], after adjusting for age, sex, BMI, and season (58). A stronger association between the risk allele and vitamin D insufficiency has also been reported by Voipio et al. (56) in a study among Finish adults (OR = 2.08, 95% CI = 1.66–2.60). In a study among European Americans, the C allele of rs2282679 was associated with lower circulating 25(OH)D levels (b = −0.05, $p = 0.001$) (6). In studies among Koreans (33) and African-Americans (49) individuals carrying the G minor allele had lower 25(OH)D concentrations, as compared to those carrying the T allele.

7-Dehydrocholesterol Reductase Related Variants

Cholecalciferol and cholesterol are synthesized through the skin formation of 7-dehydrocholesterol (7-DHC). The DHCR7 gene on chromosome 11 encodes the enzyme 7-dehydrocholesterol reductase, which converts 7-DHC to cholesterol, reducing thus the availability of this precursor (7-DHC) for the synthesis of vitamin D (5). The UV-B radiation and the enzyme possibly antagonize in the conversion of 7-DHC and as a result, 25(OH)D has been reported to have a varying association with serum HDL or skin cholesterol levels shaped by the interrelation between the DHCR7 enzyme in serum and the amount of sun exposure (79).

Several studies have shown that the DHCR7 variant (rs12785878) is associated with 25(OH)D concentrations (overall $p = 2.1 \times 10^{-27}$, combined discovery and replication samples) (58) The rs12785878 variant is located in an intron of a different gene known as NADSYN1 and as a result this SNP is often indicated as the NADSYN1/DHCR7 locus. Homozygotes (GG) have lower mean 25(OH)D levels than heterozygotes (GT), who in turn on average have lower mean 25(OH)D levels than major homozygotes TT (58). A study among 2.204 Finnish individuals confirmed the association of the G minor allele of rs12785878 with lower 25(OH)D concentrations (b = −2.10, SE = 1.01) and the increased risk of vitamin D insufficiency (<50 nmol/L, OR = 1.31, 95% CI = 1.00–1.70) compared to the T allele

and after adjusting for vitamin D intake, nutrient supplement use, body mass index, physical activity, and lifestyle factors (56). In a multi-center study including individuals of Kazak ethnicity, the DHCR7/NADSYN1(rs12785878) has also been significantly associated with vitamin D deficiency (25(OH)D levels <20 ng/mL) (OR = 2.44, 95% CI = 1.22–4.87), adjusted for sex, age, BMI, and study center (60). The negative association between G allele of rs12785878 (DHCR7) and 25(OH)D levels was also reported in a study of healthy Korean adults (33).

CYP2R1 & CYP24A1 Related Variants

The human CYP2R1 gene (on chromosome 11p15.2) encodes a member of the cytochrome P450 superfamily of enzymes, the microsomal vitamin D 25-hydroxylase, involved in vitamin D activation through hydroxylation, to the vitamin D receptor ligand. The CYP24A1 (on chromosome 20q13.2) encodes the vitamin D 24-hydroxylase, participating in the inactivation process of vitamin D metabolites. Several GWAS have identified SNPs in the CYP2R1 and CYP24A1 genes associated with 25(OH)D concentrations (i.e., rs10741657, rs1993116, rs12794714, and rs10766197 for CYP2R1 and rs6013897 for CYP24A1) (32).

The rs10741657 (G>A) SNP is located in the non-coding region 5′-UTR, which may regulate gene expression and therefore modulate the expression and activity of 25-hydroxylase. In a large GWAS, Wang et al. (58) reported an association between the rs10741657 polymorphism and the 25(OH)D levels (overall $p = 3.3 \times 10^{-20}$, combined discovery and replication samples). In their recent meta-analysis, Duan et al. (19) confirmed this association and further reported that the GG genotype was associated with lower 25(OH)D levels when compared with the AA (reference) genotype [standardized mean difference SMD = −2.31, 95% CI = (−4.42, −0.20) overall and SMD = −3.46, 95% CI = (−6.60, −0.33) and SMD = −0.24, 95% CI = (−0.51, −0.03) for Caucasian and Asian groups, respectively]. Consequently, the risk G allele has been associated with an increased risk of vitamin D deficiency (<20 ng/mL or 50 nmol/L) compared to no-risk allele A in both Caucasian and Asian populations (OR = 1.09; 95% CI = 1.03–1.15). Under the dominant model (GG+AG vs. AA), Duan et al. (19) reported a 42% higher risk of vitamin D deficiency (95% CI = 11–83%); nevertheless, under the recessive model (GG vs. AG+AA), the positive association remained but lost significance (OR = 1.28; 95% CI = 0.89–1.84).

Among African-Americans, the strongest association with 25(OH)D levels has been observed for the CYP2R1 rs12794714 variant located in exon 1 (G>A). The minor (A) allele has been significantly associated with lower 25(OH)D levels (b = −0.4, $p = 0.01$) (6), a finding also reported by Wang et al. (58) (overall $p = 2.7 \times 10^{-9}$). Among European-Americans the most relevant determinant of vitamin status was the CYP2R1 rs1993116 SNP, located in intron 1 (C>T) with the minor A allele was found to be associated with higher serum 25(OH)D levels (b = 0.04, $p = 0.0006$) (6). This association has also been observed in two large GWAS by Wang et al. (58) (overall $p = 6.3 \times 10^{-11}$) and Ahn et al. (3) (risk A allele b = 0.25, $p = 2.9 \times 10^{-17}$). Associations of CYP2R1 rs12794714 and rs1993116 variants with vitamin D status have also been found significant in Chinese subjects (45). Furthermore, the rs10766197 variant, located in the promoter of the CYP2R1 gene has been associated with the 25(OH)D levels in the discovery cohort of healthy Caucasian subjects and remained significant after further replication and analysis of the pooled dataset and after adjusting for age, sex, BMI, habitual vitamin D supplementation and season. The minor A allele of rs10766197 has been inversely associated with serum 25(OH)D levels (b = −4.53, adjusted empirical P-value = 0.002, based on the pooled dataset analysis) (12).

A genetic risk score (GRS) was calculated by Wang et al. (58) combining three confirmed genetic variants related to circulating vitamin D levels (i.e., DHCR7 rs12785878; the CYP2R1 rs10741657; and, the GC rs2282679). Individuals with a "genotype score" in the top quartile had a 2-fold higher odds of vitamin D insufficiency (25(OH)D levels below 50 nmol/L) in comparison to the lowest quartile (OR = 1.92, 95% CI = 1.70–2.16, $P \leq 1 \times 10^{-26}$)]. After adjusting for age, sex, body mass index, and season, the odds of vitamin D deficiency (i.e., 25(OH)D levels below 20 nmol/L) increased by 43% (adjusted OR = 1.43, 95% CI = 1.13–1.79) among individuals in the top quartile of this score in comparison to those in the lowest quartile. In the same study, Wang et al. (58) further reported that the rs6013897 SNP was also associated with serum 25(OH)D concentrations (overall $p = 6 \times 10^{-10}$ combined discovery and replication samples).

The VDR Related Variants

Two VDR SNPs—the rs2228570 and rs10783219—have been associated with serum 25(OH)D concentrations. Both the rs2228570 and the rs10783219 SNPs have been associated with lower 25(OH)D levels in a longitudinal population-based twin study and in a sub-population of a cross sectional family study, respectively (24, 44). In general, the way in which the VDR gene variants could influence the 25(OH)D concentrations (38) has not yet been elucidated; in their review of genetic association studies, Jolliffe et al. (32) suggested that genetic variation in VDR is strongly related to the phenotype rather than circulating 25(OH)D concentrations.

VITAMIN E

Vitamin E is a fat-soluble vitamin, existing in biologically different forms, the tocopherols (α, β, γ, δ) and the tocotrienols (α, β, γ, δ), that possess different antioxidant activity. In humans, α-tocopherol is the most abundant and physiologically active form of vitamin E. Since the food content of tocopherols and tocotrienols is converted to *α-tocopherol equivalents*, the terms "vitamin E" and "α-tocopherol" are used interchangeably. The α-tocopherol, acting as a free radical scavenger preventing DNA oxidative damage, belongs to the antioxidant defense system. It notably protects polyunsaturated fatty acids (PUFAs) within plasma lipoproteins and membrane phospholipids, preserving thus the cellular membrane integrity (e.g., of erythrocytes, central and peripheral nerves). The α-Tocopherol has also been linked to cancer prevention through inhibiting cell proliferation and angiogenesis. The main dietary sources of α-tocopherol include

vegetable oils and derivatives, some fatty fish, egg yolk, nuts, seeds, and whole grain cereals. The more abundant form in food are the α and γ-tocopherols (80).

Metabolism of Vitamin E

As a lipid-soluble vitamin, upon intake, vitamin E follows intestinal absorption, hepatic and cellular uptake similar to those of lipids and other lipophilic components (81). About 75% of a usual α-tocopherol intake is absorbed by the human body and efficient absorption requires the presence of fat (80). Proteins involved in the vitamin E uptake (in the enterocyte) include the scavenger receptor class B member 1 (SCAR-BI), the Niemann–Pick disease type C1 protein (NPC1) and the cluster determinant 36 (CD36) molecule, also known as scavenger receptor class B member 3 (SRB3). All these also participate to the transmembrane transport of cholesterol and other lipophilic components (10, 22). Scavenger receptor class B member 1 (SCAR-B1) is a multi-ligand membrane receptor expressed in many cell types. It is not only involved in α-tocopherol uptake by enterocytes, but also in its transport from enterocytes to the blood, transfer of α-tocopherol from high-density lipoprotein (HDL) cholesterol to tissues and in the biliary excretion of α-tocopherol. The SCAR-B1 also acts as a plasma membrane receptor for HDL and mediates cholesterol transfer to and from HDL (11). The CD36 is a membrane glycoprotein involved in the uptake of fatty acids and in binding native and oxidized lipoproteins contributing thus directly or indirectly to the transport of vitamin E (22).

After a-tocopherol is absorbed in the intestine, it is integrated into chylomicrons and along the lymphatic system chylomicrons are secreted into circulation. Part of the a-tocopherol in chylomicrons is incorporated into tissues by lipoprotein lipase (LPL), while the remaining is transferred to the liver. In the liver, the α-tocopherol transfer protein (α-TTP) binds a-tocopherol with the highest affinity. The a-TTP is responsible for a-tocopherol incorporation into the preliminary very low density lipoproteins (VLDLs) which are secreted by the liver into the circulation and then distributed to body tissues. VLDLs are converted into intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDLs) by the action of LPL, and to HDLs (81). Plasma lipoproteins (VLDL, LDL, and HDL) are the major carriers of vitamin E. Thus, proteins involved in lipoprotein synthesis and metabolism play a crucial role in forming lipoproteins for vitamin E transport. In particular, apo-lipoproteins, LPL, hepatic lipase, phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), and lecithin cholesterol acyltransferase are indirectly involved in vitamin E metabolism. Among the main apo-lipoproteins involved are apo A-IV and apo AV (10). Amounts of α-tocopherol not bound to α-TTP is catabolized in the liver by the hepatic enzyme cytochrome P (CYP)4F2 ω-hydroxylase. Both α-TTP and ω-hydroxylase have a significant contribution to α-tocopherol metabolism, especially to the liver balance (storage vs. catabolism) and to vitamin E systemic level (82).

Biomarkers of Vitamin E Status

Fasting plasma or serum α-tocopherol concentration has been commonly used to assess vitamin E status. Since there is no precise cut-off value above which adequate status is characterized, plasma/serum α-tocopherol levels below 12 μmol/L may indicate deficiency. In α-tocopherol deficiency, oxidative stress can damage red blood cells (RBCs) (80).

a-Tocopherol concentrations within the range of 2.5–12 μmol/L have been described in primary or secondary deficiency (83, 84). Mutations in the a-TTP gene result in primary α-tocopherol deficiency and related neurological symptoms, including ataxia (85). Secondary a-tocopherol deficiency is present in individuals suffering from conditions including abetalipoproteinaemia, cholestatic liver diseases, severe malnutrition, or fat malabsorption (83, 86). Low a-tocopherol dietary intake has not been reported to cause deficiency with clinical manifestations in healthy individuals (80).

Genetic Variations and Vitamin E Status

Vitamin E status is known to be affected by factors such as age, eating habits, oxidative stress (e.g., through smoking), absorption efficiency, and catabolism (10, 11). Genome-wide and candidate gene association studies have identified genetic variations that can influence vitamin E status, impairing its metabolism, absorption/uptake, transport and liver storage, or catabolism balance (11, 22). Borel and Desmarchelier (11) provide a comprehensive diagram illustrating the majority of genes encoding key players in the vitamin E status.

Vitamin E Uptake Related Variants

There is strong evidence that SNPs in SCAR-B1 gene on chromosome 12q24.31 affect vitamin E metabolism and α-tocopherol levels (10, 11). Rs5888, which is also known as A350A, is a variant located on exon 8 of the SCAR-B1 gene including an exchange of the minor allele (C) for (T). TT carriers have the lowest plasma α-tocopherol levels as compared to CC or CT carriers and CT have shown the highest levels compared to the other two counterparts; these associations were significant ($p < 0.05$) in men after adjustment for cholesterol levels [mean a-tocopherol levels μmol/L (SD) per pair of alleles; TT: 23.47 (6.99)<CC: 26.14 (6.22)<CT: 28.07 (6.87)] (10). The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study was a randomized, double-blind, placebo-controlled intervention trial and evaluated cancer prevention after supplementation with either alpha-tocopherol, or beta carotene or both among male smokers (37). In a GWAS of serum α-tocopherol concentrations within the ATBC cohort, Major et al. (36) reported that the presence of the minor (A) variant allele of another SCAR-B1 SNP (rs11057830) was significantly associated with plasma α-tocopherol concentration ($b = 0.04$, $p = 2.0 \times 10^{-8}$) in male smokers independent of their age, BMI, cholesterol levels, and cancer status and with high response in serum concentrations to long-term a-tocopherol supplementation in the same population ($b = 0.03$, $p = 2.9 \times 10^{-3}$).

The CD36 SNPs might also influence plasma α-tocopherol concentrations. So far, two relevant SNPs (rs1761667 and rs1527479) have been identified and are found in high linkage

disequilibrium. Individuals homozygous for the minor allele of rs1761667 (G) or rs1527479 (A) have lower plasma α-tocopherol concentrations than do carriers of the major allele (A of rs1761667, G of rs1527479) at about 22.9%, $p = 0.046$, and 23.7% $p = 0.0061$, respectively, especially among men after adjustment for age and BMI. The relationship between rs1527479 and a-tocopherol levels maintained its significance even after correction for multiple testing (p threshold < 0.0071). Furthermore, lower a-tocopherol levels were observed particularly among individuals carrying the A minor allele of rs1527479 with low triglyceride (p for trend $= 0.013$) or PUFA concentrations (p for trend $= 0.005$) than their corresponding GG individuals. The lower plasma a-tocopherol levels detected in individuals homozygous for the minor alleles may be related to a higher CD36 expression and a subsequent higher vitamin E and fatty acid transport. Dietary recommendations for higher vitamin E intakes among these individuals may be relevant to counteract the CD36 protein excess (35).

Vitamin E Transport Related Variants

Apo-AV is a minor apo-lipoprotein almost exclusively expressed in liver that plays a significant role in the regulation of plasma triglycerides. The most studied SNP located on the Apo-AV promotor is rs662799 (1131T>C) in which the minor variant (C) has been associated with both higher plasma vitamin E and VLDL-TGs levels in diabetic patients. In particular, among TT carriers the mean (SD) vitamin E levels in blood were 40.32 mmol/L (10.47), whereas in TC carriers were 45.48 mmol/L (8.20) ($p = 0.02$). Moreover, plasma triglyceride (TG) concentration was 21% higher in carriers of the TC genotype ($p = 0.04$), because of higher TG in VLDL [mean TG (SD); 0.96 mmol/L (0.78) for TT carriers and 1.33 mmol/L (1.11) for TC carriers, $p = 0.043$] and in HDL [mean TG mmol/l (SD); 0.14 mmol/L (0.05) for TT carriers and 0.17 mmol/L (0.03) for TC carriers, $p = 0.017$) (87).

In a GWAS investigating the circulating α-tocopherol phenotype, Ferrucci et al. (25) reported that the rs12272004 SNP, close to the apo-AV gene, was associated with higher plasma α-tocopherol concentrations. In particular, the A allele of rs12272004 was associated with a 0.07 SD higher vitamin (95% CI = 0.05–0.10). When analysis was adjusted for TG levels, the strength of the association was reduced (b = 0.055, 95% CI = 0.020–0.091). Major et al. (36) identified a significant association between serum α-tocopherol and rs964184 on chromosome 11q23.3, within or near the gene cluster Apo-AI/CIII/AIV/AV. The rs964184 minor G variant allele was associated with increased a-tocopherol levels (b = 0.04, SE = 0.01, $p = 2.7 \times 10^{-10}$) after adjustment for age, BMI, non-HDL cholesterol concentrations, and cancer status (36) and with high serum response to long term vitamin E supplementation (b = 0.07, SE = 0.01, $p = 2.6 \times 10^{-12}$) (37).

Based on findings from a candidate gene association study, a variant of the apo A-IV was also found to be associated with vitamin E status. ApoA-IV is secreted in the intestine and is associated with chylomicrons. A polymorphism in the gene located on chromosome 11 result to an A to T substitution that changes the threonine residue at position 347 to serine ApoIVSer-347 (rs675). Women carrying the A allele (AT+AA) of rs675 were found to have significantly ($p < 0.05$) lower plasma concentrations of a-tocopherol than women homozygous for the T allele. [(mean a-tocopherol (SD) levels TT 34.12 μmol/L (8.68) > AA+AT 26.40 μmol/L (6.59)]. However, this association did not remain significant after adjustment for blood cholesterol levels (10). The same study confirmed the previously described association between plasma a-tocopherol and Apo-E variants. The human ApoE gene is polymorphic (derived from the combination of polymorphisms rs429358 and rs7412), which results in three major isoforms/alleles (ε2, ε3, and ε4), particularly the ApoE-ε2 (Cys112, Cys158), ApoE-ε3 (Cys112, Arg158), and ApoE-ε4 (Arg112, Arg158) alleles. Apo-lipoprotein E is a multifunctional protein involved in the catabolism of lipoprotein particles. In humans, the lowest plasma vitamin E concentrations were found in ApoE-ε2/ε2 genotype [mean a-tocopherol (SD); 18.68 μmol/L (8.45), $p < 0.05$], whereas the presence of the ε4 allele was associated with the highest vitamin E levels in plasma (ApoE-ε4/ε2 genotype) [mean (SD); 32.81 μmol/L (10.54) $p < 0.05$] (10).

Rare mutations in the a-TTP gene on chromosome 8q13 have been linked with severe vitamin E deficiency which cause an autosomal recessive neurologic disorder and ataxia in humans, also known as ataxia with isolated vitamin E deficiency (AVED) (85). Furthermore, the presence of the TT genotype of the rs6994076 TTPA polymorphism (980A/T) has been found to be associated with decreased protein activity and ∼3% lower α-tocopherol levels ($p = 0.03$) compared to AA genotypes based on data collected among the control group of the ATBC trial (59). A lower (25%) response to vitamin E supplementation was observed in serum a-tocopherol concentration ($p = 0.002$, in multivariate model) in individuals (males) with the TT genotype compared to those homozygous for the major A allele, AA genotype (59).

As previously indicated, CYP4F2 encodes for cytochrome P450 4F2, which is involved in the vitamin E catabolism. The SNP rs2108622 on CYP4F2 gene results in a valine for methionine substitution. Subjects homozygous for minor T allele (TT genotype) were found to have reduced ω-hydroxylation activity and increased serum α-tocopherol (36). The TT genotype has also been significantly associated with increased serum response (b = 0.04, $p = 2.2 \times 10^{-7}$) to long-term (3 years) α-tocopherol supplementation, adjusted for age, BMI, non-HDL cholesterol concentrations, and cancer status (37).

VITAMIN C

Vitamin C or ascorbic acid is a water-soluble vitamin. Vitamin C acts as a free radical scavenger and operates as an enzyme cofactor for various biochemical reactions. Vitamin C is also involved in the biosynthesis of collagen, synthesis of carnitine and catecholamines and in the metabolism of cholesterol to bile acids. Vitamin C is naturally present mainly in fruit, vegetables, and potatoes. Exposition to oxygen or high temperatures affects its stability and results in vitamin C oxidation (88).

Vitamin C Metabolism

Ascorbic acid (ascorbate) is the functional form of vitamin C. Two transporter proteins (SVCT1 and SVCT2) encoded by the sodium-dependent vitamin C transporter genes, SLC23A1 and SLC23A2, respectively, are responsible for vitamin's absorption by the gastrointestinal tract and reabsorption by renal system through active transport across membranes. Vitamin C concentrations and body homeostasis are regulated primarily by SVCT1 (89). The expression of SVCT1 in the kidney is important for the regulation of vitamin C status (41). The SVCT2 regulates vitamin C levels within specific metabolically active tissues (89). It promotes the accumulation of vitamin C into brain, eyes, and adrenals as it binds ascorbate with high-affinity (41). Dehydroascorbate, DHA, the oxidized form of ascorbate, is transferred into the cell by some glucose transporters, including GLUT1, GLUT3, and GLUT4. Within cells, DHA is recycled back to ascorbate, sustaining the intracellular ascorbate uptake (41). In plasma, the free anion of vitamin C is distributed to tissues. The body's absorption efficiency depends on the level of vitamin C intake. Vitamin C is excreted in urine, which is the main route of elimination, at an approximate proportion of 25% of the ingested amount for an intake of 100 mg/day (88).

Biomarkers of Vitamin C Status

Ascorbate concentrations in plasma and leukocytes are considered suitable biomarkers of body stores and status, within the usual range of intakes and independently of recent vitamin C intake (88, 90).

A plasma ascorbic acid value of 50 μmol/L is indicative of an adequate status and values below 11 μmol/L indicate severe deficiency (biochemical and/or clinical symptoms including those related to connective tissue defects; scurvy). Plasma levels between 11 and 23 μmol/L (0.2–0.4 mg/100 mL) reflect marginal status, thus moderate risk of developing deficiency. The vitamin C physiological levels largely depend on the analytical methods used, limiting comparisons among laboratories. For instance, the interpretation of leukocyte vitamin C concentrations is complicated by the different concentrations of vitamin C in various leukocyte cell fractions—mononuclear contain up to 2- or 3-fold higher concentrations than polymorphonuclear cells (91).

Genetic Variations and Vitamin C Status

Polymorphisms in the genes encoding sodium-dependent SVCTs of the SLC23 family are strongly associated with vitamin C status due to their roles in direct transport, absorption, and vitamin accumulation in tissues (41, 92). Genetic variants in protein-coding genes known to play a role in oxidative stress, including haptoglobin, glutathione-S-transferases, and manganese superoxide dismutase, may also influence vitamin C status, but they have not been a priori associated with ascorbic acid (41). A diagram outlining vitamin C metabolism and related genes is provided by Michels et al. (41).

In a cross-sectional study of 1.046 volunteers including Caucasian and East Asian populations, Cahill and El-Sohemy (15) reported that individuals may differ in their blood vitamin C levels, regardless of their dietary intake, due to genetic variation in SVCT1 (rs4257763). Overall, average serum vitamin C levels were lower in individuals with the GG genotype than the AA; with the GA genotype being intermediate ($p = 0.002$). In a sub-population analysis, differences remained but were significant only among Caucasian subjects ($p = 0.02$) and not among East Asians ($p = 0.14$).

Timpson et al. (53) performed a large-scale analysis (using a discovery cohort, the British Women's Heart and Health Study BWHHS and a series of follow-up cohorts and meta-analysis) to assess the relationship between variation at SLC23A1 (rs33972313) and circulating levels of L-ascorbic acid in over 15,000 Caucasian participants from five longitudinal studies. A pooled analysis of the relationship between rs33972313 (C/T) and vitamin C status across all (discovery and replication) studies showed that each additional minor allele (T) was associated with a reduction in plasma levels by 5.98 μmol/L (95%CI = −8.23, −3.73) per minor allele. The rs33972313 variant in SLC23A1 results in a valine to methionine substitution at position 264 of SVCT1 and in decreased transport activity. In the discovery cohort, two SNPs showed positive association with vitamin C status. For each additional minor allele of SNPs rs10063949 (T/C) and rs6596473 (G/C), there was an increase in circulating ascorbic acid levels [b = 1.91 μmol/L, 95% CI = 0.47–3.34 and b = 2.86 μmol/L, 95% CI = 1.39–4.33 per minor allele (C), respectively]. In the first stage replication study [the European Prospective Investigation of Cancer Norfolk Study (EPIC-Norfolk)], results were consistent with those found in the discovery cohort regarding the rs6596473 variant (mean difference in L-ascorbic acid = 1.01 μmol/L, 95% CI = 0.14–1.87, $p = 0.02$, per minor allele) but there was no association between rs10063949 and vitamin C status [mean difference in L-ascorbic acid = −0.05 μmol/L, 95% CI = (−0.90, 0.80), $p = 0.9$ per minor allele]. Among the polymorphisms examined, rs6596471 (A/G) in BWHHS showed a notable, albeit not significant, increase in plasma vitamin C levels [b = 0.95, 95% CI = (−0.63, 2.53) per minor G allele] (53).

In a case–control study, nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort of vitamin C transporter gene variants and gastric cancer risk (21), authors evaluated genetic variants of SVCT as predictors of vitamin C plasma concentrations in a subsample of participants. Authors applied a multiple linear regression model to assess associations between the SLC23A1 and SLC23A2 SNPs and the log-transformed plasma levels of vitamin C, adjusting for age, sex, country, smoking status, *H. pylori* infection, and season of blood collection. Two SNPs in SLC23A1 (rs33972313, rs11950646) and two SNPs in SLC23A2 (rs6053005, rs6133175) were statistically significantly associated with plasma vitamin C. In agreement with the findings of Timpson et al. (53), being a heterozygote (GA) in the rs33972313 SNP in this study was associated with lower plasma vitamin C levels, (GA vs. GG or AA in a codominant model, b = −0.28, 95% CI = −0.54, −0.016). Variant rs11950646 was also associated with lower plasma vitamin C, in a dominant model [GG or AG vs. AA, b = −0.14, 95% CI = (−0.26, −0.011)] and per G allele in an allelic or log-additive model [b = −0.11, 95% CI = (−0.20, −0.017)]. In the case of the SLC23A2 gene, higher plasma vitamin C

concentrations were associated with rs6053005 TT homozygotes b = (0.21, 95% CI = 0.058–0.37) and rs6133175 GG homozygotes (b = 0.22, 95% CI = 0.029–0.40) in a recessive model (21).

Another SNP in SLC23A2 gene (rs1279683, A>G) was found to be significantly associated with plasma concentrations of vitamin C. In a case-control study including patients of primary open-angle glaucoma (POAG), homozygous subjects for the G allele (GG) had significantly lower plasma vitamin C levels than the other genotypes (AA + AG) (9.0 ± 1.4 vs. 10.9 ± 1.6 μg/mL, $p < 0.001$ in cases and 10.9 ± 1.6 vs. 12.1 ± 1.8 μg/mL, $p < 0.001$ in controls) even after multivariate adjustment for sex, age, BMI, smoking and alcohol, and after applying Bonferroni corrections for multiple comparisons (61). Unlike SLC23A1, genetic variation in SLC23A2 has little influence on vitamin C homeostasis and status, but controls the accumulation in tissues. Furthermore, studies are also lacking since most of the SNPs studied to date are located in intronic or untranslated regions and do not directly affect the SVCT2 protein coding (41).

Haptoglobin is a hemoglobin-binding protein, participating in iron metabolism and encoded by a polymorphic gene (haptoglobin gene, Hp) located on chromosome 16. In humans, Hp is characterized by a polymorphism with two alleles (Hp1 and Hp2) forming three main phenotypes: homozygous for the Hp1 allele (Hp 1-1 phenotype), heterozygous (Hp 2-1) and homozygous for the Hp2 allele (Hp2-2). Unlike Hp1, the hemoglobin protein derived from Hp2 binds insufficiently to hemoglobin and individuals expressing the Hp2-2 phenotype (Hp2-2 homozygotes) produce a less active protein and have increased circulating levels of iron compared to Hp1-1 (41). As a result, Hp 2-2 individuals are more susceptible to vitamin C deficiency since their plasma ascorbate is less stable (93). Na et al. (43) investigated the relation between haptoglobin genetic variants, vitamin C, and iron status in 110 Chinese subjects and reported that serum vitamin C was lower in Hp2-2 healthy Chinese male participants compared to both Hp2-1 and Hp1-1 individuals ($p = 0.028$). In the same study, vitamin C was also affected by iron status (ferritin levels) (43). It is however uncertain whether variations in vitamin C status related to haptoglobin polymorphism associate with iron dysregulation (affected by low dietary iron intake or anemia) especially among Hp2-2 individuals (41).

According to Cahill and Sohemy (16) individuals homozygous for the Hp2 allele had lower vitamin C concentrations compared to those with the Hp1 allele when their dietary vitamin C intake was low. In a cross-sectional examination of free-living adults enrolled in the Toronto Nutrigenomics and Health Study, individuals with the Hp2–2 genotype who did not meet the US Recommended Dietary Allowance for vitamin C had a significantly increased risk of ascorbic acid deficiency <11 μmol/L (adjusted OR = 4.77, 95% CI = 2.36–9.65) compared to those who did. Conversely, the risk of deficiency for carriers of the Hp1 allele (Hp2-1 + Hp1-1 phenotype) was lower and not significant (OR = 1.69, 95% CI = 0.80–3.63) after adjusting for BMI, sex, energy intake, use of oral contraceptives, C-reactive protein levels, plasma α-tocopherol, ethnicity, and season (16). Based on this evidence, Schwartz (46) concluded that the required daily vitamin C intake in Hp 2-2 individuals should be higher than other Hp phenotypes.

The glutathione S-transferases (GSTs) are partof a large family of enzymes involved in the detoxification of detrimental compounds. Genetic variations in GST may affect vitamin C status through their impact on reactive oxygen species and glutathione status. Deletion polymorphisms in the GSTM1 and GSTT1 genes, found on chromosome 1 and chromosome 22, respectively, are generally present in Caucasian populations and result in absence of enzyme function (41) Subjects with the GSTM1-null genotype appeared to have increased vitamin C concentrations compared with carriers of the functional gene variant (8). Horska et al. (30) reported lower plasma vitamin C levels in subjects with deletion of GSTM1 compared with subjects carrying the functional gene variant ($p = 0.042$). In the cross sectional evaluation of participants in the Toronto Nutrigenomics and Health Study, however no differences in vitamin C status were observed (14). GSTM1 genotype and vitamin C status may be characterized by a more complex relation, taking into account dietary vitamin C intake and other environmental exposures such as smoking (41).

Dietary intake, GST genetic variants and serum ascorbic acid concentrations were determined for about 900 non-smoking men and women. When compared to individuals who met the Recommended Dietary Allowance for vitamin C, individuals who did not adhere to vitamin C recommendations and possessed the GST null genotypes had an increased risk of vitamin C deficiency. The odds ratio (95% CI) for deficiency among individuals with the GSTM1 null (0/0) genotypes and functional GSTM1 (1/1+1/0) not meeting the RDA values for vitamin C were 4.03 (2.01, 8.09) and 2.29 (0.96, 5.45), respectively as compared to those with intakes in accordance to recommended levels. For GSTT1 variant, the odds ratio (95% CI) for deficiency were 2.17 (1.10, 4.28) for functional and 12.28 (4.26, 33.42) for null genotypes who did not comply with recommendations as compared to those who did (14). Individuals with GSTM1/GSTT1 deletion (null genotypes) and low intake of vitamin C from their diet had lower plasma vitamin C concentrations than those with functional enzymes at the same level of intake. This may suggest that these enzymes protect against deficiency when the vitamin C intake is insufficient (14, 30). Cahill et al. (14) also reported that subjects with the GSTT1-null genotype had decreased serum ascorbate. However, these findings were not confirmed by Block et al. (8), in which no statistically significant association was observed ($p > 0.05$). A loss of GSTT1 function appears to affect cellular ascorbate levels but not plasma concentrations compared to other GSTs (41). In general, associations between vitamin C and glutathione S-transferases are complex and not well-understood yet.

The superoxide dismutase (SOD) is considered a significant antioxidant enzyme in the body and exists in three SOD isoforms including the mitochondrial SOD manganese dependent (MnSOD), encoded by the gene SOD2 located on chromosome 6q25, in humans. A common SOD2 polymorphism, the Ala16Val (rs4880) results in a mutation at codon 16 and an alanine-to-valine substitution (GCT->GTT). The Val variant may be present at a lower concentration in the mitochondria and

individuals homozygous (Val/Val) may have decreased resistance to oxidative stress (41). Duarte et al. (20) have studied the association of MnSOD variants with plasma vitamin C levels. Healthy homozygous for the valine variant individuals have higher levels of serum vitamin C ($p < 0.05$). In contrast, subjects with hypercholesterolemia and at least one copy of the val variant had lower serum vitamin C concentrations compared to subjects homozygous for the alanine variant, indicating an increase in the oxidative stress in subjects with the VV genotype for MnSOD and hypercholesterolemia (20).

FOLATE AND VITAMIN B12

From the group of B vitamins, folate (vitamin B9) and cobalamin (vitamin B12) have been primarily studied in relation to genetic variants affecting their body status. Therefore, this section focuses on the presentation of evidence related to these two B-vitamins. Folate is used to collectively describe a family of water-soluble compounds which are included in the B-complex vitamins. They are essential co-factors in one-carbon metabolism pathway (94), which involves three major interrelated metabolic cycles in the cells' cytosol and is essential in multiple biochemical processes, including: (a) amino acid metabolism and homeostasis, (b) *de novo* nucleotide synthesis (purines and thymidine; precursors for DNA and RNA), and (c) the process of methylation. Furthermore, the folate pathway is closely related to homocysteine metabolism (95, 96). Vitamins B12 and B6 (pyridoxine) are also important enzyme cofactors or substrates in one carbon metabolism (94).

Folate is present in high amounts in dark green leafy vegetables, legumes, orange and grapefruit (juice), and nuts (peanuts, almonds). Although folate in meat is generally found in low amounts, liver and kidney are particularly rich sources. Dairy products, fish, eggs, and potatoes are also sources of folate intake (96). Principal sources of cobalamin include products of animal origin (meat, fish, dairies, eggs, and liver) and it can also be added to foods and food supplements (97).

Metabolism and Functions of Folate and Vitamin B12

Dietary folate mainly exists as polyglutamates, which are hydrolyzed to monoglutamates by folyl poly-γ-glutamate carboxypeptidase (FGCP) found in the intestinal mucosa. In the intestinal cells, folate is usually reduced and methylated to be absorbed in the small intestine by the high-affinity proton-coupled folate transporter (PCFT1). In the circulation, the prominent form of folate is 5-Methyl-tetrahydrofolate (5-methyl-THF) monoglutamate. About 50% of folate is bound to albumin, in plasma about one-third is in a free form and a small proportion is bound to the plasma folate receptor. Folate molecules enter the cell through folate receptors (FRs) via endocytosis, with FR-α having a high affinity for the monoglutamate 5-methylTHF (42).

The 5,10-methylenetetrahydrofolate (5,10 methyl-THF), one of the one-carbon substituted forms of tetrahydrofolate present in the cell, plays a central role in the folate and methionine cycles, as it can be used for thymidylate synthesis, or converted to 5-methyl-THF in the methionine synthesis cycle, or oxidized to 10-formyl-tetrahydrofolate for purine synthesis. The reduction of 5,10 methyl-THF to 5-methyl-THF is achieved by the methylenetetrahydrofolate reductase (MTHFR), which is a riboflavin-dependent enzyme. In the methionine cycle, 5-methyl-THF is used by methionine synthase (MTR) for the vitamin B12-dependent conversion of homocysteine to methionine and the formation of tetrahydrofolate (THF). Methionine synthase reductase encoded by MTRR gene is required for the reactivation and proper function of MTR (47).

Vitamin B12 in food is bound to proteins and is released in the stomach under the influence of hydrochloric acid and pepsin. The thus released vitamin initially binds to dietary proteins, including haptocorrin (transcobalamin I, TC-I). Cobalamin is released from its complex with TC-I by pancreatic enzymes in the duodenum and then free vitamin binds to gastric intrinsic factor (IF), a glycoprotein produced in the stomach. After IF-cobalamin complex is absorbed through receptor-mediated endocytosis in the lower part of small intestine, cobalamin is released into the blood stream primarily in the form of methyl-cobalamin and the IF is degraded in lysosomes. A protein called transcobalamin II (TC-II) combines with cobalamin to form holotranscobalamin-holoTC (metabolic active cobalamin) and the complex is transferred to the cells after binding to the transcobalamin receptor TC-R. The vitamin's dietary source, its ability to be released from food and bind appropriately to IF determine cobalamin's absorption. The main fraction of plasma cobalamin (70–90%) binds to haptocorrin, while holoTC accounts for 10–30% of total plasma levels (47, 97).

Vitamin B12 is involved in the cytosolic transmethylation of homocysteine to methionine by the enzyme methionine synthase. Cobalamin is also required as coenzyme to form succinyl coenzyme A (CoA) from methylmalonyl CoA in propionate metabolism by methylmalonylCoA mutase in mitochondria (97).

Biomarkers of Status

A sensitive and short-term indicator of folate dietary intake is serum or plasma folate concentration (98), while red blood cell (RBC) folate concentration is a marker of long-term intake responding gently to changes—and the most reliable biomarker of status, as it indicates tissue folate stores (96). The assessment of folate status should include either multiple measurements of serum/plasma folate over several weeks or a single measurement combined with other biomarkers of status (RBC folate). This combination is recommended by a World Health Organization (WHO) Technical Consultation on folate and cobalamin deficiencies, at the population level (96, 99).

Folate adequacy is described by serum/plasma and red blood cell folate concentrations of higher than 10 nmol/L (4.4 ng/mL) and 340 nmol/L (150 ng/mL), respectively. Serum folate and RBC concentrations of less than 6.8 and 317 nmol/L, respectively, are indicative of folate deficiency. Folate deficiency adversely affects DNA replication and synthesis and thus cell division. As a result, bone marrow may produce uncommonly large cells with abnormal maturation of nuclei and

development of megaloblastic anemia (96). Furthermore, folate deficiency impairs the methionine cycle functions, resulting in elevated plasma homocysteine and insufficient production of the S-adenosylmethionine (SAM) which is extensively used in methylation reactions (100). The association of folate status during pre-conceptional period and appearance of developmental anomalies, including neural tube defects (NTD), has long been established (101).

Serum **cobalamin** is the most widely used biomarker of vitamin B12 status, reflecting both the metabolically active cobalamin bound to TCII and the inert fraction bound to haptocorrin. However, since its concentration associates weakly with the biomarkers of cobalamin function, serum holoTC is the most specific biomarker for assessment of adequate vitamin B12 status. Other biomarkers studied (e.g., methylmalonic acid and homocysteine) are elevated in cobalamin deficiency but are influenced by numerous dietary and lifestyle factors or conditions (in poor renal function, for instance) (99, 102). Thus, their interpretation requires a more complete assessment of the body nutritional status (99).

In clinical settings, serum total **vitamin B12** is used to assess adequacy, with serum total B12 concentrations below 148 pmol/L indicating deficiency. Notwithstanding the limitations indicated in the previous section, the concentrations of homocysteine and methylmalonic acid in blood have also been used to assess vitamin B12 status, with homocysteine levels above 15 μmol/L and methylmalonic acid above 750 nmol/L frequently used in adults to diagnose cobalamin deficiency. Clinical cobalamin deficiency is most frequently associated with megaloblastic anemia, as well as with neurological symptoms (97).

Genetic Variations and Folate Status

Several polymorphisms in genes encoding enzymes and transport proteins of folate metabolism are reported to affect folate status, homocysteine levels, and health outcomes. Folate-mediated one-carbon metabolism and related genes have been illustrated (101). To date, most studies have reported that the MTHFR C677T (rs1801133) SNP is related to both biomarkers of status and disease risk (54, 103–105). Together with rs1801131, they are the most well-known genetic factors influencing folate status that have been studied in great detail in terms of molecular mechanism and impact on disease risk (29, 42). However, the 1298A-C MTHFR variant (rs1801131), which is in strong linkage disequilibrium with the 677C-T variant, has no effect on biomarkers of folate status (42, 48). The distribution of the polymorphism shows a substantial variation worldwide and across ethnic groups. The frequency of the MTHFRrs1801133 TT SNP is described to be high in Europeans, Asians, Central, and South Americans (10–32%) and low in several African populations (0–3%) (106). Binia et al. (107) reported that the TT genotype presents at about 25 and 57% in Mexican Mestizo and American-Indian populations, respectively.

The common rs1801133 variant is a C to T transition at position 677, resulting in substitution of alanine with valine. MTHFR is a flavoprotein incorporating loosely bound flavin adenine dinucleotide (FAD). This substitution results in weaker binding affinity for the riboflavin (vitamin B2) cofactor and increased loss of the FAD cofactor, creating a mildly dysfunctional thermolabile protein (108). Homozygosity for the T allele (TT genotype) is associated with reduced enzyme activity (109), lower serum and RBC folate and higher plasma homocysteine levels compared with the 677CC (31, 110, 111). The well-documented association between MTHFR rs1801133 and folate status has also been evaluated in more recent studies (13, 18, 48).

In their meta-analysis of the association between the MTHFR rs1801133 polymorphism and blood folate concentrations (plasma/serum, RBC), Tsang et al. (54) reported a steady difference in serum/plasma and RBC folate concentrations across MTHFR rs1801133 genotypes showing a distinct pattern of CC > CT > TT. The percentage difference was highest for the CC vs. the TT genotype [S/P = 13%, 95% credible interval (CrI) derived from Bayesian statistics = 7–18%] [RBC = 16%; 95% CrI = 12–20%] followed by CC vs. CT (S/P = 7%; 95% CrI = 1–12%] [RBC = 8%; 95% CrI = 4–12%) and CT vs. TT (S/P = 6%; 95% CrI = 1–11%] [RBC = 9%; 95% CrI = 5–13%]. The inheritance of one recessive allele (i.e., the CT genotype) is related to concentrations intermediate to the CC and TT genotypes, as described by the additive model (54).

Hyperhomocysteinemia is known to be most pronounced if the TT genotype occurs in combination with low nutritional status of either folate (110) or riboflavin (31, 39). Several studies have reported that individuals with the homozygous TT genotype exhibit decreased response of folate biomarkers to folate intervention compared to those with the homozygous CC genotype, suggesting a higher requirement for folate (29). The EFSA NDA Panel has taken this polymorphism into account in setting the dietary reference values for folate, applying a coefficient of variation of 15% to address additional variability (96). Moreover, genetically at-risk adults (TT homozygotes) also have higher riboflavin requirements in order to maintain adequate enzyme function (39, 40). Among TT genotypes, riboflavin supplementation results in decreased homocysteine concentrations by 22% overall ($p = 0.03$) and 40% among individuals with lower riboflavin status at baseline ($p = 0.010$) (40).

Genetic Variations and Vitamin B12 Status

In a recent publication, Surendran et al. (51) reviewed candidate gene studies and GWAS published until May 2017 and conducted primarily among Caucasian populations, in order to identify associations between SNPs in genes related to vitamin B12 pathway and their impact on the circulating cobalamin levels. Authors present a comprehensive description together with schematic presentations of SNPs in genes related to co-factors, regulators of the vitamin transport, membrane cobalamin transporters or factors involved in enzymatic reactions of the one-carbon cycle (e.g., MTHFR and MTRR).

In their review, Surendran et al. (51) present, among others, the fucosyltranferase 2 gene. The FUT2 or secretor (Se) gene is located on chromosome 19 coding the a(1,2) fucosyltransferase enzyme that is involved in the production of H antigens (common precursors for the blood group A and B antigens expressed on secretory glands and digestive mucosal surfaces)

and may also influence B12 absorption at the gastric level. In a meta-analysis of studies among US Caucasian populations Hazra et al. (27) concluded that the SNP rs601338, also known as 428 G/A non-secretor variant, was significantly associated with plasma vitamin B12 concentrations. In particular, the A allele was positively associated with circulating vitamin B12 levels (β = 0.06 pg/mL, SE = 0.01). There is a heterogeneity in associations between FUT2 polymorphism and cobalamin concentrations, as the distribution of the minor allele of rs601338 considerably varies among ethnicities. Hazra et al. (27) reported that the rs492602 SNP is in complete linkage disequilibrium with rs601338. The G allele of the SNP rs492602 variant was associated with lower vitamin B12 levels (β = 0.06 pg/mL, SE = 0.01) Interestingly, in another study among 3114 Canadian adults, the G allele of the same SNP was found to be associated with a lower risk (OR = 0.60, 95% CI = 0.54–0.70) of vitamin B12 deficiency (<148 pmol/L), compared to A allele (62). The most commonly studied FUT2 variant however is the rs602662 SNP, which is also reported to be in linkage disequilibrium with the rs601338 SNP. Carriers of the A allele in the rs602662 variant are at lower risk (OR = 0.61, 95% CI = 0.47–0.80) of vitamin B12 deficiency (<148 pmol/L), compared to those carrying the G allele (62). Genetic variations in the FUT2 gene have also been implicated in alterations of the composition of the gut microbiome and individuals with the non-functional FUT2 phenotype (non-secretors) are susceptible to Helicobacter pylori infection and subsequent vitamin B12 malabsorption (51).

Another variant described in the Surendran et al. (51) review is the transcobalamin 2 (TCN2) gene, located on chromosome 22, which encodes transcobalamin II (vitamin B12 binding protein). The most commonly reported association between TCN2 polymorphism and vitamin B12 levels in Caucasians is the SNP rs1801198, characterized by C to G substitution at nucleotide 776 (TCN2 776C>G) and an exchange of proline to arginine at codon 259. In a candidate gene association study among 613 Irish men (50), individuals with the TCN2 776CC genotype were associated with lower serum vitamin B12 levels compared to those with 776 CG (adjusted $P = 0.03$) and 776GG genotypes (adjusted $P = 0.045$). In contrast, in a cross-sectional study of 122 individuals from Portugal, holotranscobalamin (Holo-TC) levels were significantly associated with the SNP rs1801198; carriers of the G allele had lower Holo-TC concentrations than C carriers ($P < 0.05$) (17), a finding which has also been reported in an earlier cross-sectional study (57).

Cubulin (CUBN), CD320 and methionine synthase reductase (MTRR) genes are also vitamin B12 related genes described in the Surendran et al. (51) review. CUBN is known as the intrinsic factor-cobalamin (IF-B12) receptor located on chromosome 10 and variants within this gene have been associated with B12 status, but results are often conflicting. Hazra et al. (27) reported that the A allele of the rs1801222 (Ser253Phe) variant was associated with lower cobalamin status (β = −0.05 pg/mL, SE = 0.01) in 4763 US individuals, while subjects homozygous for the rs1801222 G allele had higher vitamin B12 concentrations. In contrast, Zinck et al. (62) described that the G allele of the rs1801222 was associated with an increased risk of cobalamin deficiency (OR = 1.61, 95% CI = 1.24–2.09).

The CD320 or "CD320 molecule" gene encodes the transcobalamin receptor (TCblR) and is located on chromosome 19. The most commonly studied variant is the rs2336573 variant characterized by a glycine to arginine change, at codon position 220. Zinck et al. (62) reported that the C allele of this variant was associated with a lower risk (OR = 0.62, 95% CI = 0.45–0.86) of inadequate vitamin B12 levels (<220 pmol/L). An earlier study among Icelandic and Danish individuals ($n = 45{,}571$ adults), however, reported that the "T" allele was associated with increased vitamin B12 concentrations (effect β = 0.22–0.32 pmol/L; $P = 8.4 \times 10^{-59}$) (23).

Genetic variants in methionine synthase reductase (MTRR) gene, located on chromosome 5, have also been associated with vitamin B12 levels in healthy individuals. Among them, SNP MTRR rs162036 (Lys350Arg) was found to be associated with vitamin B12 levels in a study of 2424 twins of North European descent (Caucasians) ($p = 0.04$) (4).While the majority of candidate gene association studies did not provide evidence ($P > 0.05$) for an association between the MTHFR gene polymorphisms (rs1801131 and rs1801133) and cobalamin concentrations, Thuesen et al. reported that the TT genotype of the rs1801133 variant was associated with a higher risk of vitamin B12 concentrations below 148 pmol/L compared with the CC genotype (OR = 1.78, 95% CI = 1.25–2.54) in a study among Danish participants (52).

DISCUSSION

We have undertaken a comprehensive narrative review of the literature available until March 2020 in order to identify genetic variants extensively studied that could impair the body's vitamin status. The fat-soluble vitamins D and E and the water-soluble vitamins C, B9 (folate), and B12 (cobalamin) prevail in the corresponding literature and have therefore been presented in this review. Based on the outcomes of candidate gene studies, GWAS and the meta-analysis thereof, several SNPs have been identified to impair enzymes, carrier proteins, cell membrane channels and similar routes and substances that can subsequently affect the absorption, release in the blood stream and cell uptake of the vitamins under study. Therefore, through possible impacts on the vitamins' physiology, metabolism, and functionality, several of these genetic variants could determine the individual's vitamin status following an intake through the diet. Evidence is however not always consistent and we opted for the presentation of both converging and diverging results which could be attributed to either the heterogeneity among the populations studied, lack of comparability between methods and different biomarkers of vitamin status or a combination of all.

Furthermore, for the vast majority of these polymorphisms, prevalence rates in the general population are not known and cannot be used for setting advice on dietary requirements at the population level or design public health actions. An exception, however, holds for the European dietary reference values for folate in which the MTHFR genotype (rs1801133) has been

taken into consideration. In particular, after setting the Average Requirement for folate, the EFSA Panel of Nutrition, Novel Foods and Food Allergens (96) considered evidence on the prevalence of the various genotypes in the European population and on the higher folate requirements among individuals with the MTHFR 677TT, compared to those with the MTHFR 677CC genotype, and selected a larger coefficient of variation to be applied in setting the Population Reference Intake.

Next to their impact on vitamin status, several of the polymorphisms presented have also been associated with disease risk. Variants in the vitamin D-related VDR gene have commonly been associated with the risk of a range of skeletal and non-skeletal health outcomes, including infections from the respiratory syncytial virus, the hepatitis B virus; tuberculosis; cancer, and autoimmune diseases such as systematic lupus erythematosus and rheumatoid arthritis (32, 112). Polymorphisms in the SLC23A1 and SLC23A2 genes as well as in other genes encoding the vitamin C transporter have also been associated with the risk of chronic diseases, including cancer, inflammatory bowel disease, preterm delivery, coronary heart disease, and glaucoma (113).

Several studies indicated gene-diet interactions between folate intake, MTHFR C677T genotype and risk of breast, colorectal, and lung cancer (114). With respect to colorectal cancer, meta-analyses of case-control studies provided evidence that the 677TT genotype is associated with a lower risk of colorectal cancer when compared to the CC genotype (105, 115). In another meta-analysis of case-control studies including CHD patients, individuals with the 677TT genotype had a significantly higher risk of CHD (OR = 1.16, 95% CI = 1.05–1.28) compared to the CC genotype, particularly when combined with low folate status (OR = 1.44, 95% CI = 1.12–1.83) (116). Cronin et al. (104) reported a dose-response association between the MTHFR 677T allele and the risk of ischemic stroke (T allele pooled OR = 1.17, 95% CI = 1.09–1.26 and TT genotype pooled OR = 1.37, 95% CI = 1.15–1.64, respectively). Homozygosity for the MTHFR C677T polymorphism (TT genotype) has also been associated with an increased risk for NTD-affected pregnancies (103), which provides additional support to the well-reported link between folate status and NTD risk. The presentation of such gene-disease associations were out of the scope of the present manuscript.

Variants related to vitamin E transport have also been associated with disease risk. Apo-AV is a minor apo-lipoprotein almost exclusively expressed in liver that plays a significant role in the regulation of plasma triglycerides. The most studied SNP in Apo-AV is rs662799 where the minor variant (C) has been associated with both higher plasma vitamin E and VLDL-TGs levels in diabetic patients. Guardiola and Ribalta (117) reported that in patients with metabolic syndrome or type 2 diabetes, apo-AV variants increase TG levels and minor allele carriers present an altered lipoprotein profile including large VLDL and small LDL and HDL particles that characterize atherogenic dyslipidemia.

Some considerations are crucial when reporting and interpreting genetic studies in relation to vitamin status. Confounding generated through the use of less appropriate comparators can lead researchers to spurious findings. Ethnicity and race are for instance important characteristics that can confound the associations observed (118). In our review, we generally selected to present studies that controlled for self-reported ethnicity. Misclassification due to either the biomarker of vitamin status used or the analytic validity of genetic markers is an additional issue to consider. As regards the vitamin-related biomarker, we have solely relied on studies presenting associations between genetic variants and conventional biomarkers of vitamin status that have been widely used by national and international scientific bodies (64, 68, 80, 88, 90, 96, 97). With respect to the validity of the genetic markers used in the studies we report in this review, unless errors are systematic, any misclassification would have probably attenuated the association between a dichotomous biomarker and the risk of low vitamin status, but it would probably not generate an association when in reality this does not exist. Studies of genetic epidemiology making use of numerous gene variants suffer from an increased possibility for Type I and Type II errors, particularly when authors overemphasize on statistical testing. In our presentation of results, we selectively described the outcome of methods that addressed the problem of false positive associations resulting from multiple comparisons (e.g., *p*-values after the application of Bonferroni correction or Bayesian methods, when available). Next to statistical significance, biological relevance needs to be also taken into account in combination or as a separate criterion to establish causality.

The strengths of our narrative review lie in the provision of a collective description of converging and diverging evidence regarding genetic variants that influence vitamin status in response to dietary intake. Furthermore, our review summarizes studies among individuals from the general population, free of prevalent diseases that could affect their nutritional status addressing thus issues related to the public health. Our review is limited by the fact it focuses on vitamin deficiencies. However, it should be pointed out that the excess of water-soluble vitamins is physiologically controlled through homeostatic mechanisms and health professionals are primarily concerned with the deficiency rather than the excess of lipo-soluble vitamins (vitamins D and E, in our case).

The ongoing miRNA research is providing additional evidence on gene expression, body function and many other biological processes. miRNAs have been reported to target vitamin-related genes and human circulating vitamin levels (119). Nevertheless, the currently published research primarily explores such associations among patients of degenerative diseases/conditions. The demonstration of possible interactions among free-living, generally healthy individuals is yet to be accumulated and will substantially enhance our knowledge on disease progression.

In conclusion, our review summarizes current findings on how genetic variants could shape inter-individual differences in response to dietary intakes. Capturing these differences in intakes and mitigate sources of bias that could confound any association observed has traditionally been the goal of objective dietary assessment studies. The consideration

of genetic variants has its own share in improving our understanding of the diet-disease inter-relationship. The individual genetic profile could add an extra layer of personalization in nutritional evaluation and offer a more comprehensive dietary assessment. Next to their association with vitamin status, genetic variants have also been implicated in underlying needs for differential vitamin intake to prevent chronic, diet-related diseases. The early knowledge of those needs could drive personalized nutritional advice favoring prevention. Well-designed studies addressing biological relevance next to statistical significance are however needed if the identification of genetic variants it to be included in future dietary assessments.

AUTHOR CONTRIBUTIONS

ANi performed the literature review, in collaboration with VK. ANi drafted the paper. All authors contributed in the development of the submitted manuscript. ANa has the primary responsibility for the manuscript's final content.

ACKNOWLEDGMENTS

The manuscript has been prepared in the context of a collaboration between the Dept. of Hygiene, Epidemiology and Medical Statistics of the NKUA School of Medicine and the DNANUTRICOACH®.

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Conflict of Interest: VK is the founder of MEDOLIALI SL, a nutrigenetic consulting and supporting SME.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 14 January 2021
doi: 10.3389/fnut.2020.577720

The Potential of Multi-Biomarker Panels in Nutrition Research: Total Fruit Intake as an Example

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Dietary and food intake biomarkers offer the potential of improving the accuracy of dietary assessment. An extensive range of putative intake biomarkers of commonly consumed foods have been identified to date. As the field of food intake biomarkers progresses toward solving the complexities of dietary habits, combining biomarkers associated with single foods or food groups may be required. The objective of this work was to examine the ability of a multi-biomarker panel to classify individuals into categories of fruit intake. Biomarker data was measured using ^{1}H NMR spectroscopy in two studies: (1) An intervention study where varying amounts of fruit was consumed and (2) the National Adult Nutrition Survey (NANS). Using data from an intervention study a biomarker panel (Proline betaine, Hippurate, and Xylose) was constructed from three urinary biomarker concentrations. Biomarker cut-off values for three categories of fruit intake were developed. The biomarker sum cut-offs were ≤4.766, 4.766–5.976, >5.976 μM/mOsm/kg for <100, 101–160, and >160 g fruit intake. The ability of the biomarker sum to classify individuals into categories of fruit intake was examined in the cross-sectional study (NANS) (N = 565). Examination of results in the cross-sectional study revealed excellent agreement with self-reported intake: a similar number of participants were ranked into each category of fruit intake. The work illustrates the potential of multi-biomarker panels and paves the way forward for further development in the field. The use of such panels may be key to distinguishing foods and adding specificity to the predictions of food intake.

Keywords: dietary biomarkers, nutrition, dietary assessment, food intake, metabolomics, multi-biomarker panel

OPEN ACCESS

Edited by:
John Draper, Aberystwyth University, United Kingdom

Reviewed by:
Carla Ferreri, National Research Council (CNR), Italy
Jose Paulo Andrade, Universidade do Porto, Portugal

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *29 June 2020*
Accepted: *17 December 2020*
Published: *14 January 2021*

Citation:
McNamara AE, Walton J, Flynn A, Nugent AP, McNulty BA and Brennan L (2021) The Potential of Multi-Biomarker Panels in Nutrition Research: Total Fruit Intake as an Example. Front. Nutr. 7:577720. doi: 10.3389/fnut.2020.577720

INTRODUCTION

Dietary assessment is important for the elucidation of diet-disease relationships; however, traditional dietary assessment techniques are subject to some well-documented limitations (1). The identification of dietary and food intake biomarkers offers the potential of accurate and objective measure of food intake (2, 3). Food intake biomarkers are single metabolites, or a combination of metabolites, reflecting the consumption of either a specific food or food group, displaying a clear time- and dose-response after intake (4). An extensive range of putative intake biomarkers

of commonly consumed foods have been identified to date (5) but more work is needed toward confirming the utility of such biomarkers. To be efficient indicators of dietary intake, biomarkers need to be validated including analysis of sensitivity, specificity, and dose-response (6). Selecting a single intake biomarker to represent exact dietary intake is difficult due to the overlapping range of nutrients, non-nutrients, and bioactives present in foods. However, previously investigated food intake biomarkers have proven to be good predictors of consumption for classes within food groups, such as fruits and vegetables (7). As the field of food intake biomarkers progresses, and the need to elucidate the complexities of dietary habits arises, combining biomarkers associated with single foods or food groups may be required (8). It is possible that by combining two or more biomarkers together into multi-biomarker panels could result in more sensitive and specific estimates of intake.

Development of validated and comprehensive multi-biomarker panels have the potential to add value to the assessment of dietary intake by enabling the capture of a broad range of dietary exposure including bioactive compounds, foods, food groups, and complex dietary patterns (9). Multi-biomarker panels have been previously used to classify individuals into banana consumers and non-consumers more robustly compared to individual biomarkers (10). In another example a multi biomarker panel, composed of beer ingredient and food processing biomarkers was capable of distinguishing beer consumption from urine samples collected before and up to 12 h after intake of beer with excellent specificity and sensitivity (11). Other examples of successful use of multiple biomarkers include the SU.VI.MAX study, where the sum of urinary flavonoid biomarkers demonstrated higher correlations with fruit and fruit juice consumption than any of the included biomarkers individually (12). A biomarker panel containing the wine biomarkers ethyl glucuronide and tartrate outperformed individual markers when predicting wine consumers and non-consumers [90.7% receiver operating characteristics (ROC) curves area under the curve (AUC) compared to 86.3% for ethyl glucuronide and 85.7% for tartrate]. This panel was validated in an epidemiological study and was capable of estimating whether or not participants had consumed wine in the previous 3 days (13).

Multi-biomarker panels can be applied to classify and monitor adherence to dietary patterns. This was recently illustrated in post-menopausal women where a biomarker panel was capable of discriminating between high and low quintiles of adherence to four diet scores [the alternate Mediterranean diet score (aMED), alternate Healthy Eating Index (AHEI)-2010, Dietary Approaches to Stop Hypertension (DASH) diet, and the Healthy Eating Index (HEI)-2015] (14). Another interesting application of multiple biomarkers is the interrogation of the relationships between food intake and diseases incidence. In this context previous work has employed a biomarker score, derived from multiple biomarkers of fruit and vegetable intake, as a proxy for intake to examine the relationship with diabetes incidence (15). This biomarker score demonstrated an inverse association with diabetes incidence, with odds ratio (OR) of incidence decreasing with increasing intake of fruit and vegetables (highest quartile of intake compared to lowest OR: 0.13; 95% CI: 0.08, 0.21) (15). Collectively, these studies highlight the potential for combinations of multiple biomarkers in determining intake of foods or dietary patterns and assessment of relationships with health outcomes.

Fruit is an important component of a healthy diet and previous work has investigated biomarkers of various fruit. A recent systematic review of the literature conclude that we have limited knowledge for biomarkers of pome and stone fruit (16) with many biomarkers requiring validation in terms of relating to fruit intake. With respect to apple intake the most promising biomarker identified was phloretin and phloretin glucuronide. The biomarker arbutin is promising for pear intake but again requires more validation. A review of biomarkers of tropical fruit intake also highlighted a dearth of information (17), concluding clearly that there is a need for further research in the area. Proline betaine is a well-established biomarker of citrus intake with previous work indicating that urinary proline betaine concentrations can give quantitative food intake information (18, 19). However, as most consumers eat more than one fruit, there is an interest to examine total fruit intake and thus combining multiple biomarkers could be a useful approach to estimate total fruit intake.

Although previous work has indicated the potential of multiple biomarkers in the form of multi biomarker panels, combining of biomarkers to give quantitative information on food intake is not trivial. Previous studies have highlighted the potential of multi biomarker panels in terms of classification into consumers and non-consumers. However, more work is needed in the field to examine the potential of such biomarker combinations for use in assessment of consumption of different quantities of food. Therefore, the objective of this work was to examine the ability of a multi-biomarker panel to classify individuals into categories of fruit intake.

MATERIALS AND METHODS

National Adult Nutrition Survey (NANS) Study—Cross-Sectional Study

Details of the NANS study have been published elsewhere (https://www.iuna.net/) (20). Ethical approval for this study was granted by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals [ECM 3 (p) 4 September 2008] and recruitment began in May 2008. Briefly, NANS collected data on habitual food and beverage consumption, lifestyle, health indicators, and attitudes to food and health in 1,500 adults, representative of the population during 2008–2010 in Republic of Ireland. A subset of this population ($N = 565$) was randomly selected, to ensure equal numbers of men and women across the age ranges (18–90 years) for metabolomics analysis as previously described (20). A 4 days semi weighed dietary record was used to collect dietary data over 4 consecutive days. Detailed information on the type and amount of all foods, drinks and nutritional supplements consumed over the 4 days was recorded by participants. Where possible, participants were asked to weigh foods and encouraged to retain

food packaging for further information on foods consumed, but where weights were not recorded a photographic food atlas was used to estimate food weights (18). Dietary data was analyzed using WISP software (Tinuviel Software, Anglesey, UK), which uses data from McCance and Widdowson's The Composition of Foods, fifth and sixth editions and all supplementary editions to generate nutrient data (21–24). Dietary data was coded into 2552 individual food codes and grouped into one of 68 food groups. For the purpose of this analysis any fruit containing food groups were collapsed into a single "Total Fruit Intake" group and the mean daily fruit intake over the 4 days was calculated. Biological samples were collected at the end, or as close to as possible, of the dietary recording period, including a fasting first void urine sample in a sterile 50 mL tube which was chilled until processing. All urine samples were centrifuged at 1,800 × g for 10 min at 4°C. Aliquots of 1 mL were stored at −80°C for later analysis.

A-Diet Validation Intervention Study

Ethical approval for this A-Diet study was granted by the UCD Sciences Human Research Ethics Committee (LS-17-16-Brennan). Recruitment commenced at the end of March 2017 via advertisement (posters, flyers, and emails) and finished in November 2017. The recruitment process is outlined in **Supplementary Figure 1**. Details of the study design are published elsewhere (25). Briefly, inclusion criteria included healthy, non-pregnant/lactating and non-smoking individuals, between 18 and 60 years old, and with a body mass index (BMI) between 18.5 and 30 kg/m^2, without any diagnosed health condition (chronic or infectious diseases), no consumption of medications/nutritional supplements or any allergies/intolerances to the test foods. Once informed consent was acquired, participants were assigned to either a lunch (N = 27) or dinner (N = 34) test meal group and invited to partake in a 5-weeks study. Each test week participants were provided with four portions of a test meal and asked to consume this test meal for 4 consecutive days. During these 4 days, participants were also asked to avoid consuming any other foods related to the test meal ingredients. Participants were also asked to record a 4 days dietary record for each test week, to ensure compliance. The focus of this analysis is the amount of fruit consumed as part of these test meals. The low, medium, and high portions of fruit provided was 50, 100, 300 g and 80, 160, 320 g for apples and oranges, respectively.

Fasting first void urine was collected after an overnight 12 h fast at the end of each test week and chilled until processing. All urine samples were centrifuged at 1,800 × g for 10 min at 4°C. Aliquots of 1 mL were stored at −80°C for later analysis.

Metabolomic Analysis of Urine Samples

Metabolomic analysis was performed using nuclear magnetic resonance (NMR) spectroscopy. Urine samples were first defrosted and then prepared by addition of 250 μL phosphate buffer (0.2 mol KH_2PO_4/L, 0.8 mol K_2HPO_4/L) to 500 μL urine. After centrifugation at 5,360 × g for 5 min at 4°C, 10 μL sodium trimethylsilyl [2,2,3,3-2H$_4$] propionate (TSP) and 50 μL deuterium oxide (D_2O) were added to 540 μL of the supernatant. Spectra were acquired on a 600 MHZ Varian Spectrometer (Varian Limited, Oxford, United Kingdom) by using the first increment of a nuclear Overhauser enhancement spectroscopy pulse sequence at 25°C. Spectra were acquired with 16,384 data points and 128 scans. Water suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms). All ^{1}H NMR urine spectra were referenced to TSP at 0.0 parts per million (ppm) and processed manually with the Chenomx NMR Suite (version 7.7) by using a line broadening of 0.2 Hz, followed by phase and baseline correction. Three metabolites were chosen to demonstrate the proof-of-concept that combinations of biomarkers could be used to predict total fruit intake. The biomarkers chosen were xylose, proline betaine, and hippurate. Identification and quantification of metabolites was achieved using the Chenomx library. To confirm correct assignment, a urine sample was spiked with an analytical standard and a ^{1}H NMR spectrum acquired.

Osmolality was measured by using an Advanced Micro Osmometer model 3300 (Advanced Instruments). Aliquots of urine were measured for osmolality with the use of micro-osmometry by freezing point depression, with values reported as the number of solute particles, in moles, dissolved in a kilogram of urine (mOSM). Metabolite concentrations were normalized to osmolality for quantifying urinary concentrations of xylose, proline betaine and hippurate.

Statistical Analysis

Statistical analysis was performed using IBM SPSS software package version 24.0 for windows (SPSS Inc. Chicago, IL, USA) and SIMCA-P software (version13; Umetrics). One-way analysis of variance was performed to compare tertiles of self-reported intake and urinary concentrations of three biomarkers. Spearman's correlations were used to assess association between mean daily self-reported total fruit intake and biomarkers. The urinary concentrations of each biomarker were summed together to create a single combined biomarker value for each individual. Using the intervention data cut-offs were developed for certain fruit intake categories.

RESULTS

Examining Relationship Between Biomarkers and Self-Reported Fruit Intake

Urinary biomarkers of interest were quantified by ^{1}H NMR analysis in the NANS cross-sectional study. The following concentrations were obtained: xylose (range: 0.07–2.19 mM), proline betaine (range: 0.04–2.13 mM), and hippurate (range: 0.13–15.87 mM). Participants were grouped into tertiles based on self-reported mean daily intake of total fruit from semi-weighed food records (**Table 1**, **Supplementary Table 1**). All three urinary biomarker concentrations increased as the intake of fruit increased with significant increases observed for proline betaine and hippurate. The urinary concentrations of all three biomarkers were also significantly correlated with mean daily intake of fruit. The main contributors to total fruit intake were apples, bananas, oranges, pears, strawberries, and pineapple, all of which increased significantly across tertiles of intake except for pineapple.

TABLE 1 | Urinary food intake biomarker concentrations across tertiles of fruit intake in cross-sectional study (NANS).

	Tertile 1 ($N = 180$)		Tertile 2 ($N = 181$)		Tertile 3 ($N = 185$)			Spearman's correlation^{+}	
	Mean	***SD***	**Mean**	***SD***	**Mean**	***SD***	***p*-value**	**Rho**	***p*-value**
Metabolite (μM/mOsm/kg)									
Xylose	0.596	0.34	0.614	0.35	0.637	0.42	0.574	0.078	0.070
Proline betaine	0.285	0.22	0.415	0.34	0.569	0.44	<0.001	0.400	<0.001
Hippurate	3.600	2.78	3.991	2.87	4.727	3.20	0.001	0.181	<0.001
Mean daily intakes (g/d)									
Total fruit	28.42	24.61	127.80	32.61	315.19	115.44	<0.001		
Apples	4.94	13.50	23.93	30.76	58.15	65.78	<0.001		
Banana	7.17	15.28	26.86	31.77	43.07	42.70	<0.001		
Oranges	2.49	9.86	29.56	42.55	94.14	105.97	<0.001		
Pears	0.52	3.70	4.46	15.61	22.18	47.46	<0.001		
Strawberry	1.03	5.41	2.48	9.34	4.44	13.40	0.005		
Pineapple	0.47	3.73	1.20	7.47	2.75	16.91	0.129		

All values presented are mean and standard deviation (SD) unless stated otherwise. ANOVA, analysis of variance; MDI, Mean daily intake. N = 546.
$^{+}$Spearman's correlation between each biomarker and total mean daily fruit intake.

Further analysis was performed using total fruit consumers only ($N = 509$) (**Table 2**). Similar trends were observed for the urinary biomarker concentrations, increasing across of tertiles of intake with significant values for proline betaine and hippurate.

Using Multiple Biomarkers to Classify Individuals Into Categories of Intake

The multi-biomarker panel was used to create a combined biomarker value by summing the individual biomarkers. In the A-Diet validation dataset ($N = 160$) the combined biomarker values were used to determine cut-offs for classification of individuals into categories of intake. Participants were categorized into one of three groups of intakes (0–100, 101–160, and >160 g/d) based on the fruit consumed as part of the intervention. The average sum of biomarkers for participants who consumed ≤100 g/day of fruit (4.766 μM/mOsm/kg) was set as cut-off point 1. Cut-off 3 was calculated as the average biomarker sum (5.976 μM/mOsm/kg) of participants who consumed >160 g of fruit in the A-diet study. The middle cut-off was set at any value in between cut-off 1 and 3 (**Table 3**).

To examine the ability of these biomarker sum values to categorize participants into categories of intake the method was applied to NANS study. Using the NANS ($N = 546$) self-reported dietary data participants were categorized into three groups of fruit intake (**Table 4**). Independently using the urinary biomarker concentrations, participants were assigned to a category of intake using the biomarker sum cut-offs. Excellent agreement between the two methods was observed with for example, 97 participants self-reporting intakes between 101 and 160 g/d, and 86 participants assigned into this category based on the biomarker panel data (**Table 4** and **Supplementary Table 2**). Assessment of the data split by gender revealed similar agreement trends (**Table 5**).

In order to examine other biomarker combinations, the biomarker values that increased across categories of intake were selected (**Supplementary Table 3**). The ability to categorize participants into categories of intake was examined with all biomarkers and the sum of proline betaine and hippurate providing the best agreement (**Table 6**).

DISCUSSION

This study developed a biomarker panel that was capable of classifying individuals into categories of fruit intake. Examination of the approach in a free-living cross-sectional study revealed excellent agreement with self-reported intake. Similar number of participants were ranked into each category of fruit intake. The work illustrates the potential of multi-biomarker panels and paves the way forward for further development in the field.

The three biomarkers chosen to build the multi-metabolite panel in this research have frequently been found in previous research to be associated with fruit intake. Xylose was previously identified as a food intake biomarker associated with apple intake and capable of ranking participants in an observational study by increasing intake (25). Proline betaine is a well-established, robust, and quantitative biomarker of citrus intake (18, 26). Proline betaine was previously included as part of a biomarker panel for the investigation of orange juice intake and juice quality (27). Hippurate, has often been associated with consumption of polyphenol rich plant foods, such as citrus fruit, bananas, and berries (10, 26, 28–30). This research combined these three food intake biomarkers into a biomarker score for the successful ranking of fruit intake. It is important to acknowledge that there are other potential biomarkers of fruit intake; however, examination of the full panel of potential biomarkers was beyond the scope of the current work where the emphasis was on the demonstration of the potential of combination of biomarkers.

Previous multi-metabolite panels have focused on associating the panels with intake of certain foods or achieving a dichotomous classification of consumers and non-consumers. An example of previous work demonstrated good associations

TABLE 2 | Urinary food intake biomarker concentrations across tertiles of fruit intake in cross-sectional study (NANS), total fruit consumers only.

Mean daily intakes (g/d)	Tertile 1 ($N = 168$)		Tertile 2 ($N = 168$)		Tertile 3 ($N = 173$)		One-way ANOVA	Spearman's correlation^{+}	
	Mean	*SD*	Mean	*SD*	Mean	*SD*	*p*-value	Rho	*p*-value
Metabolite (μM/mOsm/kg)									
Xylose	0.601	0.35	0.604	0.35	0.649	0.43	0.424	0.090	0.043
Proline betaine	0.298	0.24	0.425	0.33	0.581	0.45	<0.001	0.382	<0.001
Hippurate	3.622	2.63	3.957	2.82	4.771	3.24	0.001	0.187	<0.001
Mean daily intakes (g/d)									
Total fruit	42.67	26.44	139.58	32.28	323.36	114.98	<0.001		
Apples	8.66	18.54	25.60	33.86	59.09	66.35	<0.001		
Banana	10.05	17.90	29.61	32.63	43.12	43.46	<0.001		
Oranges	4.18	13.92	33.34	43.80	97.75	108.18	<0.001		
Pears	1.11	6.39	4.24	15.46	23.71	48.71	<0.001		
Strawberry	1.36	6.01	2.42	9.45	4.74	13.81	0.008		
Pineapple	1.07	7.08	1.14	7.21	2.55	16.75	0.399		

All values presented are mean and standard deviation (SD) unless stated otherwise. ANOVA, analysis of variance; MDI, Mean daily intake. N = 509.
$^{+}$Spearman's correlation between each biomarker and total mean daily fruit intake.

TABLE 3 | Cut-off points for each of the fruit intake categories derived from the Intervention Study.

Fruit intake category (g/d)	Biomarker cut-offs (μM/mOsm/kg)
0–100	<4.766
101–160	4.766–5.974
>160	≥5.975

Cut-offs were calculated using sums of biomarker concentrations in each intake category from the A-Diet intervention study.

TABLE 4 | Classification into categories of fruit intake based on biomarker data or based on self-reported intake data in the cross-sectional study (NANS).

Fruit intake category (g/d)	Self-reported data (N)	Biomarker data (N)
0–100	227	306
101–160	97	86
>160	222	154

Participants were classified into one of 3 categories based on either (1) Self-reported dietary data or (2) biomarker data. The number of participants into each category is reported, N = 546.

TABLE 5 | Distribution of participants classified into each category of intake based on sum of urinary biomarker concentrations compared to reported intake data in the cross-sectional study, split by gender.

	NANS			
	Self-reported		Predicted	
Gender	M	F	M	F
N	278	268	278	268
Intake category				
0–100 (g/d)	128	99	170	136
101–160 (g/d)	42	55	42	44
>160 (g/d)	108	114	66	88

Participants (N = 546) categories based on cut-offs were calculated using sums of biomarker concentrations in each intake category from the A-Diet intervention study. ADV, A-Diet Validation Study; NANS, National Adult Nutrition Survey; M, Male; F, Female; N, Number of participants.

between biomarkers of fruit and vegetables intake and self-reported dietary records (12). When 24 h urinary concentrations of isorhamnetin, hesperetin, naringenin, kaempferol, and phloretin were combined in a panel they were correlated with fruit intake ($r = 0.27$, $p = 0.06$), fruit juice intake ($r = 0.28$, $p = 0.04$), and intake of total fruits and fruit juices ($r = 0.38$, $p = 0.006$). The correlations for the fruit related panel improved when examined in spot urine samples: fruit intake ($r = 0.34$, $p = 0.01$), fruit juice intake ($r = 0.44$, $p = 0.001$), and total fruit and juice intake ($r = 0.47$, $p = 0.0004$). The authors concluded that this combination of flavonoids could be used as a reliable biomarker of total fruit and juice intake, however, to the best of our knowledge there is no demonstration that the biomarkers could predict intake. Our work is an important advancement as it clearly demonstrates that the multi-biomarker approach is capable of a classification of intake into a range of categories. However, it should be noted that the present work did not make the distinction between whole fruit and fruit juices and the biomarker panel classification was based on total fruit intake including juices.

Our previous work identified four food intake biomarkers of sugar sweetened beverages (formate, citrulline, taurine, and isocitrate) using heat-map analysis of metabolomic urinary profiles from the NANS study (31). These markers were confirmed by food analysis of the sugar-sweetened beverage and an acute intervention study. The markers were combined in a panel and ROC curves demonstrated that the panel could discriminate between consumers and non-consumers of sugar-sweetened beverages (AUC = 0.8) and was more

TABLE 6 | Urinary biomarker classification of fruit intake compared to reported intake in the cross-sectional study (NANS).

Classification method	Self-reported intake	Sum of proline betaine and hippurate	Sum of xylose and proline betaine	Sum of all biomarkers
Average fruit intake (g/d)	*N*	*N*	*N*	*N*
0–100	227	304	306	306
101–160	97	70	194	86
>160	222	172	46	154

Number of participants (N = 546) classified into each intake category was based self-reported intake and on combinations of urinary biomarker concentrations.

predictive of intake than the individual biomarkers themselves (AUCs ranging from 0.5 to 0.7). A recently published study used data from the KarMeN study to identify five metabolites [methoxyeugenol glucuronide (MEUG-GLUC), dopamine sulfate (DOP-S), salsolinol sulfate, 6-hydroxy-1-methyl-1,2,3,4-tetrahydra-β-carboline sulfate, and xanthurenic acid] that were discriminative between high and non-consumers of banana (10). Individually, DOP-S had the best prediction ability [AUC = 0.84, error rate (ER) = 0.25] for classifying high consumers against non-consumers but was not as robust as a combination of all five metabolites (AUC = 0.90, ER = 0.13). However, the best predictive ability was a combined panel of MEUG-GLUC and DOP-S with the lowest error rate of misclassification. This research demonstrates how a panel of food intake biomarkers which individually were not robust enough, when combined can be used to classify recent banana intake. Our research takes the application of biomarker panels a step further by classifying participants into categories of fruit consumed and moves beyond the dichotomous classification of consumer/non-consumer.

While the above work demonstrates the potential for multi-biomarkers in terms of estimating food intake such panels can also be used to assess the relationship between food and diet-related diseases. A combined biomarker-score was developed using the standardized plasma values of vitamin C, β-carotene, and lutein, all of which were previously related to fruit and vegetable intake (15). This score was inversely associated with odds of incidence of type 2 diabetes (OR: 0.13; 95% CI: 0.08, 0.21) even after adjustment for lifestyle factors and demographics. An identified plasma biomarker panel representative of dietary habits consisted of β-alanine (beef intake), alkylresourcinols (wholegrain/rye), eicosapentaenoic acid and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (fish), lauric acid (saturated fats), linoleic acid (seeds, nuts, and vegetable oils), oleic acid (olive and rapeseed oil), and α and γ tocopherol (32). This combined panel was capable of predicting new cases of type 2 diabetes over a 5-years follow-up period with a specificity and sensitivity similar to classic diabetes predictors (serum adiponectin, insulin resistance, impaired glucose tolerance and impaired fasting glycaemia). Collectively, these and other studies highlight the potential of a multi-metabolite panel for the assessment of the relationship between diet and health/disease.

If future studies develop comprehensive and validated multi-biomarker panels, they could add value to the assessment of dietary intake by enabling the capture of a broad range of dietary exposures including bioactive compounds, foods, food groups, and complex dietary patterns. Panels could then be used in epidemiological research to elucidate the mechanisms and metabolic pathways of diet-related diseases and to validate self-reported dietary data. Further development of more comprehensive panels could enable measurement of adherence to specific dietary patterns, such as the Mediterranean diet. Future challenges for the field will be finding the simplest combination of metabolites to accurately determine exposures as well as validating these panels to a standard where they can be applied in nutritional research and public health surveys (9). As the field develops further, there will be a need to develop new statistical tools to integrate multiple biomarkers with self-reported data. Our work has recently developed calibration equations based on biomarker-predicted citrus intakes to gain a more accurate and objective measure of true intake (33). Using biomarker data to correct self-reported data for food intake is a promising option and could be adapted to include a biomarker panel. Further work is to develop the statistical tools to achieve this.

This study has strengths and limitations. A limitation worth noting is the fact that we examined the ability of the biomarker panel to categorize fruit intake in a cross-sectional study where intake was estimated with self-reported data. Future studies where comparison is performed in large intervention studies would be useful to examine relationships with true food intake. On the other hand, including the development of a food intake biomarker panel with testing in cross-sectional study is a strength as it demonstrates that the panel was capable of ranking fruit intake at a population level, against the background of exposure to various other foods. This research did not examine the potential impact of gut microbiome on the biomarker panel. Future work is warranted to address this.

To conclude, this study successfully demonstrated the utility of a panel of biomarkers for estimating fruit intake. The identification of comprehensive and validated multi-biomarker panels related to certain foods will be important as this field develops. The use of such panels may be key to distinguishing foods and adding specificity to the predictions of food intake. Combining such panels with self-reported measures will be important for increasing the accuracy of dietary assessment methods. Furthermore, there is potential for the use of such panels in large epidemiological studies to examine the relationships between diet and disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**,

further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UCD HREC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM conducted the A-Diet Validation study, acquired, analyzed the data, and prepared the manuscript. LB designed the research, analyzed the data, and prepared the manuscript. BM, AN, JW, and AF contributed the data from the NANS study. All authors read and accepted the final version of the manuscript.

FUNDING

This work was supported by a H2020 European Research Council (647783).

ACKNOWLEDGMENTS

The authors would like to thank all volunteers for their commitment and patience during the study. The authors would like to acknowledge the help of Dr. Helena Gibbons and Dr. Diana González-Peña.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.577720/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

CONCEPTUAL ANALYSIS
published: 14 January 2021
doi: 10.3389/fnut.2020.519444

Estimating Dining Plate Size From an Egocentric Image Sequence Without a Fiducial Marker

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Despite the extreme importance of food intake in human health, it is currently difficult to conduct an objective dietary assessment without individuals' self-report. In recent years, a passive method utilizing a wearable electronic device has emerged. This device acquires food images automatically during the eating process. These images are then analyzed to estimate intakes of calories and nutrients, assisted by advanced computational algorithms. Although this passive method is highly desirable, it has been thwarted by the requirement of a fiducial marker which must be present in the image for a scale reference. The importance of this scale reference is analogous to the importance of the scale bar in a map which determines distances or areas in any geological region covered by the map. Likewise, the sizes or volumes of arbitrary foods on a dining table covered by an image cannot be determined without the scale reference. Currently, the fiducial marker (often a checkerboard card) serves as the scale reference which must be present on the table before taking pictures, requiring human efforts to carry, place and retrieve the fiducial marker manually. In this work, we demonstrate that the fiducial marker can be eliminated if an individual's dining location is fixed and a one-time calibration using a circular plate of known size is performed. When the individual uses another circular plate of an unknown size, our algorithm estimates its radius using the range of pre-calibrated distances between the camera and the plate from which the desired scale reference is determined automatically. Our comparative experiment indicates that the mean absolute percentage error of the proposed estimation method is ∼10.73%. Although this error is larger than that of the manual method of 6.68% using a fiducial marker on the table, the new method has a distinctive advantage of eliminating the manual procedure and automatically generating the scale reference.

Keywords: wearable device, fiducial marker, dining plate size, egocentric image, technology-based dietary assessment

OPEN ACCESS

Edited by:
Natasha Tasevska, Arizona State University Downtown Phoenix Campus, United States

Reviewed by:
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *11 December 2019*
Accepted: *04 December 2020*
Published: *14 January 2021*

Citation:
Jia W, Wu Z, Ren Y, Cao S, Mao Z-H and Sun M (2021) Estimating Dining Plate Size From an Egocentric Image Sequence Without a Fiducial Marker. Front. Nutr. 7:519444. doi: 10.3389/fnut.2020.519444

INTRODUCTION

Many chronic diseases, such as heart diseases, cancer and diabetes, are associated with unhealthy diet. A recent study by the Global Burden of Disease found that poor diet accounted for ∼20% of adult deaths in 2017 (1). As diet-related health risks are high, it is important to conduct dietary assessment among individuals' with, or in an emerging stage of, chronic diseases. Traditionally,

this assessment depends on individuals' self-report, which is subjective and often inaccurate (2). In recent years, as microelectronic and mobile technologies advance, image-based dietary assessment has emerged (3, 4). The images of food are acquired from an individual either actively or passively. In the active approach, the individual takes pictures of his/her food before and after each eating event (5). Although this method is inexpensive (because of the wide availability of the smartphone) and the image quality is high, picture-taking must be volitionally initiated, which depends on the individual's memory. In the passive approach, the individual is provided with a small electronic wearable device, such as the eButton in the form of a chest pin [**Figure 1A**, (6, 7)]. This device is equipped with a wide-angle camera aiming at the food on the table during the eating process. Rather than taking pictures manually, a sequence of images is acquired automatically at a pre-set rate (4–6 s between images). For a complete dietary assessment, the device can be activated for the entire day, producing a large amount of data saved on the device. Once the data are uploaded to a computer, they are first screened using the Artificial Intelligence (AI) technology (8). This screening automatically filters out all image segments not containing foods or beverages, both reducing the burden of data examination by human experts and mitigating the related privacy concerns. The AI approach also allows objective studies of snacking and a wide range of other diet-related activities, such as food shopping, storage, preparation, cooking, and post-eating events. This work is in the domain of the passive approach.

Although image-based dietary assessment has many advantages over the traditional self-report method, it requires a scale reference within each image. The scale reference is extremely important, analogous to the importance of the scale bar in a map which enables the determination of the distance between any two points on the map or the area of any geological region covered by the map. Likewise, the volumes of foods and beverages on a dining table in the scope of an image cannot be determined without the scale reference. Currently, the scale reference is provided by a fiducial marker which is an object of known dimensions, such as a checkerboard or a business card (2, 5, 9). This method requires the individual to physically carry the card, place it on the dining table before the eating process and retrieve it afterwards. Clearly, these tasks are inconvenient and contradicts the goal of passive dietary assessment. In order to eliminate these tasks, we previously developed a method to use the dining plate itself as the scale reference (10, 11). Since a circular plate appears in the image as an ellipse and the eccentricity of the ellipse depends on the viewing angle of the wearable device, the coordinate transformation between the image pixel coordinates and the world coordinates can be established, under the condition that the radius of the plate is known. Although this method eliminates the need to carry, place and retrieve the fiducial marker, it requires a measurement of the plate radius, which is still a manual procedure and a significant burden to the participant. Eliminating this manual procedure would lead to a true passive dietary assessment, removing the last bottleneck that undermines the passiveness. Because of the high importance of this problem, considerable effort has been spent by the research community, and several approaches have been reported, such as using two cameras for a stereo view (12), adopting a depth camera (13), and using a laser

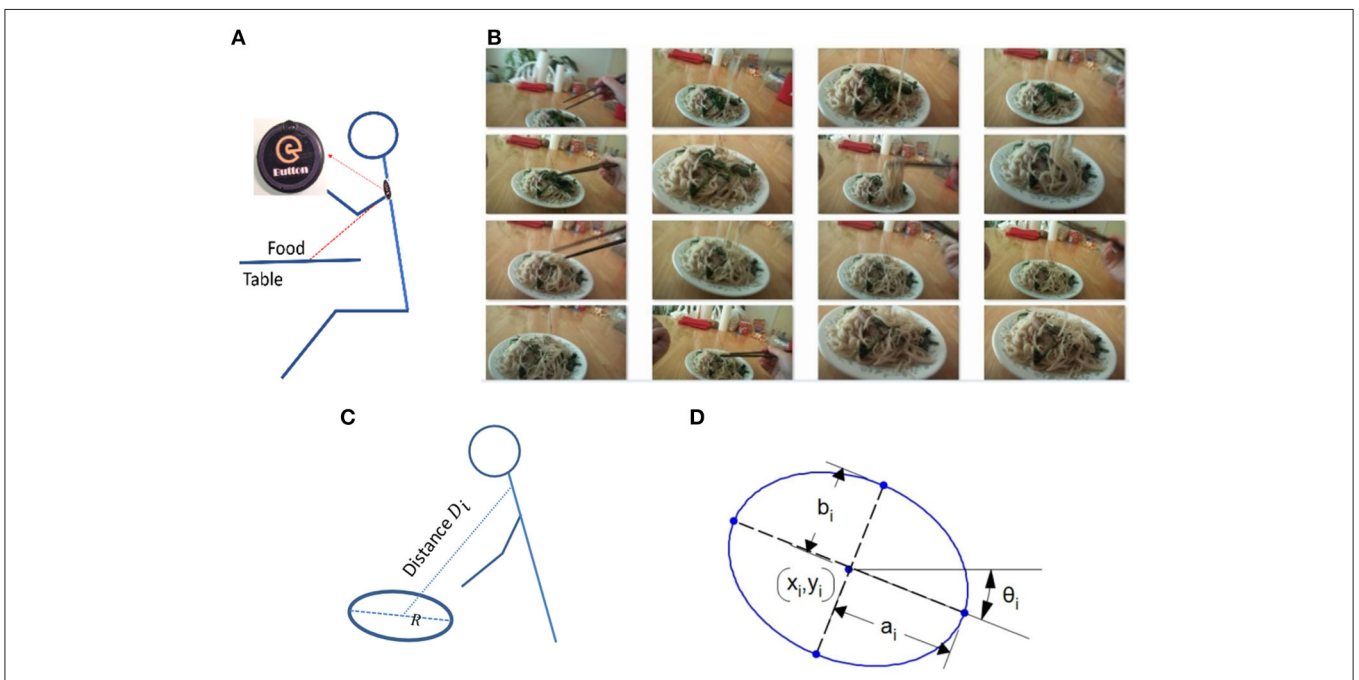

FIGURE 1 | (A) A skeletal representation of a person wearing eButton during a meal; **(B)** Part of an egocentric image sequence acquired by the eButton showing quasiperiodic variations of the ellipses of the plate; **(C)** Definition of D_i; **(D)** Parameters of an ellipse.

reference produced by an add-on device (14, 15). Although these solutions are effective, the extra power consumption, enlarged wearable device size and increased cost have hampered their practical utility.

Unfortunately, elimination of the manual procedure and automatic determinization of the scale reference based purely on image contents represent an extremely difficult problem. The theory of computer vision has indicated that it is impossible to estimate the real size of an object in a single 2D image without providing the scale information (16). However, we will show, in this work, that this theoretical constraint can be circumvented if we use a sequence of images as the input and meet the following assumptions: (1) the heights of the dining table and chair at each dining location are fixed, (2) a one-time calibration is performed at each dining location using a circular plate of known size, (3) the individual uses the same wearable device affixed at the same body location to capture images, and (4) one of the food containers on the dining table is a circular plate. Then, we show that the desired scale reference can be determined automatically from the circular plate. Here we point out that, as in the case of a map where the scale bar is applicable to all geological regions covered by the map, this scale reference, once obtained, is applicable to all foods, beverages and other objects on the dining table. As a result, their lengths and volumes can be estimated from the image.

The rest of the paper is organized as follows. Section Methods presents the details of our method including the concepts utilized, the formulation of the method, and the plate radius estimation procedure. Section Experimental Results summarizes the experimental data and analysis results. In section Discussion, several issues of this method are discussed. Finally, limitations and future work are described in section Limitation and Future Work and conclusions are drawn in section Conclusion.

METHODS

System Design Concepts

In real life, most individuals follow a certain eating pattern. With exceptions of traveling or "eating out," they usually use fixed locations to have meals, for example, the kitchen or dining room at home for breakfast and dinner, and the office desk, a cafeteria, or a favored restaurant for lunch. At each location, the heights of the dining table and chair are usually fixed. Additionally, when a wearable device is used for dietary assessment, the location of the wearable device is usually fixed also, such as the chest location of the eButton (**Figure 1A**). All these factors indicate that, during eating events, the imaging environment of the individual at each dining location does not change drastically regardless of the food served and utensils utilized.

Although, as indicated previously, the theory of computer vision prohibits the determination of plate radius from a single image alone without the scale information, the estimation becomes possible when a sequence of images is captured by a wearable camera. Our key approach is to investigate the variation in the size of the observed dining plate in the image sequence (see an example in **Figure 1B**) as the result of the individual's repeated motion for reaching and fetching food. Although this body motion is not truly periodic (hence we call it "quasiperiodic motion") involving considerable irregularities in the camera-to-plate distance, it is reasonable to assume that the statistical range of camera-to-plate distance variations remains the same for all eating events if the eating environment is fixed. From our previous studies (10), we know that the camera-to-plate distance can be calculated when a circular plate presents in the image and the plate size is known. If a one-time calibration with a plate of known size is conducted for an individual, the range of camera-to-plate distances during all future eating events of this person can be considered known. Then, the radius of an unknown plate can be estimated using this known range of the distances if his/her eating happens at the same location. These represent the key concepts of our method.

Our method, to be detailed below, for estimating the radius of an unknown plate from the image sequence is highlighted as follows. First, the relationship between the image of the plate (i.e., an ellipse) and the camera-to-plate distance is investigated and simplified. Then, a set of lines is generated to represent such relationship for different plate sizes. Next, from these lines, a particular line (i.e., the optimal line) is determined that best-matches the known range of the camera-to-plate distances obtained during the calibration process. The radius of the unknown plate is determined to be the radius represented by that line.

Modeling Camera-to-Plate Distance

Let D_i be the distance (unit: mm) between the lens of the wearable device to the center of the plate, where subscript i denotes the i^{th} image in the image sequence (**Figure 1C**). We have previously shown (10) that D_i can be determined from the ellipse (representing the plate) in the image if the actual radius of the plate is measured, and the intrinsic parameters of the camera, including the focal length and pixel size of the semiconductor chip, are provided. **Figure 2A** illustrates the change of camera-to-plate distance (red dots) during an eating episode. The mathematical expression for D_i is derived based on intersecting a cone (with its vertex located at the optical center of the camera) by the surface of the tabletop, where the circular plate (assuming that its height can be ignored) coincides with the intersection contour (10, 17). While the mathematical details of the expression are quite complex, here we write it as g, given by

$$D_i = g\left(x_i, y_i,\ a_i, b_i, \theta_i, R\right), \tag{1}$$

where (x_i, y_i) denote the coordinate of the center for the ellipse in the image; (a_i, b_i, θ_i) represent the length of the semimajor axis, the semiminor axis, and the major axis angle of the ellipse, respectively (shown in **Figure 1D**); and R is the radius of the plate (unit: mm). Among the six variables of g, R is the only one that has a physical size in the world coordinates. With the ellipse parameters, the orientation and location of the dining table where the plate is placed on can be determined.

Model Simplification

To simplify Equation (1) and make the relationship between D and (x, y, a, b, θ, R) more intuitive, we start with a simple case assuming that the optical axis of the camera goes through

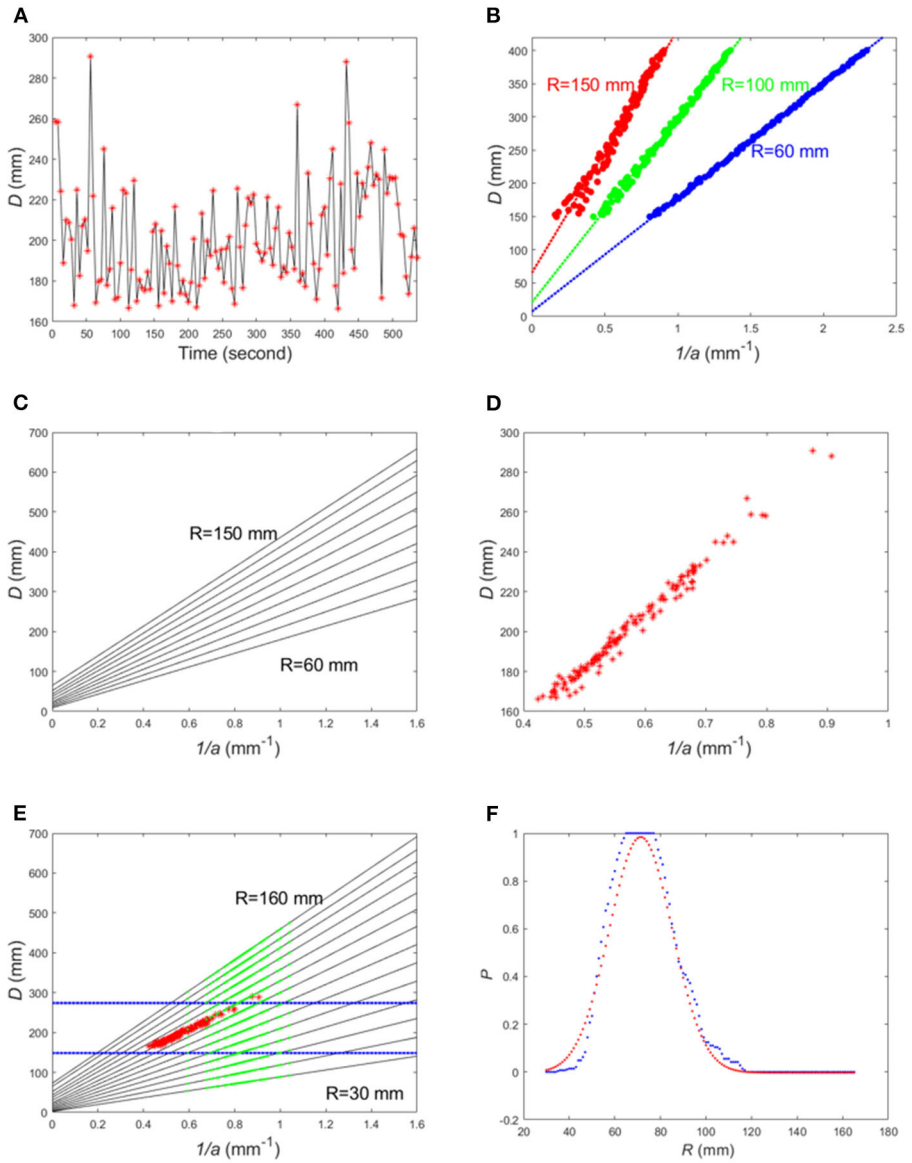

FIGURE 2 | (A) Plot of change of camera-to-plate distance (red dots) during an eating episode; **(B)** Simulated camera-to-plate distance vs. $1/a$ using Equation (3); **(C)** A set of fitted curves for $R = 60,\ 70,\ \cdots, 150$ mm; **(D)** Camera-to-plate distance D vs. $1/a$ plot for the real data in **(A)**; **(E)** The two dotted blue lines represent the distance range $[D_l, D_u]$ obtained from calibrated data (red dots). The black lines represent the fitted lines for different plate radii. The green dots are the corresponding D_i to each $1/a_i$ obtained from the image with unknown plate. **(F)** Plot of P and fitted Gaussian function.

the center of the plate and the camera is level (the bottom of the camera is parallel to the horizon) but tilting downwards by an angle γ to capture the food on table. Under these assumptions, we explicitly derive function g based on a pin-hole camera model. Even with these simplifications, the derivation is still complex. It is thus not included here. Interested readers are referred to the **Supplementary Material** (attached). The final relationship between camera-to-plate distance D and the reciprocal of semimajor axis a of the observed ellipse is given by:

$$\frac{1}{a} = \frac{1}{f}\sqrt{\left(\frac{D}{R}\right)^2 - \cos^2\gamma}, \tag{2}$$

where R is the plate radius, γ is the tilting angle, and f is the camera's focal length. The reason that the semiminor axis b is not included is also discussed in the **Supplementary Material**. Note that the unit of a is millimeter in the image plane (i.e., the sensor chip) within the camera. The conversion between the pixel coordinates in the image and the real-world coordinates in the image plane can be made through the intrinsic parameters of the camera (such as focal length, pixel size) (16).

With Equation (2), we can find the relationship between $1/a$ and D when R and γ are given. In practice, angle γ changes during eating due to human body's movement for reaching, fetching and delivering the food to the mouth (exemplified in

Figure 1B as snapshots of this process). Thus, γ is set to a uniformly distributed random number between 20 and 70°. By simulation, a large number of pairs of $(1/a_i, D_i)$, $i = 1, 2, \cdots, N$, using Equation (2) can be generated for different R values (see examples in **Figure 2B**). The red dots represent the data points for $R = 150$ mm, green dots for $R = 100$ mm and blue dots for $R = 60$ mm. It can be seen that the relationship between $1/a$ and D can be approximated by a linear function. By least-square fitting of the simulated data points for each R_t according to the following criterion

$$\min_{m,n} \sum_{i=1}^{N} \left(D_i - \frac{m}{a_i} - n \right)^2, \tag{3}$$

the fitting parameters m, n corresponding to the given radius R_t $(t = 1, \ldots, T)$ can be obtained, as shown in **Figure 2B**. Here T is the total number of simulated fitting lines. Then, we have

$$D_i \approx \frac{m}{a_i} + n \qquad (i = 1, \ldots, N). \tag{4}$$

Figure 2C illustrates a case for $T = 10$ where each line represents a different value of R. Thus, for each R value, the ellipse parameter $1/a$ can be calculated from camera-to-plate distance D. Conversely, if D is known, we can determine R. Although D varies during the eating process as stated previously, the range of D is known from pre-calibration. If the calibrated range of D is $[D_l, D_u]$ and the extracted ellipse parameters from the image sequence are $\{1/a_i\}$, for $i = 1, \ldots, N$, the problem of estimating the unknown plate size becomes finding the optimal line among all the simulated (or pre-tabulated) lines that best-maps the set of $\{1/a_i\}$ into the range of $[D_l, D_u]$.

Although, in this simplification, the requirement that the optical axis of the camera goes through the plate center cannot be met normally, our data indicate that the approximate linear relationship between $1/a$ and camera-to-plate distance D still hold for real image sequences obtained during eating events (exemplified in **Figure 2D**). This demonstrates that the simplified model is generally acceptable. In some cases, however, the quasiperiodic body movement of the individual during eating is interrupted because of certain activities related or unrelated to the eating process (e.g., reaching a can of drink far away from the individual or operating a TV remote control). These activities result in sudden large changes in the positions and/or orientations in the observed sequence of ellipses. These changes do not fit our model but can be easily identified from the image sequence and discarded as data outliers.

System Calibration and Plate Radius Estimation

In the following, we will first describe the calibration procedure. Then, we will provide two different estimates for the camera-to-plate distance, one by analytic calculation and the other by simulation. Finally, these two estimates are combined to estimate the unknown plate radius based on the result of calibration.

Calibration Procedure

A one-time calibration is required for each subject at each eating location. This calibration is nothing more than having a meal by the individual at the location with a circular plate of known radius R. From the calibration image sequence, the ellipse parameters are extracted from the i^{th} image and thus the camera-to-plate distance can be computed using Equation (1) which specifies the relationship between D_i and ellipse parameters. Although the mathematical expression for Equation (1) is complex, an analytic solution has been reported and can be computed using ellipse parameters (10, 17). From the whole image sequence, we can obtain a set of $\{D_i\}$ and a set of ellipse parameters $\{x_i, y_i, a_i, b_i, \theta_i\}$. Thus, the range of D_i, defined as $[D_l, D_u]$, can be estimated from the distribution of $\{D_i\}$. Due to the limited number of images in a sequence and the noisy nature of the experimental data, the minimum and maximum value of D_i, $i = 1, 2, \cdots, N$, may not reflect the actual distance range. We thus manipulate the histogram of $\{D_i\}$ to obtain the distance range, which will be described in section Data Analysis.

Camera-to-Plate Distance by Simulation

For a new image sequence including an unknown plate, the ellipse parameters $(x_i, y_i, a_i, b_i, \theta_i)$ can also be extracted for each image in the sequence. Then, we set R to be a variable and equally sample this variable to form R_t, $t = 1, 2, \cdots, T$ with a sufficient range and resolution (e.g., from $R_1 = 30$ mm to $R_T = 165$ mm with an increment of 1 mm). Next, the fitting parameters m,n corresponding to the given radius R_t $(t = 1, 2, \ldots, T)$ are obtained using Equation (3) as illustrated in **Figure 2B**. By substituting a_i to the simplified form of Equation (1), i.e., Equation (4), the camera-to-plate distances D_i, $i = 1, 2, \cdots, N$, for each R_t, $t = 1, 2, \cdots, T$, can be simulated (i.e., pre-tabulated), defined as $\{D_i^1\}$.

Calculated Camera-to-Plate Distance

Since the available data obtained from an eating event are usually limited (N is usually <100), we calculate another set of $\{D_i\}$ called $\{D_i^2\}$ for the same values of R_t, $t = 1, 2, \cdots, T$, using Equation (1) although the calculation is complicated (10, 17). The main reason of adding this part of calculation is to double the number of data points that can be used to make the estimation more reliable.

Plate Size Estimation

After combining the two sets of $\{D_i\}$ as $\{D_i\} = \{D_i^1\} \cup \{D_i^2\}$ for each R, the number of $\{D_i\}$ that fall into the calibrated range $[D_l, D_u]$ can be counted. An index representing how close each R is to the actual radius can be calculated as $P = |\{D_i \in [D_l, D_u]\}| / |\{D_i\}|$ (see **Figure 2F** for an example), where the vertical bars "$|\cdot|$" represent the number of elements in a set. Finally, we fit the curve with a Gaussian function, and the estimated R corresponds to this maximum point (i.e., the mean of the Gaussian distribution).

EXPERIMENTAL RESULTS

To validate our plate radius estimation method, we conducted experiments in real-world settings. In this section, we describe the details of our experiments, including human subjects, experimental procedure, data analysis, and experimental results.

Human Subjects

With an approval by the Institutional Review Board at the University of Pittsburgh, three human subjects participated in the experimental study. In order to satisfy the assumptions presented in section Model Simplification, these subjects were selected based on the following criteria: (1) they were healthy with normal body posture at both sitting and standing positions; (2) they followed a regular daily routine during the study (e.g., traveling was excluded); and (3) their dining locations were mostly fixed.

Experimental Procedure

The subjects were first trained for using the eButton to record their dining events. They were instructed to comply with the following requirements: (1) using circular plates as the food container for serving; (2) wearing the eButton at a fixed chest location; and (3) keeping the heights of dining table and chair at each dining location unchanged. The subjects were instructed to follow their regular dietary patterns without restrictions on types of food and activities while sitting at the table (e.g., listening to music, watching TV, making a phone call, or interacting with people). No limitation was imposed on food types and utensils.

In each meal during the experiment, the subject wore the eButton and had meals normally using the pre-measured plate. The measured values were used either for the calibration process or as the gold standard for assessing the accuracy of our plate radius estimation algorithm.

Data Analysis

After the study, the subjects returned the eButton to our laboratory where the recorded data were read from the microSD card within the device. The following data analysis steps were implemented.

Image Screening and Ellipse Extraction

All the images in each eating event were visually examined by a researcher. The images that contained no plate or a plate with most of its boundary missing were regarded as outliers and excluded from data analysis. For each image, the contour of the plate edge, observed as an ellipse in the image, was first extracted automatically using an automatic algorithm developed by us previously (18). In some cases, the automatic method failed due to occlusion or shadowing. In these cases, we used interactive method in which six points on the ellipse were manually selected. In either case, the parameters of each ellipse (e.g., semimajor axis a) were extracted by a least-squares fitting of the ellipse boundary.

Distance Range From Calibrated Image Sequence

For each image in the calibrated image sequence, all $\{x_i, y_i, a_i, b_i, \theta_i\}$, $i = 1, 2, \cdots, N$, where N is the number of images in an eating event after eliminating outliers, were extracted. Then, distance D_i corresponding to each image was obtained using Equation (1) with the pre-measured R. The red dots in **Figures 2D,E** represent the pairs of $\{1/a_i, D_i\}$ calculated from the calibrated image sequence. To determine the range of $\{D_i\}$ reliably, the histogram of $\{D_i\}$ was calculated and the values in the two extreme bins were removed if the frequency in either bin was small (i.e., less than half of the average frequency). After that, the maximum and minimum values of the remaining D_i were set to $[D_l, D_u]$. Examples are shown in **Figure 2E**.

Simulation of the Relationship Between Ellipse Parameter and Camera-to-Plate Distance

The simulation was described in section System Calibration and Plate Radius Estimation. Simulated lines represent the relationship between D and $1/a$, as shown in **Figure 2C**. In our experiment, the range of was chosen from 30 to 165 mm with an increment of 1 mm.

Plate Radius Estimation From the Image Sequence With Unknown Plate Size

For each human subject at each dining location, we collected data containing different eating events using plates of different radii. We call this collection of data "eating episodes" in which each episode is a particular event in the collected dataset. We took each plate as the calibration/reference plate sequentially from the dataset and the radii of the remaining plates were estimated using the procedure described in section System Calibration and Plate Radius Estimation. Our experiment resulted in $M(M-1)$ estimates of plate radii for each human subject where M is the number of plates utilized by the subject during the experiment.

Statistical Analysis

To observe the estimation error statistically, we calculated the percentage error for the estimated plate radius in each eating episode using different plates for calibration. Then, we calculated several statistical measures, including the mean Percentage Error (mPE), mean absolute Percentage Error (maPE), mean relative Root Mean Square Error (mrRMSE), defined as follows:

$$Percentage\ Error\ \ (PE) = \frac{R_{k,j} - R_j}{R_j}$$

$$mean\ Percentage\ Error\ (mPE) = \frac{1}{M(M-1)} \sum_k \sum_{j \neq k} \frac{R_{k,j} - R_j}{R_j}$$

$$mean\ absolute\ Percentage\ Error\ (maPE) = \frac{1}{M(M-1)} \sum_k \sum_{j \neq k} \left| \frac{R_{k,j} - R_j}{R_j} \right|$$

$$mean\ relative\ Root\ Mean\ Square\ Error\ (mrRMSE) = \sqrt{\frac{1}{M(M-1)} \sum_k \sum_{j \neq k} \left(\frac{R_{k,j} - R_j}{R_j} \right)^2}$$

where R_j is the true radius of the plate in the jth episode, $R_{k,j}$ is the estimated radius of plate in the jth eating episode using the plate in the kth episode as the reference plate for calibration, and M is the total number of episodes. Note that each error calculation is represented in the percentage value.

Results

In our experiments, a total of 37 eating episodes (15 for Subject 1, 12 for Subject 2, and 10 for Subject 3) were recorded, and the plate radius used in each episode was measured as the ground truth. One episode of Subject 3 was removed from further analysis because the number of images in this episode was insufficient. Thus, 36 episodes were analyzed, and 15 different circular plates with different radii and heights were used in this study. Typical images are illustrated in **Figure 3A**, where one image is shown for each episode. In these eating episodes, the foods consumed included beef, rice, noodle, dissert, bread, Chinese pancake, pasta, and different kinds of vegetables. Chopsticks, forks, knifes, and spoons were used as utensils.

While our results for all subjects are summarized in **Figure 3**, specific values of estimated plate radii for Subject 3 are provided in **Table 1** as an example. Total nine tests were conducted for this subject. In this table, the values in each row (denoted by "Test #") represent estimated radii of different plates using the same reference, while the values in each column (denoted by "Plate #") represent estimated radii of the same plate using different references. The boldfaced values along the diagonal lines are true radii, which are actually measured values. The calculated mPE, maPE, mrRMSE using the formula in section Data Analysis for each subject are listed in **Figure 3B**. A set of statistical measures is provided in **Figure 3C**, including distributions of percentage errors for all subjects, 25th percentiles of errors, 75th percentiles of errors, and median errors.

In order to compare the accuracies of our automatic and the traditional manual methods, we conducted an additional experiment using a fiducial marker, which was a rectangular checkerboard of 6×7 cm. Ten circular plates with different radii and heights were utilized in this comparative experiment. The range of the plate radii was identical to that in the previous experiment. The checkerboard card was manually placed next to each plate before taking pictures with an eButton. Since the thickness of checkerboard was small, its surface can be considered as the same surface of the table. Due to the plate height, the plane of table surface estimated from the checkerboard in the image was different from the plane of the plate border, causing a small amount of error in plate radius estimation. For a fair comparison with our method, we assumed that the plate height was standard, which was the height of the reference plate, the same as the assumption made in our method. Under this assumption, each of the ten plates was taken as the reference plate and the remaining nine plates were estimated. Thus, total 90 plate radius estimates were obtained. In each estimate, five images in different viewing distances and angles were processed, and the five results were averaged. Example pictures, the data processing algorithm, estimated values and estimation errors are included in the **Supplementary Material**. Finally, the estimation errors were studied using the same statistical measures (i.e., mPE, maPE, mrRMSE, and boxplot), as in the previous experiment. The results of this comparative experiment are summarized in **Figures 3B,C**. It can be observed that our automatic method has a larger error than the manual method using a fiducial marker (10.73% vs. 6.68% in terms of the mean absolute percentage error). This is not surprising since the fiducial marker provides a scale reference directly in the image. Although a larger error is involved, the new method has a distinctive advantage of eliminating the manual procedure and automatically generating the scale reference.

DISCUSSIONS

In this work, we develop a new method to eliminate the requirement for a fiducial marker in egocentric image based dietary assessment. We take advantage of the fixed environment at the dining location to model the eating behavior of an individual. Our study yields a new method to estimate the dining plate radius automatically. If there is only one plate of food in the image, the plate radius (or diameter) is sufficient to serve as the scale reference. In cases where the captured image shows multiple foods on the table, we need to go only one step further. Using this radius and the orientation information obtained from the observed elliptic shape of the plate, a plane equation for the tabletop can be determined which serves as the desired scale reference. This plane equation is easy to obtain because the ellipse in the image provides the orientation (or the norm vector) of the plate, the circle of the plate is in or close to the plane of the tabletop, and the radius provides the scale in a real-world unit (e.g., mm). Analogous to a map where the sizes of all regions in the map can be estimated using the scale bar, the sizes or volumes of any foods (within containers of any forms or shapes or even without containers) or beverages on the table can be estimated using the scale reference. Compared with the existing methods using additional sensors and laser emitters, our method requires no added cost. A simple, once-for-all calibration is the only requirement to implement our method.

Our method is built upon a number of assumptions: (1) it is applicable only to each individual, (2) the heights of the dining table and chair at the dining location are invariant, (3) the device-wearing position on the body is fixed, and (4) the range of body rotation during normal eating is invariant. Clearly, these are strong assumptions which may not be met exactly in a real-world setting. However, making such assumptions is a key step to simplify the complex six-variable relationship (Equation 1) into a single-variable linear equation (Equation 4). Our experimental results have indicated that, even if the assumptions are not met completely, the mean absolute percentage error of plate radius estimation is $<11\%$. Nevertheless, attention should be paid to the validity of the data as we did in data analysis. It is strongly recommended to exclude the images with a considerable portion of the plate shifted out of the image frame. These cases can be easily identified from the image data.

In our method, estimating the range of the distance from the calibrated image sequence is an important step. However, due to the limited data points in an image sequence (e.g., the eButton acquires one image in every 4–6 s, preset by the user), the

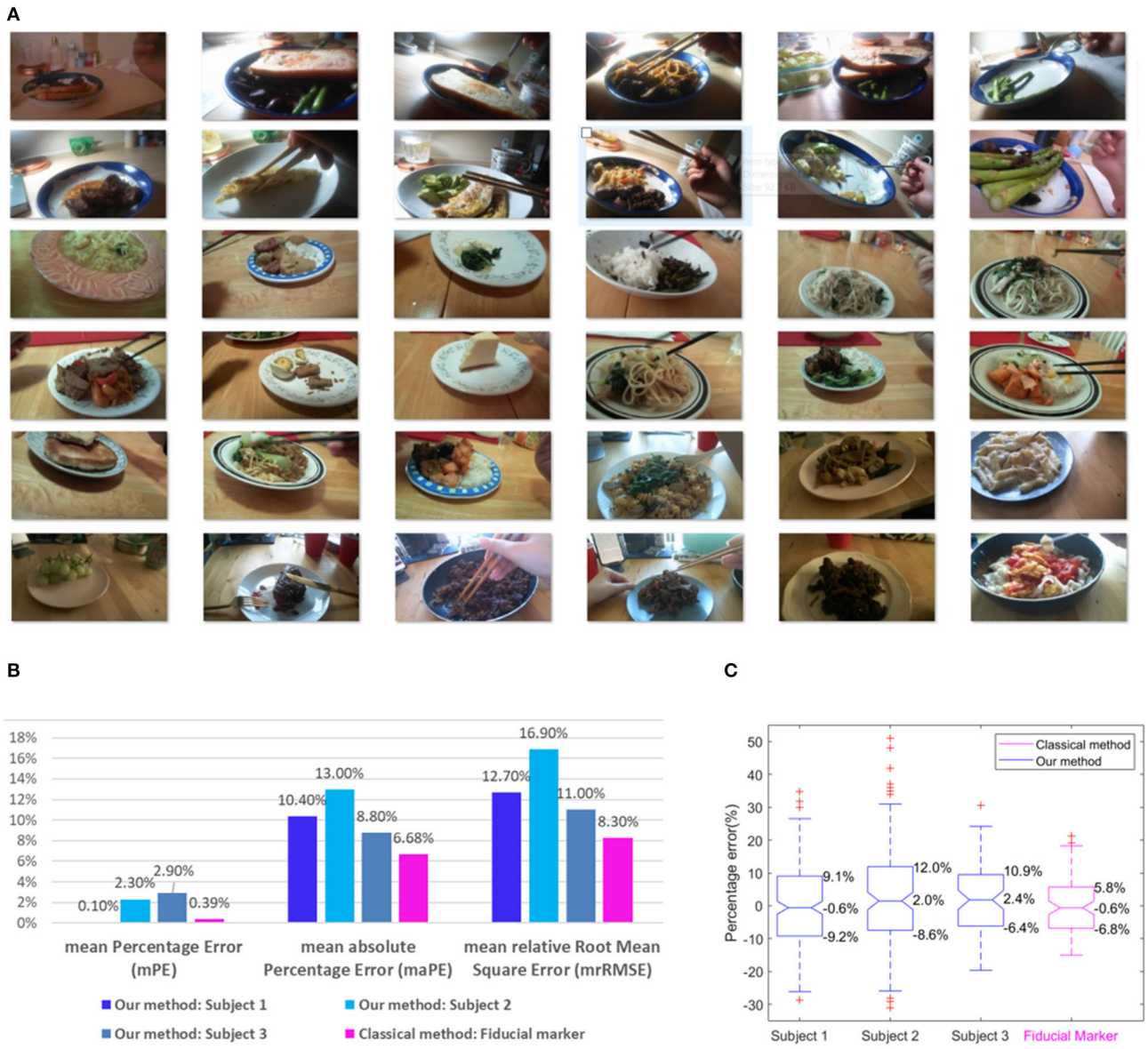

FIGURE 3 | (A) Illustrations of one typical image for each episode; **(B)** Calculated mPE, maPE, and mrRMSE for each subject and the fiducial marker method; **(C)** Box-plot of percentage errors for each subject with extreme percentage errors, 25th percentiles of errors, median, and 75th percentiles of errors marked for the three subjects and the fiducial marker method.

estimation of the distance range may not be sufficiently accurate. Increasing the frame rate of the wearable camera to obtain more images may improve the estimation.

We would also like to point out two main reasons of using a circular plate to obtain the scale reference. First, it is a commonly used utensil in most parts of the world. Second, if the plate is shallow, its top surface is close and parallel to the table surface. However, with exception of the disposable paper plate, most plates have significant heights. In our algorithm, we implicitly assume that the height of the reference plate is the height of the unknown plate, and this "standard height" is used as an offset to be considered in the plane equation for the tabletop. Nevertheless, this method involves a certain error. In some cultures, bowls are used more commonly than plates. We point out that our method can still be used by changing the reference plate to the reference bowl and use its height as the standard height, with some tolerance of the height-related error. Finally, since our method relies only one circular plate to estimate the scale reference, in our experiments, each image contains only a single plate. However, our method is applicable to images containing multiple foods in any forms of containers as long as one of them is a circular plate (or bowl if the reference is a bowl).

The result of the comparative experiment indicates that the manual fiducial marker method is more accurate than our automatic method. This is understandable because the marker provides a scale reference directly while the automatic method

TABLE 1 | Comparison of measured (ground truth) and estimated plate radii for Subject 3.

	Plate#1	Plate#2	Plate#3	Plate#4	Plate#5	Plate#6	Plate#7	Plate#8	Plate#9
Test#1	**130**	131	107	120	97	100	133	104	98
Test#2	130	**125**	103	115	93	96	128	99	95
Test#3	121	117	**95**	108	88	90	120	93	89
Test#4	148	146	118	**127**	107	110	147	114	109
Test#5	141	135	111	124	**100**	103	139	107	102
Test#6	154	153	124	141	113	**112**	154	121	115
Test#7	139	135	109	123	100	102	**130**	106	101
Test#8	132	128	105	118	95	98	131	**100**	97
Test#9	130	126	103	116	94	96	129	100	**95**

The numbers in each row (Test #) represent estimated radii of different plates using the same reference, while the numbers in each column (Plate #) represent estimated radii of the same plate using different references. The boldfaced numbers on the diagonal lines are true radii (actually measured values).

does not have such information. However, in the fiducial marker method, a checkerboard card must be carried by the individual, placed on the tabletop next to the food before eating, and retrieved after eating for the next use. These procedures are unwelcome and can be forgotten easily.

LIMITATIONS AND FUTURE WORK

Our method provides an automatic way to estimate the size of a circular plate. Therefore, as long as there is a plate on the table and the assumptions about the fixed eating environment are satisfied, we will be able to obtain a scale reference for all items on the table based on a one-time calibration procedure. If there are bowls, glasses/cups and/or snacks placed on the same table, in theory, their volumes can be estimated based on the scale reference that our method provides. However, the estimation is subject to various constraints, assumptions and, in some cases, availability of a certain set of knowledge (e.g., the shape of a bowl or a cup). In addition, the problem of 3D food volume estimation from a single or a series of 2D images has not yet been fully solved, and there is a strong demand to develop new computational methods using advanced technologies, such as artificial intelligence (AI). Even though this volume estimation problem is fascinating, its discussion would be lengthy, beyond the scope of this paper which is focused solely on automatic plate radius estimation to generate a scale reference. We emphasize again that this reference is a fundamental requirement regardless the technologies to be utilized.

Our method is currently limited to the dining scenarios where a circular plate or bowl is used as a food container. For the cases where only non-circular plates or bowls are present in the image, we have not found an effective method to estimate their parameters without pre-measurements. These types of containers are still the subjects of further investigation.

CONCLUSION

We have developed a new method to estimate the radius of a dining plate in a sequence of egocentric images acquired by a wearable device thus a scale reference can be obtained automatically. This method is based on mathematical analysis of the eating behavior of an individual and the invariance of the eating environment (i.e., the heights of the table and chair are fixed at each dining location). Unlike the traditional methods that use a fiducial marker or require measurement of plate radius for every meal, our method requires only a once-for-all radius measurement of a single plate. After this calibration step, the radius of arbitrary plate can be estimated. Due to the elimination of a fiducial marker, our method greatly reduces the research burden for research participants, making the dietary assessment passive and objective.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Pittsburgh Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS, ZW, and WJ were responsible for concept formulation and methodological design. ZW, YR, SC, and WJ conducted data collection and image processing. WJ, YR, ZW, Z-HM, and MS contributed to the algorithm for data analysis, final drafting, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institutes of Health grant No. R56DK113819 and the Bill & Melinda Gates Foundation Contract IDOPP1171395.

ACKNOWLEDGMENTS

We also appreciate the support from all research participants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.519444/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 09 April 2021
doi: 10.3389/fnut.2021.604296

Dietary Inflammatory Index Is Associated With Inflammation in Japanese Men

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Background: Dietary components are known to affect chronic low-grade inflammation status. The dietary inflammatory index (DII®) was developed to measure the potential impact of a diet on an individual's inflammatory status, and it has been validated mainly in Western countries.

Objective: This study aimed to examine the validity of the energy-adjusted DII (E-DII™) using high-sensitivity C-reactive protein (hs-CRP) concentration in Japanese men and women.

Methods: In total, 6,474 volunteers from a cancer-screening program (3,825 men and 2,649 women) completed a food frequency questionnaire (FFQ) and their hs-CRP concentrations were evaluated. E-DII scores were calculated on the basis of 30 food parameters derived from the FFQ. Higher E-DII scores reflect a greater pro-inflammatory potential of the diet. The associations between E-DII quartiles and hs-CRP concentration were assessed using regression models adjusted for age, body mass index, smoking status, and amount of physical activity.

Results: Mean E-DII in men and women was + 0.62 ± 1.93 and −1.01 ± 2.25, respectively. The proportion of men and women who had hs-CRP concentration >3 mg/L was 4.7 and 3.1%, respectively. A significant positive association was observed between E-DII score and hs-CRP concentration in men; geometric mean of hs-CRP concentration in the lowest and highest E-DII quartiles was 0.56 mg/L and 0.67 mg/L ($P_{trend} < 0.01$), respectively. The odds ratio (95% confidence interval) of having an elevated hs-CRP concentration (>3 mg/L) was 1.72 (1.10–2.67) in the highest E-DII quartile ($P_{trend} = 0.03$) in men. However, no association was observed between E-DII score and hs-CRP concentration in women, except in those not taking prescription medications.

Conclusions: DII was associated with inflammation status in Japanese men, but the association was limited in Japanese women.

Keywords: dietary inflammatory index, food frequency questionnaire, inflammatory biomarker, high-sensitivity C-reactive protein, Japanese

OPEN ACCESS

Edited by:
Megan A. McCrory, Boston University, United States

Reviewed by:
Roberta Masella, National Institute of Health (ISS), Italy
Parvin Mirmiran, Research Institute for Endocrine Sciences, Iran

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *09 September 2020*
Accepted: *09 March 2021*
Published: *09 April 2021*

Citation:
Kotemori A, Sawada N, Iwasaki M, Yamaji T, Shivappa N, Hebert JR, Ishihara J, Inoue M and Tsugane S (2021) Dietary Inflammatory Index Is Associated With Inflammation in Japanese Men. Front. Nutr. 8:604296. doi: 10.3389/fnut.2021.604296

INTRODUCTION

Low-grade chronic inflammation promotes the development of lifestyle-related chronic diseases such as cancer (1–3), cardiovascular disease (4), diabetes (5), and depression (6). High-sensitivity C-reactive protein (hs-CRP) is a well-known inflammatory biomarker, and previous studies have reported that elevated concentrations of hs-CRP are associated with an increased risk of cancer and the incidence of other chronic diseases (1–3, 7, 8).

Dietary components are one of the key factors affecting an individual's inflammatory status (9). High intake of dietary fiber has been shown to be associated with low hs-CRP concentrations, whereas high intake of saturated fatty acids leads to elevation in hs-CRP concentration (10). In addition, a healthy dietary pattern, characterized by high intake of fruits, vegetables, and fish, has been associated with lower hs-CRP concentrations (11). Mediterranean diet also has been shown to reduce hs-CRP concentrations in randomized controlled trials (12, 13). In contrast, Western dietary patterns, characterized by high intake of red and processed meat, is associated with high hs-CRP concentrations (11). These reports suggest that dietary components and dietary patterns may have a contrasting effect on inflammatory status. Therefore, a comprehensive index is required to understand the potential impact of whole diet on inflammatory status.

The Dietary Inflammatory Index (DII®) is a literature-based dietary score that was developed to measure the potential impact of a diet on the inflammatory status of an individual; a high DII score reflects pro-inflammatory potential of the diet, whereas a low DII score reflects the anti-inflammatory potential of the diet (14, 15). To date, 30 validation studies have been performed between DII and various inflammatory markers, mainly in Western countries (15–18). In addition, the DII has also been shown to be associated with an increased risk of many chronic diseases including cancer (19–21). In Japan, one study reported that a higher DII score increased the risk of upper aerodigestive tract cancers (22). Among the 30 construct validations performed throughout the world, two have been performed in Japan (18, 23), the results of which are inconsistent with regard to sex-specific analyses. Dietary habits and inflammatory status are considerably different between Japanese and Western populations (23–26). Therefore, it is important to determine the utility of the DII to quantify the inflammatory potential of diet in Japanese men and women.

Previously, we conducted a cross-sectional validation study in a subsample from the Japan Public Health Center-based prospective (JPHC) study but could not identify a positive association between the DII and inflammatory status in women (23). The underlying reasons for the null result could be the small sample size of the study and the fact that women consume a more anti-inflammatory diet than do men (23). Therefore, the present study aimed to assess the associations between DII scores and hs-CRP concentrations in a large number of Japanese men and women.

Abbreviations: BMI, body mass index; CI, confidence interval; CV, coefficient of variation; DII, dietary inflammatory index; E-DII, energy-adjusted dietary inflammatory index; FFQ, food frequency questionnaire; hs-CRP, high-sensitivity C-reactive protein; MET, metabolic equivalent; OR, odds ratio; SD, standard deviation.

METHODS

Study Design and Participants

The National Cancer Center of Japan started a cancer-screening program in February 2004. The total number of participants in the present study was 7,919 healthy volunteers (4,664 men and 3,255 women) aged 40–69 years who had participated in the cancer-screening program from May 2009 to December 2013. The blood samples of participants were collected during cancer screening. They also answered self-administered questionnaires for demographic, lifestyle, and dietary information. This study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan (approval number G15-01 and 2016-165). The study aims and protocols were explained to all participants, and each participant provided written informed consent before enrolment in the study.

Self-Administered Questionnaire

The self-administered questionnaire was designed to obtain participants' demographic, lifestyle, and dietary information, including information on height and weight at the time of examination, smoking status, physical activity, and past medical history of cancer, stroke, and myocardial infarction. Dietary information was obtained using a validated food frequency questionnaire (FFQ), which had questions on the consumption of 188 food and beverage items other than supplements. Energy and nutrient intakes were calculated by taking the sum of the products of eating frequency, portion size, and energy and nutrient content of each food, while referring to the Standard Tables of Food Composition in Japan, Fifth Revised and Enlarged Edition (27). A validation study for the energy and nutrient intake had already been conducted in a subsample of the examinees of the cancer screening program, wherein FFQ-derived data were compared with the data derived from four-day weighed dietary records. The correlation coefficients of energy intake in men and women were 0.53 and 0.34, respectively, and the median correlation coefficients of 45 nutrients were 0.57 and 0.47, respectively. More detailed information has been described elsewhere (28).

Calculation of DII Score

The DII is a literature-based dietary index calculated from 45 nutrients and food components to assess the potential impact of a diet on the inflammation status; a high score indicates pro-inflammatory potential of the diet and a low score indicates anti-inflammatory potential of the diet. The index was developed by reviewing and scoring 1,943 peer-reviewed publications, which included cell culture experiments, animal experiments, and human studies, and examined the associations between various dietary components and six inflammatory biomarkers [interleukin (IL)-1β, IL-4, IL-6, IL-10, Tumor Necrosis Factor α, and CRP] (14). Inflammatory effect scores for each of the DII

components were determined by considering the direction of the effect on inflammation, weight of study design, and number of publications. The inflammatory effect scores are available from the publication (14). To calculate an individual's DII score, dietary intake of the DII components was standardized as a Z-score using global daily mean intake and converted into proportion scores in the study population. The global daily mean intake was calculated from a national database of dietary intake in eleven countries including Japan (14). Then, the individual's DII score was calculated as the sum of the products of the centered proportion score and the inflammatory effect score for each of the DII components.

In the present study, we computed the energy-adjusted DII (E-DII^{TM}) using the energy-adjusted intake of the following 30 DII components (out of 45 possible components) (23): protein, total fat, saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, n-3 fatty acid, n-6 fatty acid, cholesterol, carbohydrate, magnesium, iron, zinc, retinol equivalent, beta-carotene, vitamin D, alpha-tocopherol, vitamin B_1, vitamin B_2, niacin, vitamin B_6, vitamin B_{12}, folate, vitamin C, total dietary fiber, isoflavone, ethanol, onion, green or black tea, and caffeine. Some of the components (such as thyme or oregano and rosemary) are not commonly consumed by the Japanese (23). Energy adjustment was done using the density method, and the amount per 1,000 kcal was used for E-DII calculation.

Blood Sampling and Measurement of Inflammatory Biomarkers

Fasting blood samples were collected along with the self-administered questionnaire data before any cancer screening procedures on the first day of screening. Venous blood was drawn into a vacutainer tube without anticoagulant, and the samples were centrifuged to obtain serum.

Serum hs-CRP concentrations were measured using a commercial reagent kit (Nanopia CRP, Sekisui Medical Co., Ltd., Tokyo, Japan) with an automated analyzer JCA-BM6070 (Jeol Ltd., Tokyo, Japan) at National Cancer Center, Tokyo, Japan. The intra-assay coefficient of variation was 1.8% for 1 mg/L, 1.3% for 2 mg/L, and 0.7% for 82 mg/L of CRP concentration ($n = 20$ each) according to the manufacturer's data (29). The detection range of the kit was 0.2 mg/L−420 mg/L. In case the values were lower than the lower limit of quantification, we assigned a value of 0.1 mg/L.

Statistical Analysis

Among the 7,919 participants, 1,445 were excluded on the basis of following criteria: BMI< 14 or >40 kg/m^2 ($n = 13$); missing data on smoking status ($n = 1$); missing data on metabolic equivalents ($n = 1$); participants with a history of any cancer, stroke, and myocardial infarction ($n = 877$); extreme energy intake identified in the upper and lower 2.5%-tile ($n = 349$); missing data on alcohol consumption ($n = 120$); and missing data on hs-CRP or hs-CRP concentration >10 mg/L ($n = 84$). Therefore, data from 6,474 participants (3,825 men and 2,649 women) were included in the statistical analysis. Cohen's effect size f^2 and noncentral F distribution $F(df_{Reg}, df_{Res}, \lambda)$ were used to calculate sample size n for multiple regression analysis; where $f^2 = \frac{R^2}{1-R^2}$, R^2 is the coefficient of determination, df_{Reg} is the degree of freedom for regression (= k), df_{Res} is the degree of freedom for residual (= n – k – 1), and the noncentral parameter λ is $\lambda = f^2 n$ (30). When we set the effect size = 0.01, k = 12, power = 0.8, and significance level = 0.05, the sample size n was calculated to be 1,745.

Participants' characteristics were summarized using percentages for categorical variables; mean and standard deviation (SD), as well as median and interquartile range for continuous variables; and geometric mean and coefficient of variation (CV) for log-transformed hs-CRP. The CV was also calculated with the log-transformed value using the formula: CV = $(e^{SD}-1)^{1/2}$ (31). Comparison of characteristics between men and women was done using the chi-square test for categorical variables. The Mann–Whitney U test was used for all continuous variables, as the Kolmogorov–Smirnov test revealed non-normal distribution of all variables except men's height.

Multiple regression analyses were performed for the associations between the E-DII score and food group intake. Associations between the E-DII scores and hs-CRP concentration were evaluated using multivariable linear regression models. Linear trends across E-DII quartiles were calculated using natural log-transformed hs-CRP concentration as a dependent variable. To interpret a partial regression coefficient estimated using this model, e (Euler's number) should be exponentiated with that coefficient. The models were adjusted for age (years; continuous), BMI (kg/m^2; continuous), smoking status (current, past, and never), regular prescription medicine use (yes or no), and daily total physical activity level (MET-h/d; continuous). Sensitivity analyses were performed stratified by age, BMI, and regular prescription medicine use. In addition, logistic regression models were applied to estimate the odds ratio (OR) of having elevated hs-CRP concentration (>3 mg/L) across quartiles of E-DII score because this is a clinically relevant cut-off point for chronic inflammation status (32). All statistical analyses were performed using Statistical Analysis Systems software, version 9.3 (SAS Institute Inc., Cary, NC, USA), and the significance level was set at $p < 0.05$.

RESULTS

Characteristics of the Participants

Table 1 shows the characteristics of the participants stratified by sex. BMI and the proportion of smokers and regular prescription medicine users were significantly higher in men than in women. The amount of physical activity was significantly higher in women than in men. The E-DII score was significantly higher in men (+0.62 ± 1.93) than in women (−1.01 ± 2.25) ($p < 0.001$). Hs-CRP concentrations also were significantly higher in men than in women; mean (CV) was 0.58 mg/L (1.21) and 0.44 mg/L (1.24), respectively ($p < 0.001$). The proportion of participants who had hs-CRP concentration >3 mg/L was also significantly higher in men than in women (4.7 and 3.1%, respectively; $p < 0.001$). The comparisons between the prescription medication non-users and users are shown in **Supplementary Table 1**. In both sexes, users were older and had higher BMI, lower DII, and higher hs-CRP concentrations than non-users did.

TABLE 1 | Characteristics of the volunteers of the cancer-screening program from May 2009 to December 2013 at National Cancer Center Japan.

	Men		Women		*p*-value
	(*n* = 3825)		(*n* = 2649)		
	Mean (SD)	Median (interquartile range)	Mean (SD)	Median (interquartile range)	
Age (years)	56.7 ± 8.3	58 (50, 64)	56.5 ± 8.2	58 (50, 64)	0.45
Height (cm)	169.5 ± 5.9	169.6 (165.5, 173.4)	156.4 ± 5.5	156.4 (152.5, 160.0)	<0.001
Weight (kg)	68.6 ± 9.5	67.6 (62.2, 74.2)	53.7 ± 8.1	52.7 (48.5, 57.8)	<0.001
BMI (kg/m^2)	23.9 ± 2.9	23.7 (21.9, 25.5)	21.9 ± 3.1	21.5 (19.9, 23.5)	<0.001
Smoking status (%)					
Current	15.4		5.5		<0.001
Former	52.1		15.2		
Never	32.4		79.3		
Regular prescription medicine user (%)	48.1		44.7		0.008
Physical activity (MET-h/d)	36.6 ± 3.2	35.9 (34.6, 37.6)	38.0 ± 3.7	37.1 (35.6, 39.3)	<0.001
E-DII (/1000 kcal)[a]	0.62 ± 1.93	0.88 (−0.62, 2.07)	−1.01 ± 2.25	−1.00 (−2.71, 0.63)	<0.001
Crude hs-CRP (mg/L)	0.91 ± 1.14	0.5 (0.3, 1.0)	0.72 ± 1.04	0.4 (0.2, 0.8)	<0.001
hs-CRP (mg/L)[b]	0.58 ± 1.21	—	0.44 ± 1.24	—	<0.001
>3 mg/L of hs-CRP (%)	4.7		3.1		<0.001

Chi-square test and Mann–Whitney U test are used for statistical analyses.

[a]E-DII is calculated from dietary intake converted per 1000 kcal.

[b]Geometric mean and coefficient of variation were presented for log-transformed inflammatory biomarkers. Coefficient of variation is calculated using the formula: CV = $(e^{SD}-1)^{1/2}$.

BMI, body mass index; E-DII, energy-adjusted dietary inflammatory index; hs-CRP, high-sensitive C-reactive protein; MET, metabolic equivalent.

Associations Between Food Group Intake and E-DII Score

Table 2 shows the results of multiple regression analyses between E-DII score and food group intake, stratified by sex. The findings suggest that a higher E-DII score was associated with a higher intake of sugar, meat, and confectioneries in men, while a higher E-DII score was associated only with higher sugar intake in women.

Association Between E-DII Score and hs-CRP Concentration

Table 3 shows the mean of E-DII score and the geometric mean of hs-CRP concentration according to the E-DII quartiles. The mean E-DII score increased from −2.04 to 2.85, and the geometric mean of hs-CRP concentration increased from 0.56 mg/L to 0.67 mg/L in men, according to the E-DII quartiles. For women, the mean E-DII score increased from −3.93 to 1.88, but the geometric mean of hs-CRP concentration remained unchanged across E-DII quartiles. A significant positive association was observed between E-DII quartiles and hs-CRP concentration in men (partial regression coefficient = 0.064, $P_{trend} < 0.01$) but not in women (partial regression coefficient = 0.012, $P_{trend} = 0.44$). This means that for every increase in E-DII quartiles in men, there was a 6.6% ($e^{0.064} = 1.066$) increase in the geometric mean concentration of hs-CRP.

In the sensitivity analysis stratified by age, although a positive association was not observed in participants aged 40–49 years ($P_{trend} = 0.31$), significant positive associations were observed in those aged 50–59 years ($P_{trend} = 0.01$) and 60–69 years ($P_{trend} < 0.01$) in men. Upon stratification by BMI, positive associations were consistently observed in underweight (14 kg/m^2 ≤ BMI < 18.5 kg/m^2, $P_{trend} = 0.01$), lower normal-weight (18.5 kg/m^2 ≤ BMI < 22 kg/m^2, $P_{trend} = 0.01$), higher normal-weight (22 kg/m^2 ≤ BMI <25 kg/m^2, $P_{trend} < 0.01$), and overweight and obese (25 kg/m^2 ≤ BMI < 40 kg/m^2, $P_{trend} = 0.08$) men. Even when stratified by medication status, a significant association was observed in men (non-users, $P_{trend} < 0.01$; users, $P_{trend} < 0.01$). In contrast, no significant associations were observed between E-DII quartiles and hs-CRP concentration, stratified by age and BMI, in women. Similarly, when stratified by medication status in women, no association was observed in users; however, there was a significant positive association for women non-prescription-drug users (non-users, $P_{trend} = 0.034$; users, $P_{trend} = 0.267$, see the **Supplementary Table 2**).

Table 4 shows the association between E-DII quartiles and higher hs-CRP (>3 mg/L) concentration. Men in the highest quartile of E-DII had 72% higher odds of having CRP >3 mg/L than men in the lowest quartile of E-DII did [OR: 1.72, 95% confidence interval (CI): 1.10–2.67, $P_{trend} = 0.03$]. In contrast, no significant association was observed among women (OR for the highest vs. lowest: 0.92, 95% CI: 0.47–1.82, $P_{trend} = 0.96$).

DISCUSSION

The DII was developed to evaluate the inflammatory potential of people's diets. The present study was conducted to validate the E-DII with a hs-CRP concentration in a large number of Japanese participants. The findings of our study suggest that there are significant positive associations in all men and only in women who are prescription drug non-users.

TABLE 2 | Standardized beta coefficient with 95% confidence interval between E-DII score and food groups' intake by multiple regression analyses in Japanese men and women aged 40–69 years.

	Men		Women	
	Standardized beta coefficient[a]	**(95% CI)**	**Standardized beta coefficient[a]**	**(95% CI)**
Cereals (g/day)	−0.056	(−0.076, −0.036)	−0.112	(−0.144, −0.079)
Potatoes and starch (g/day)	−0.075	(−0.091, −0.058)	−0.137	(−0.154, −0.119)
Sugar and sweetener (g/day)	0.023	(0.011, 0.035)	0.042	(0.023, 0.061)
Pulses (g/day)	−0.240	(−0.255, −0.224)	−0.227	(−0.243, −0.210)
Nuts and seeds (g/day)	−0.079	(−0.092, −0.065)	−0.091	(−0.108, −0.074)
Vegetables (g/day)	−0.386	(−0.404, −0.369)	−0.394	(−0.414, −0.373)
Fruits (g/day)	−0.194	(−0.210, −0.179)	−0.220	(−0.239, −0.202)
Mushroom (g/day)	−0.110	(−0.131, −0.090)	−0.060	(−0.076, −0.044)
Algae (g/day)	−0.079	(−0.095, −0.063)	−0.089	(−0.106, −0.071)
Fish and Shellfish (g/day)	−0.241	(−0.254, −0.227)	−0.291	(−0.310, −0.273)
Meat (g/day)	0.020	(0.004, 0.035)	−0.003	(−0.027, 0.021)
Egg (g/day)	−0.030	(−0.043, −0.017)	−0.042	(−0.059, −0.025)
Milk (g/day)	−0.042	(−0.058, −0.025)	−0.050	(−0.073, −0.027)
Fats and oil (g/day)	−0.087	(−0.104, −0.070)	−0.055	(−0.075, −0.034)
Confectioneries (g/day)	0.028	(0.011,0.045)	−0.009	(−0.028, 0.010)
Alcoholic beverages (g/day)	−0.085	(−0.102, −0.068)	−0.100	(−0.134, −0.067)
Non-alcoholic beverages (g/day)	−0.181	(−0.194, −0.167)	−0.188	(−0.205, −0.172)
Seasoning (g/day)	−0.087	(−0.100, −0.075)	−0.103	(−0.122, −0.084)

Food groups' intake adjusted by energy intake using the residual method.
[a]Multiple regression analysis is performed with energy-adjusted dietary inflammatory index value (continuous) as the dependent variable and energy-adjusted food groups' intake as the independent variable.
CI, confidence interval.

TABLE 3 | Adjusted geometric mean and 95% confidence interval of high-sensitivity C-reactive protein (hs-CRP) concentration in serum (mg/L) according to quartile of energy-adjusted dietary inflammatory index (E-DII)[a].

	n	**E-DII**	**hs-CRP**	**Partial regression coefficient**	P_{trend}
		Mean ± SD	**GM (95% CI)[b,c]**		
Men					
Q1	956	−2.04 ± 1.17	0.56 (0.53, 0.59)	0.064	<0.01
Q2	956	0.20 ± 0.42	0.57 (0.54, 0.60)		
Q3	956	1.46 ± 0.34	0.62 (0.59, 0.66)		
Q4	957	2.85 ±0.57	0.67 (0.63, 0.71)		
Women					
Q1	662	−3.93 ± 0.83	0.46 (0.43, 0.50)	0.012	0.44
Q2	662	−1.83 ± 0.48	0.45 (0.41, 0.48)		
Q3	662	−0.16 ± 0.46	0.48 (0.44, 0.51)		
Q4	663	1.88 ± 0.93	0.47 (0.44, 0.51)		

[a]E-DII is calculated from dietary intake converted per 1000 kcal.
[b]The quartile values of E-DII were entered as independent variables, and hs-CRP values were entered as dependent variables. Adjusted for age, body mass index (BMI), physical activity (MET-h/d), smoking status, and regular prescription medicine use.
[c]Geometric mean is calculated by back transforming the arithmetic mean of the log-transformed values.
CI, confidence interval; GM, geometric mean; Q, quartile; SD, standard deviation.

The positive association in Japanese men observed in the present study is consistent with the results of previous studies from Western countries (15–17) and Japan (18, 23). The dietary habits of the Japanese are different from those of the Western people, and the CRP concentrations of the Japanese (0.6 mg/L in the men of the present study) are considerably lower than those of Westerners [typically, they are approximately 2–3 mg/L (15, 17)]. Therefore, the positive association observed in Japanese

TABLE 4 | Adjusted odds ratios and 95% confidence interval for the association between energy-adjusted dietary inflammatory index (E-DII) quartile[a] and >3 mg/L of high-sensitivity C-reactive protein (hs-CRP).

	Men			Women		
	***n* (%)**	**OR (95% CI)[b]**	P_{trend}	***n* (%)**	**OR (95% CI)[b]**	P_{trend}
Q1	38 (4.0)	1	0.03	22 (3.3)	1	0.96
Q2	45 (4.7)	1.21 (0.78, 1.89)		17 (2.6)	0.74 (0.38, 1.43)	
Q3	40 (4.2)	1.15 (0.72, 1.83)		21 (3.2)	0.94 (0.50, 1.78)	
Q4	57 (6.0)	1.72 (1.10, 2.67)		21 (3.2)	0.92 (0.47, 1.82)	

[a] *E-DII is calculated from dietary intake converted per 1,000 kcal.*

[b] *Adjusted for age, body mass index (BMI), physical activity (MET-h/d), smoking status, regular prescription medicine use.*

Number of participants who had higher hs-CRP values (>3 mg/L) is expressed as n (%).

CI, confidence interval; CRP, C-reactive protein; E-DII, energy-adjusted dietary inflammatory index; OR, odds ratio; Q, quartile.

men in the present study suggests that DII may apply to a diverse male population with considerably different dietary habits and a different range of inflammatory status.

As in previous studies, inconsistent results were observed between Japanese men and women in the present study. We could not detect any associations between E-DII scores and hs-CRP concentrations across women in this study. However, we did observe a positive association when we restricted the analyses to non-users of prescription medicine. The mean hs-CRP concentration was significantly lower in women than in men. In addition, the proportion of participants with hs-CRP concentration >3.0 mg/L was significantly lower in women than in men. Therefore, owing to the low concentration of hs-CRP in Japanese women in this study, detection of the association between E-DII score and hs-CRP concentration may be difficult. This may have also been responsible for the limited positive association between DII and hs-CRP concentration in non-users of prescription drugs among women. In this study, we excluded from the analysis subjects with a history of cancer, stroke, or myocardial infarction, which could be confounding. However, approximately half of the subjects were regular users of some prescription medication. It has been reported that hs-CRP concentrations are higher in hypertension patients than in healthy subjects (33) and that hs-CRP concentrations are higher in diabetes patients with high HbA1c concentrations (34). Considering these previous studies, hs-CRP concentrations may be high in populations with some disease taking prescribed medication. In fact, in the current study, prescription drug users of both sexes had higher hs-CRP concentrations than non-users did. The difference in the percentage of people with high hs-CRP concentration was particularly apparent in women (see **Supplementary Table 1**). Therefore, we suggest that in women, the association between DII and hs-CRP concentration may have been more clearly demonstrated in non-drug users only. Of note, studies in populations with low hs-CRP concentrations are limited; therefore, further studies are needed to determine the utility of DII in populations with low CRP concentrations, such as Japanese women.

Another underlying reason for the null finding among Japanese women may be the differences in dietary habits between men and women. The women's diets had significantly lower inflammatory potential, indicated by the negative value of mean E-DII score, than those of men, who showed a positive value of mean E-DII score. This is in line with the results of our previous study, wherein the E-DII scores of women were much lower than those of men, and no associations were observed in women in that study either (23). Further, similar results have been reported in a previous study conducted in postmenopausal women from USA, wherein mean E-DII score was a negative value (-0.62 ± 2.69), and a null association was observed between E-DII score and hs-CRP concentration despite having comparatively higher hs-CRP concentration (mean 1.36 mg/L) (35). Taking into consideration the aforementioned reports, it is possible that if the target population eats a predominantly anti-inflammatory diet, the association between DII score and hs-CRP concentration may be difficult to detect.

The dietary patterns that constitute E-DII may vary among populations. Among men with an association between E-DII and inflammatory markers in this study, the food groups positively associated with E-DII were meat and confectioneries. Similarly, in NIPPON DATA, wherein the association with inflammatory markers was confirmed, meat and confectioneries, as well as cereals and fats, were positively associated with E-DII (18). Contrastingly, in the male population of the JPHC-FFQ validation study, meat and confectioneries were not associated with E-DII. The higher the E-DII, the lower the intake of potatoes, legumes, vegetables, and seaweed (23). The study period of the current study (2009–2013) is similar to that of the NIPPON DATA2010 study (18), but it differs from that of the JPHC-FFQ validation study (1990–1993) (23). Thus, the differences in the dietary habits due to cohort effects may contribute to the differences in the dietary patterns involved in E-DII. Further studies in various populations are needed to determine the types of dietary patterns involved.

This study has several limitations. First, the participants of the present study were individuals who had voluntarily undergone cancer screening. These participants, especially women, may be particularly health conscious as indicated by their smoking rate, which was considerably lower than that observed in the National Health and Nutrition Survey in Japan: approximately 15% men and 5% women in the current study compared with 30% men and 10% women in the national survey (36). This selection bias

might have contributed to the null association between E-DII score and hs-CRP concentration, observed in women. Second, because this is a cross-sectional study, we could not account for the temporality requirement for assessing causality. Despite these limitations, the present study is a relatively large-scale study, and the results indicated gender differences in E-DII validity and reaffirmed validity in Japanese men. Thus, this justifies the use of E-DII in Japanese men.

To conclude, we conducted a validation study of E-DII using hs-CRP concentration in Japanese men and women and observed a positive association between E-DII scores and hs-CRP concentration in Japanese men, even after adjusting for age, BMI, smoking status, regular prescription medicine use, and physical activity. This indicates the utility of the E-DII in Japanese men who have different dietary habits and considerably lower-grade inflammation status than those of the Western population. However, we could not detect any association between E-DII scores and hs-CRP concentration in Japanese women, except for prescription medication non-users. Therefore, further studies are needed to clarify the utility of the E-DII in Japanese women.

DATA AVAILABILITY STATEMENT

We cannot publicly provide individual data due to participant privacy, according to ethical guidelines in Japan. Additionally, the informed consent we obtained does not include a provision for publicly sharing data. Qualifying researchers may apply to access a minimal dataset by contacting Dr. Shoichiro Tsugane, Principal Investigator, Epidemiology and Prevention group, Center for Public Health Sciences, National Cancer Center, Tokyo, Japan, at stsugane@ncc.go.jp. Or, please contact the Office of the Research Center for Cancer Prevention and Screening Program at tyamaji@ncc.go.jp.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan (approval number G15-01 and 2016-165). The study aims and protocols were explained to all participants, and each participant provided written informed consent before enrolment in the study.

AUTHOR CONTRIBUTIONS

AK, NSa, MIw, JI, and MIn designed the study. ST, JI, NSa, MIw, and MIn arranged the field survey. AK, NSa, MIw, and TY contributed to the blood analysis. NSh and JH conducted DII calculation and provided critical input to the manuscript. AK performed statistical analysis, interpreted the results, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Ministry of Health, Labour and Welfare of Japan (Grant-in-Aid for the 3rd Term Comprehensive 10-Year-Strategy for Cancer Control), the National Cancer Center Research and Development Fund (23-A-1, 26-A-1, 29-A-1), Practical Research for Innovative Cancer Control from Japan Agency for Medical Research and Development (AMED; No. JP19ck0106266h003), and JSPS KAKENHI (Grant Number: 20K15488).

ACKNOWLEDGMENTS

We are grateful to all the participants of the study and to the doctors, nurses, and administrative staff at the Research Center for Cancer Prevention and Screening who were involved in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.604296/full#supplementary-material

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Conflict of Interest: JH owns a controlling interest in Connecting Health Innovations LLC (CHI), a company planning to license the right to his invention of the dietary inflammatory index (DII), from the University of South Carolina, to develop computer and smartphone applications for patient counseling and dietary intervention in the clinical setting. NSh is an employee of CHI. The subject matter of this paper will have no direct bearing on the work of CHI, nor has any CHI-related activity exerted any influence on this project. MI was the beneficiary of a financial contribution from the AXA Research Fund as a chair holder of the AXA Department of Health and Human Security at The University of Tokyo. The AXA Research Fund had no role in the design, data collection, analysis, interpretation, manuscript drafting, or in the decision to submit the manuscript for publication.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 14 June 2021
doi: 10.3389/fnut.2021.676697

Validity and Reliability of a Water Frequency Questionnaire to Estimate Daily Total Water Intake in Adults

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Edited by:
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Reviewed by:
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Claudio Esteban Perez-Leighton, Pontificia Universidad Católica de Chile, Chile

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Specialty section:
This article was submitted to Nutrition Methodology, a section of the journal Frontiers in Nutrition

Received: *05 March 2021*
Accepted: *18 May 2021*
Published: *14 June 2021*

Citation:
Colburn AT, Johnson EC, Péronnet F, Jansen LT, Capitan-Jimenez C, Adams JD, Guelinckx I, Perrier ET, Mauromoustakos A and Kavouras SA (2021) Validity and Reliability of a Water Frequency Questionnaire to Estimate Daily Total Water Intake in Adults. Front. Nutr. 8:676697. doi: 10.3389/fnut.2021.676697

The purpose of this investigation was to assess the validity and reliability of a seven-day water frequency questionnaire (TWI-FQ) to estimate daily total water intake (TWI) in comparison to a water turnover objective reference value via deuterium oxide (D_2O). Data collection occurred over 3 weeks, with a wash-out period during week two. Healthy adults (n = 98; 52% female; 41 ± 14 y; BMI, 26.4 ± 5.5 kg·m^{-2}) retrospectively self-reported consumption frequencies of 17 liquids and 35 foods with specified volumes/amounts for weeks one and three via TWI-FQ. Standard water content values were utilized to determine the volume of water consumed from each liquid and food for calculation of mean daily TWI for each week. Diet records were completed daily during week two to estimate metabolic water production. To assess validity of the TWI-FQ, participants consumed D_2O at the start of each week and provided urine samples immediately before ingestion, the following day, and at the end of the week to calculate water turnover. Metabolic water was subtracted from water turnover to estimate TWI. TWI-FQ validity was assessed via Bland-Altman plot for multiple observations. Reliability was assessed via intraclass correlation and Pearson's correlation between weeks. TWI-FQ significantly underestimated D_2O TWI by −350 ± 1,431 mL·d^{-1} (95% confidence interval (CI): −551, −149 mL·d^{-1}). TWI-FQ TWI was significantly correlated ($r = 0.707$, $P < 0.01$) and not different (198 ± 1,180 mL·d^{-1}, 95% CI: −38, 435 mL·d^{-1}) between weeks. TWI-FQ intraclass correlation = 0.706 was significant [95% CI: 0.591, 0.793; $F_{(97, 98)} = 5.799$], indicating moderate test-retest reliability. While this tool would not be suitable for individual TWI assessment, the magnitude of bias may be acceptable for assessment at the sample-level.

Keywords: dietary assessment, hydration, nutrition methodology, self-report, underhydration, water

INTRODUCTION

A limited ability to accurately assess water intake at a population-level has likely slowed progress in elucidating the impact of water intake on health. Some evidence suggests that low water consumption and underhydration are associated with adverse health outcomes including chronic kidney disease and diabetes (1–3). Similarly, the Institute of Medicine suggests dehydration may be related to numerous detrimental health outcomes including cardiovascular strain, urinary tract infection, and diabetes dysregulation (4). Conversely, increased water intake is associated with positive health outcomes including reduced risk for kidney stones (4) and urinary tract infections (5) as well as augmented glucose regulation (6) and adolescent cognitive performance (7). Thus, there appears to be an inverse relationship between water intake and health risk. However, evidence supporting these associations is not sufficient to establish total water intake (TWI) recommendations beyond an Adequate Intake, the least specific recommendation included in the Institute of Medicine's dietary reference intakes (4). Due to the wide range of TWI volumes that allowed individuals to maintain adequate serum osmolality, the Adequate Intake is the median value of the TWI volumes observed in the Third National Health and Nutrition Examination Survey (4). These high variations have largely been attributed to differences in culture, climate, and/or physical activity (4). However, measurement error in TWI assessment and lack of a standard assessment tool could exacerbate these variations.

The TWI Adequate Intake recommendations encompass water consumed from all foods (∼20% TWI) and liquids (∼80% TWI) (4). The current recommendation is based upon the National Health and Nutrition Examination Survey 24-h diet recalls, which were conducted before utilization of the United States Department of Agriculture's Automated Multiple-Pass Method, which is a validated method for energy and nutrient intake, but not TWI (8). Additionally, trained interviewers prompt participants to choose an occasion for every food item on the record, most of which are meals (9). However, beverage intake occurs more continuously throughout the day. Individuals have 0 – 19 drinking occasions per day (of water only), with an average time of 3 h between drinking occasions (range 1–17 h) (10). This has been observed when TWI was compared between a fluid-specific tool and the United Kingdom's National Diet and Nutrition Survey, which utilizes food diaries (11). The fluid-specific tool revealed that 70% of beverage consumption occurred outside of meals. In Indonesian populations, TWI estimated from a 7-day fluid diary was significantly greater than that from the 24-h dietary recall, by 382 mL (12). Additionally, the 24-h dietary recall captured 2.2 fewer drinking occasions (6.7 vs. 8.9 occasions). Consequently, current dietary assessments which have not been validated for water intake are not necessarily suitable for drinking behavior.

To date, investigators have not been able to identify a method to estimate TWI that is comparable to an objective reference value, such as that obtained from water turnover by dilution of deuterium oxide (D_2O) (13) corrected for metabolic water, which is costly and impractical for population-level use. Recently, our group and others have begun to advance the field through development and validation of fluid-specific assessment tools (14–19). Compared to 24-h recalls, which are subject to bias from day-to-day variation in consumption, frequency questionnaires are more likely to capture usual intake (20). However, only relative validation, via dual reporting, has been assessed for prior beverage frequency questionnaires with comparison of water intake estimates against self-reported 24-h records (14–17). As the 24-h diet record and new questionnaires under assessment for validation are both self-reporting instruments, sources of error will overlap between the instruments and be correlated. Additionally, validation through dual recording will not distinguish inaccuracies if they are reported on both assessments. Dual recording could also deceptively improve accuracy of the new questionnaire, and therefore falsely show validation, as recording intake in diet records in days leading up to a frequency recall will likely improve recall accuracy.

We recently utilized D_2O to validate Liq.In^7, a 7-day fluid diary, to record all beverage intake over seven-days. While it has been shown to be an accurate recording instrument for TWI volume, the seven-days of recording impose substantial subject burden (18). Additionally, Liq.In^7 only captures water from liquids, and not TWI. However, there is limited evidence from the US and Europe supporting the current belief that TWI is ∼80% water from liquids. In fact, those with high and low TWI have been observed to consume a similar amount of water from food (∼0.6 $L \cdot d^{-1}$), resulting in substantially different contributions to TWI. For instance, water from food comprised ∼23% of TWI in those with high TWI, while ∼47% in those with low TWI (21). Consequently, liquid-only assessments may be preferable in studies where precise recording of fluid intake is important but may elucidate misleading results in terms of TWI. To address this gap, we developed a total water intake frequency questionnaire (TWI-FQ) that prompts individuals to recall water intake from food and beverages over a 7-day period. The purpose of this investigation was to assess the reliability and validity of the TWI-FQ to estimate TWI as compared to the value obtained with dilution of D_2O, corrected for metabolic water.

MATERIALS AND METHODS

Subjects

Potential healthy participants (n = 262, 18 – 65 y) were recruited from Northwest Arkansas, and provided informed consent acknowledging the risks and benefits of participating in the study (**Supplementary Figure 1**). Following completion of a medical history questionnaire, individuals were excluded if they satisfied any of the following criteria: (1) unable to understand and write English, (2) currently pregnant or breastfeeding, (3) previous surgical operation on digestive tract (excluding appendectomy), (4) drug treatment within 15-days prior to the start of the study, (5) exercise > 4 $h \cdot week^{-1}$, (6) dietary changes within the last month, or (7) changes in body weight > 2.5 kg within the last month. Volunteers with clinically relevant diseases that could alter fluid balance (i.e., relevant metabolic, cardiovascular, hematologic, hepatic, gastrointestinal,

renal, pulmonary, endocrine or psychiatric history of disease) were not enrolled.

Ultimately, 103 individuals received medical clearance, met all criteria, consented to voluntary participation, enrolled, and completed the study protocol. Data from five participants were excluded due to missing data that prevented calculation of TWI through TWI-FQ or dilution of D_2O during weeks one or three. Participant demographics are presented in **Table 1**. Data collection occurred May – December 2014 in Fayetteville, Arkansas, USA (ambient temperature, 17.2 ± 8.4°C). This protocol was approved by the University's institutional review board and biosafety committee (protocol no. 14-03-555) and was conducted in compliance with the Helsinki Declaration as revised in 1983.

Questionnaire Development

The TWI-FQ is a 59-item water intake assessment that quantitatively assesses frequency and volume of TWI within the period of a week. The first and second page of the questionnaire consisted of 24 and 35 items to assess water from liquid and food, respectively. The TWI-FQ included 17 liquid types with specified volumes (e.g., water [8 fl oz cup]). Water was further broken into eight occasions of consumption to include periods that may be forgotten in traditional meal- and snack-focused questionnaires (e.g., before breakfast, between lunch & dinner, during your sleep). Nine frequency options were included, ranging from "Never or <1 per week" to "7+ per day." The TWI-FQ also includes four overarching food categories (vegetables; fruits; cheese, egg, meats; & bread, cereal, starches). Within categories, food types were listed with specified quantities (e.g., mango, pineapple [1 cup], pizza [1 slice]). Eight frequency options were included, ranging from "Never or <1 per week" to "6+ per day." The TWI-FQ has a Flesch-Kincaid grade level of 8.4 and a completion time of ∼5 min.

The TWI-FQ is visually similar to the validated Harvard Willett Food Frequency Questionnaire (Harvard T.H. Chan School of Public Health, Department of Nutrition) (22). While this questionnaire includes a section on beverage intake, reproducibility and validation have only been established for dietary assessment of caloric intake and macro- and micronutrient intake, but not for TWI. There is only one question for plain water intake in the Harvard Willett Food Frequency Questionnaire, which only allows individuals to record a maximum of 1.5 $L{\cdot}d^{-1}$ with the allotted frequency options. This is not adequate considering the median water intake from liquids is 2.2 L for women and 3.0 L for men (4). Additionally, as mentioned previously, water consumption occurs throughout the day (10) and is often underreported on self-report tools that are not specific to beverages (11, 12). The eight occasions of consumption for plain water were included in our TWI-WFQ to accommodate individuals who drink more than 1.5 $L{\cdot}d^{-1}$ of plain water. These eight occasions also serve as a reminder for individuals to report water consumed throughout the day. Outside of plain water, all other beverages and foods were selected from the U.S. Department of Agriculture What We Eat in America Food Categories from NHANES 2009–2010 (23). Within each food category, some items included multiple foods with similar water content. For example, "mango, pineapple (1 cup)" was one item in the fruit category. The water content of 1 cup of mango and 1 cup of pineapple are 138 mL and 142 mL, respectively.

Study Design

Participants visited the lab on nine separate occasions across 22 days with the second week serving as a wash-out period (**Supplementary Table 1**). A TWI-FQ was completed on day 1 to familiarize participants with the tool. Participants ingested D_2O at the start of weeks one and three for determination of total body water and mean daily water turnover from the disappearance of D_2O in the body water pool via the slope-intercept method (13, 18). The days following completion of weeks one and three (days 8, 22), participants completed the TWI-FQ for the previous seven days. Diet records (24) were completed daily during week two and analyzed to determine metabolic water (25, 26). Estimates of TWI from weeks one and three were compared between the D_2O method and TWI-FQ method to assess the validity of the TWI-FQ. TWI estimates were compared between weeks one and three to assess reliability.

Baseline characteristics were collected on day one. Body mass was assessed with a scale, height was measured using a wall-mounted stadiometer, and body fat was measured via

TABLE 1 | Baseline sample demographics by sex and age group.

	Women			Men			All participants
Age range, *y*	**18–29**	**30–49**	**50–65**	**18–29**	**30–49**	**50–65**	**41 ± 14**
Participants, *n*	14	22	15	12	21	14	98
Height[a], *m*	1.66 ± 0.06	1.62 ± 0.06	1.64 ± 0.08	1.75 ± 0.04	1.75 ± 0.07	1.81 ± 0.07	1.70 ± 0.09
Weight[a], kg	68.9 ± 19.9	75.4 ± 17.6	69.6 ± 11.7	74.1 ± 18.1	79.2 ± 13.3	89.0 ± 14.2	76.2 ± 16.8
BMI[a], $kg{\cdot}m^{-2}$	25.0 ± 6.8	28.8 ± 6.7	25.8 ± 4.0	24.5 ± 6.5	25.7 ± 4.0	27.1 ± 3.7	26.4 ± 5.5
Total Body Water[a,b], L	34.5 ± 6.9	33.1 ± 4.7	30.7 ± 3.3	39.6 ± 5.2	44.3 ± 6.4	46.2 ± 6.0	38.0 ± 8.0
Total Body Water[a,b], %BM	51.1 ± 6.0	45.0 ± 7.0	44.6 ± 4.0	54.8 ± 7.6	56.4 ± 5.1	52.4 ± 4.8	50.5 ± 7.4

BMI, body mass index; %BM, total body water as a percentage of body mass.

[a] *Values are presented as mean ± standard deviation.*

[b] *Total body water is the average of values computed at weeks 1 and 3.*

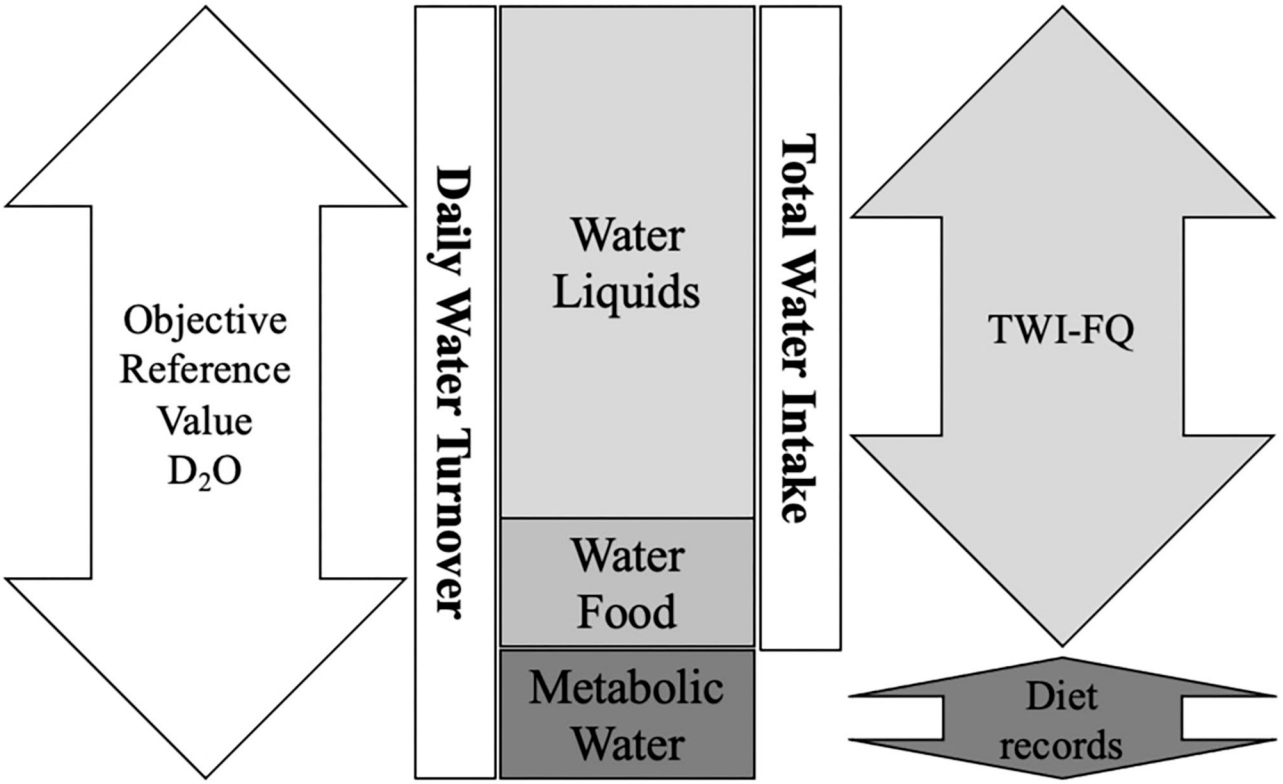

FIGURE 1 | Estimation of total water intake using deuterium oxide dilution method and the total water intake frequency questionnaire. D_2O, deuterium oxide; TWI-FQ, total water intake frequency questionnaire.

dual X-ray absorptiometry scan (Lunar Prodigy, GE Healthcare, Waukesha, WI).

Total Water Intake: Frequency Questionnaire

TWI-FQ were completed on days 1, 8, and 22. Day 1 served as a familiarization with the instrument, while participants retrospectively recalled water from liquids and foods for weeks 1 and 3 using TWI-FQs on days 8 and 22, respectively. The types and frequencies of liquids and foods consumed were entered into a customized spreadsheet and converted to mL (27). The volumes of liquids were converted to volumes of water based on standard water contents (e.g., 100 mL of milk = 89 mL water) (27). Reported volumes and frequencies were then used to determine mean daily water from liquids. Researchers converted reported quantities of foods to mL of water according to standard food water content and determined mean daily water from food based on calculated volumes and frequencies (**Figure 1**).

Water Production From Metabolism

Participants recorded all food and liquid intake in 24-h diet records (24) every day of week 2 (days 8–14). For each item consumed, participants were instructed to record timing, portion size, method of preparation, number of servings, and any other pertinent information (i.e., brand name, restaurant, nutrient descriptors such as low-fat, condiments, etc.). Diet records were completed in real time, in contrast to diet recalls which can introduce error due to reliance on memory. Furthermore, multiple diet records were completed to increase the likelihood of capturing usual intake.

Diet records were analyzed with Nutrition Data System for Research software to determine the total energy intake and the proportions of energy that corresponded with each macronutrient. These values were then used to determine the volume of water generated through macronutrient oxidation using the following formula (25, 26):

$$\text{Metabolic water } \left(\text{mL} \cdot \text{d}^{-1}\right) = \text{total energy expenditure}$$
$$\text{x } \left(\frac{1}{10^5}\right) \text{x } \left[\left(\%\text{fat x } 0.119\right) + \left(\%\text{protein x } 0.103\right)\right.$$
$$\left. + \left(\%\text{carbohydrate x } 0.150\right) + \left(\%\text{alcohol x } 0.168\right)\right]$$

Total energy expenditure was assumed to be equivalent to total energy intake. Body weights measured on the first, second, and fifth days of both weeks were assessed to confirm weight stability and therefore confirm the aforementioned assumption was met.

Total Water Intake: D_2O Dilution

Participants provided a urine sample on day 1 immediately before D_2O ingestion (0.1 g·kg^{-1} LBM, 99.9% deuterium, Cambridge

TABLE 2 | Mean daily water turnover and mean daily water intake by week and method.

	Week 1		Week 2	Week 3	
	D_2O	**TWI-FQ**	**Food diaries**	**D_2O**	**TWI-FQ**
Water turnover[a], mL·d^{-1}	3,680 ± 1,341			3,596 ± 1,275	
Metabolic water[a], mL·d^{-1}			264 ± 104		
Water from food[a], mL·d^{-1}		508 ± 258			490 ± 242
Water from liquids[a], mL·d^{-1}		2,624 ± 1,587			2,443 ± 1,358
Total water intake[a], mL·d^{-1}	**3,405 ± 1,331[b]**	**3,132 ± 1,665[c]**		**3,356 ± 1,234[b]**	**2,933 ± 1,425[c]**

D_2O, deuterium oxide dilution method; TWI-FQ, total water intake frequency questionnaire.
[a]*Values are presented as mean ± standard deviation.*
[b]*Total Water Intake = Water Turnover – Metabolic Water.*
[c]*Total Water Intake = Water from Food + Water from Liquids.*

Isotope Laboratories, Inc., Tewksbury, MA). The dose of D_2O was added to a cup with 100 mL of water. Participants consumed the diluted tracer followed immediately by two additional 100 mL volumes of water ingested from the same cup to ensure tracer was consumed and not left on the cup. Participants returned on days 2 and 8 to provide additional urine samples. This process was repeated during week 3 on days 15, 16, and 22 with a D_2O dose of 0.08 g·kg^{-1} LBM ingested at day 15 immediately after providing the urine sample. Samples were then analyzed via isotope ratio mass spectrometry (Micromass Isoprime DI, coupled with an Aquaprep system; Isoprime Ltd., Cheadle Hulme, UK) using the H_2-water equilibration method to determine the ratio of deuterium to hydrogen (13, 28). The slope intercept method (29) was then used as previously described (13) to compute the volumes of total body water for weeks one and three from the dose ingested and the ratio of deuterium to hydrogen back-extrapolated at the time of ingestion, as well as water turnover from the disappearance of D_2O from the body water pool. Finally, D_2O TWI was calculated by subtracting metabolic water from water turnover.

Sample Size Estimation

An a priori sample size of $n = 75$ was determined based on the desired accuracy of Bland-Altman limit of agreement estimates (30). Accuracy of estimates is determined by the standard error of 95% confidence intervals (CI) for the limits of agreement. Standard error (SE) was determined by $SE = \sqrt{(3\ x \frac{SD^2}{n})}$, where SD is the standard deviation of the mean difference and n is the sample size. The 95% $CI = \pm 1.96\ x\ SE$. A sample size of 75 allows for 95% $CI = \pm 0.39\ x\ SD$.

Statistical Analyses

Outcome variables were assessed for normality via Shapiro-Wilk test of normality, visual examination of the data (e.g., Q-Q plots, box plots, histograms), and skewness and kurtosis statistics. Non-normal data were analyzed non-parametrically. Analyses were conducted using commercial software (IBM SPSS Statistics Version 25.0.0). A jack-knife approach was employed using JMP Pro 15.2.0 (SAS Institute Inc.) to identify and examine the influence of outliers in the sample. Outliers were classified as mild (jack-knife distances $> 2.5–\leq 4.0$) or severe (jack-knife distances > 4.0). A $P < 0.05$ was considered statistically significant for all analyses. Data are presented as mean ± standard deviation.

Correlation and *t*-test analyses alone are not sufficient to assess validity between two measurement methods (31, 32). Therefore, we used paired *t*-tests to assess mean differences between measurements and a Bland-Altman plot to assess agreement between D_2O and TWI-FQ to estimate daily fluid intake over weeks one and three. Bland-Altman analyses were conducted in accordance with methodology specific to multiple observations in which the true value of the primary outcome variable (i.e., TWI) is expected to vary over the observation period (33). This model accounts for mean difference (bias) between methods (TWI-FQ – D_2O TWI) as well as variance in individual differences (between + within-subject variance). Individual differences between methods were plotted against the average of methods [(TWI-FQ – D_2O TWI)/2], with repeated measurements treated as independent measurements ($n = 196$). Evaluation of the Bland-Altman plot within limits of agreement allowed us to understand the significance of bias of the TWI-FQ from the objective reference value D_2O TWI. Kendall's tau was utilized to evaluate heteroscedasticity of the plot.

Reliability of the TWI-FQ to estimate TWI was assessed via related-samples Wilcoxon Signed Rank test, Spearman's correlation, and Intraclass correlation coefficient. Paired *t*-test and Spearman's correlation analyses were also conducted on D_2O TWI to provide an indication of weekly variation in true TWI. To explore systematic bias in reliability, separate Bland-Altman plots (31) were created for D_2O and TWI-FQ estimates of TWI. For each method, differences between repeated estimates of TWI (week 1 TWI – week 3 TWI) were plotted against the average of estimates from both weeks ($n = 98$).

RESULTS

Body mass was consistent within weeks (% of change in body mass: week 1, 0.05 ± 0.99%; week 2, 0.35 ± 1.19%; week 3, 0.11 ± 1.22%) with low coefficients of variance between the three measurements during all weeks (week 1, 0.54 ± 0.36%; week 2, 0.62 ± 0.41%; week 3, 0.60 ± 0.50%). Mean daily water turnover and the components that contribute to water turnover computed using data from D_2O dilution and the TWI-FQ are presented in **Table 2**. Daily caloric intake during week 2 was 2,028 ± 523 kcal (range: 911–3,430 kcal).

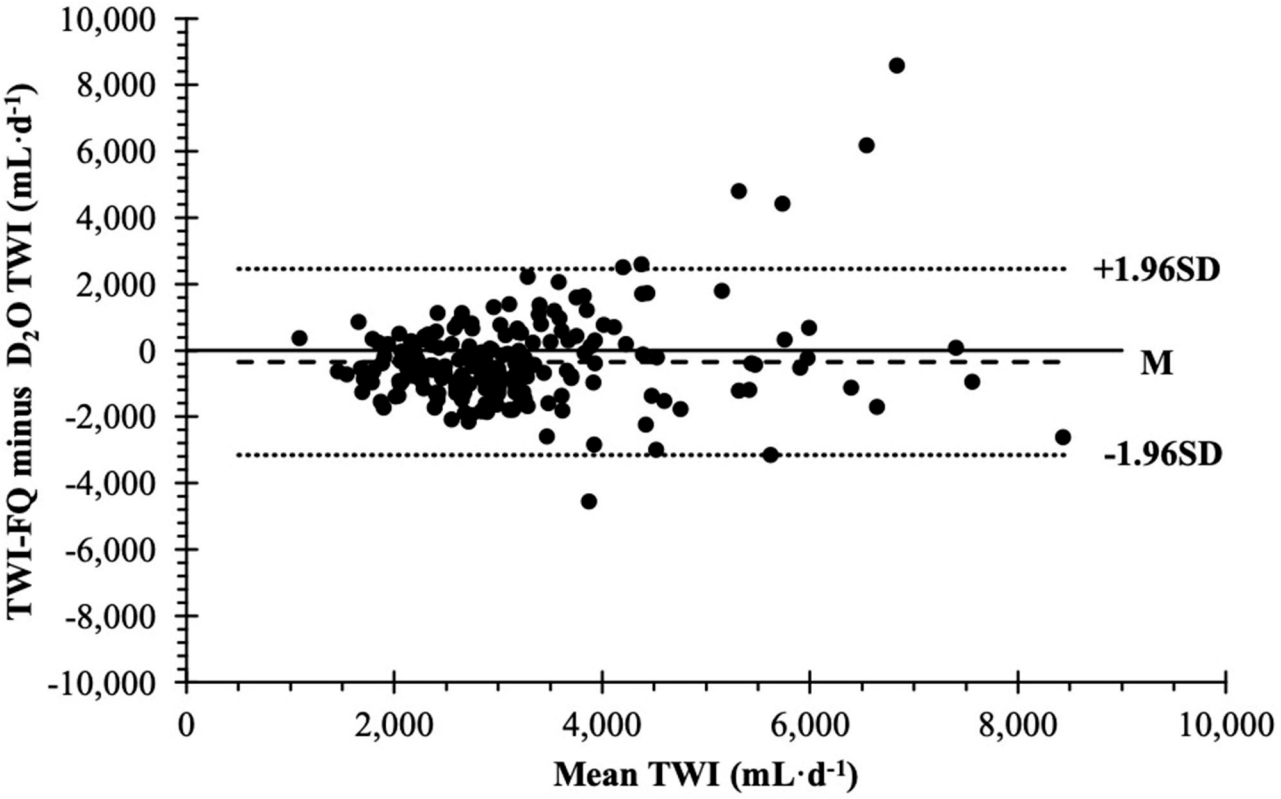

FIGURE 2 | Bland-Altman plot of agreement between total water intake frequency questionnaire and deuterium oxide dilution method to estimate mean daily total water intake during two, one-week periods ($n = 196$). TWI, mean daily total water intake; TWI-FQ, total water intake frequency questionnaire; D_2O, deuterium oxide dilution method; M, mean difference between methods (bias); SD, standard deviation of the mean difference.

The jack-knife analysis identified eight mild outliers and three severe outliers across eight participants (63% male; age, 37 ± 13 y; BMI, 26.5 ± 5.9 kg·m^{-2}) (**Supplementary Table 2**). All three severe outliers were found in males in week 1, while four mild cases were identified in each week. TWI was overestimated by the TWI-FQ in five of the eleven cases, two of which were identified as severe outliers. No outliers were excluded from validity or reliability analyses.

Questionnaire Validity

TWI estimates were not different between methods during week 1 ($t_{[97]} = 1.60$, mean difference = −269 mL·d^{-1}, 95% CI:−603, 65 mL·d^{-1}, $P = 0.1133$), but were significantly different during week 3 ($t_{[97]} = 3.71$, mean difference = −431 mL·d^{-1}, 95% CI: −661, −200 mL·d^{-1}, $P = 0.003$). Combined TWI-FQ TWI estimates from both weeks significantly underestimated D_2O estimates by −350 ± 1,431 mL·d^{-1} (95% CI: −551, −149 mL·d^{-1}; **Figure 2**). Limits of agreement for the Bland-Altman plot were −3,155 and 2,455 mL·d^{-1}. Kendall's tau was not significant ($r = 0.076$, $P = 0.112$), which indicates the data were not heteroscedastic.

Questionnaire Reliability

D_2O TWI was significantly correlated (r = 0.856, $P < 0.01$) and was not different ($P = 0.805$) between weeks. Similarly, TWI-FQ TWI was significantly correlated (r = 0.707, $P < 0.01$) and was not different ($P = 0.115$) between weeks 1 and 3. The Intraclass correlation coefficient for TWI-FQ was significant [ICC = 0.706, 95% CI: 0.591, 0.793; $F_{(97,98)} = 5.799$, $P < 0.001$], indicating moderate test-retest reliability. Based on Bland-Altman plots, the mean difference in D_2O TWI estimates between weeks was 36 ± 593 mL·d^{-1} (95% CI: −83, 155 mL·d^{-1}; **Figure 3A**). The mean difference in TWI-FQ TWI estimates between weeks was 198 ± 1,180 mL·d^{-1} (95% CI: −38, 435 mL·d^{-1}; **Figure 3B**). Systematic bias in reliability was not observed for either method.

DISCUSSION

The purpose of this study was to assess the validity and reliability of a TWI-FQ to estimate TWI as compared to the objective reference value, D_2O. The principle finding of this study is that the TWI-FQ consistently underestimated TWI. While this tool would not be suitable for individual assessment, the overall magnitude of bias may be acceptable for assessment at the sample-level. In this protocol, we utilized the isotopic tracer, D_2O, as the rate of disappearance of D_2O following enrichment is directly associated with water turnover and is not subject to homeostatic or inter-individual variations in metabolism (34). Accordingly, D_2O is an unbiased measure of water turnover that

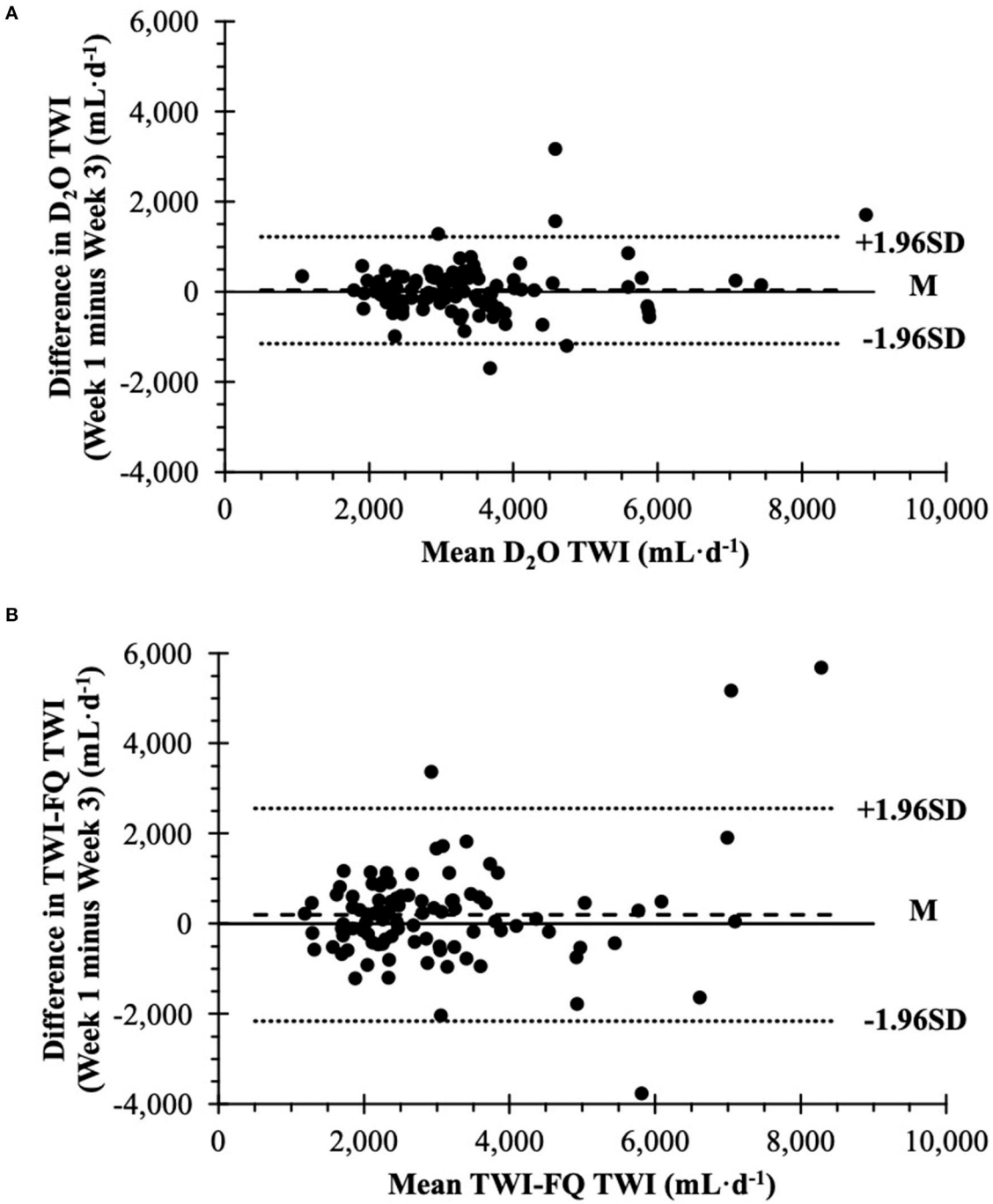

FIGURE 3 | Bland-Altman plots of agreement between repeated estimates (week 1 and 3) of mean daily total water intake via **(A)** deuterium oxide dilution method and **(B)** total water intake frequency questionnaire ($n = 196$). TWI, mean daily total water intake; TWI-FQ, total water intake frequency questionnaire; D_2O, deuterium oxide dilution method; M, mean difference between weeks (bias); SD, standard deviation of the mean difference.

is not subject to measurement error commonly seen in self-report data. Furthermore, we utilized Bland-Altman statistical analyses that accounted for variation between methods, between individuals, and between occasions (33). Most prior studies (14–17) have utilized correlation and *t*-test analyses, which are not independently sufficient to assess agreement between two methods for validity assessment (31, 32).

Due to the robustness of the D_2O dilution method, we observed total body water as a percentage of body mass estimates within the ranges reported by the Institute of Medicine (43–73%,

males 19–50 y; 41–60%, females 19–50 y) (4). Additionally, daily metabolic water production has been estimated to be ∼250–350 mL·d^{-1} for sedentary individuals (4). Although metabolic water (264 ± 107 mL·d^{-1}) was determined from diet records during the wash-out period in the current study, it still aligns with the aforementioned estimates. Confirmation of body weight stability for all participants across each week indicates that the assumption that TEE was equivalent to total energy intake was met for metabolic water calculations. Accordingly, we are confident the D_2O TWI estimates reflect actual TWI.

The TWI-FQ significantly underestimated TWI by −350 ± 1,431 mL·d^{-1} compared to D_2O. While the mean difference is clinically adequate, there is considerable variation in bias as evidenced by the standard deviation of 1,431 mL and limits of agreement allowing for underestimation of −3,155 mL·d^{-1} and overestimation up to 2,455 mL·d^{-1}. The magnitude of these differences is substantial considering the Adequate Intake for water is 2.7 L·d^{-1} for women and 3.7 L·d^{-1} for men (4). Based on visual examination of **Figure 3**, these large differences appear to be driven, in part, by individuals who consume high amounts of TWI (≥4 L·d^{-1}). In some cases, high amounts of TWI were accurately reported in the TWI-FQ (**Supplementary Table 2**). Large differences may in part be related to a learning curve as all three severe outliers were identified in the first week. Furthermore, most participants with outliers appeared to improve by week 3 (i.e., reduced from severe to mild outlier or no longer an outlier). Ultimately, outliers were a mixture of overestimation and underestimation with no clear association with subject characteristics (i.e., sex, age, BMI). Despite large variances, the TWI-FQ was still determined to be reliable due to moderate correlation between weeks ($r = 0.725$) and moderate test-retest reliability (ICC = 0.706). Systematic bias in TWI-FQ between weeks was not statistically significant as evaluated via Bland-Altman plot, in which the mean difference in TWI-FQ TWI estimates was 198 ± 1,180 mL·d^{-1}.

The mean difference (36 ± 593 mL·d^{-1}) between repeated D_2O TWI estimates was minimal and non-significant. However, the acceptable limits of agreement (-1,149, 1,221 mL·d^{-1}) are still large clinically and indicate a considerable degree of within-subject variance in week-to-week TWI. Additionally, mean D_2O TWI was distributed across a wide range of volumes, between 1,000 and 9,000 mL·d^{-1}, with the majority of mean D_2O TWI falling between 1,000 and 4,500 mL·d^{-1}. This indicates there is also a considerable degree of between-subject variance in D_2O TWI, which was also captured by the TWI-FQ, as can be seen in **Figures 3A,B**. This magnitude of variance in TWI is not surprising as daily water needs can vary greatly between and within individuals depending on age, sex, diet, physical activity behaviors, climate, and culture (4). We purposefully recruited participants who were well-distributed across sex and age. Therefore, although the limits of agreement for the TWI-FQ validity assessment were large, these data indicate that the variance observed was compounded by within- and between-subjects' differences in water consumption habits.

Previous liquid questionnaires have been developed to assess fluid intake but not TWI (14–17, 19). While this TWI-FQ was designed specifically to assess water intake volume at population levels, previous questionnaires were developed primarily to assess energy intake from liquids (16, 17), grams or fluid ounces of individual and total liquids consumed (16, 17, 19), water intake and voiding habits for treatment of urinary tract symptoms (14), and water balance (15). Additionally, validation protocols for these questionnaires utilized imperfect reference instruments, such as 24-h diet records, which are subject to intake-related bias and correlated error (35). We used methods similar to the previous study to assess validity and reliability of the Liq.in^7, which is a 7-day fluid record that required participants to record liquids and foods with high water content as they were consumed (18). Compared to D_2O, the Liq.in^7 underestimated water from liquids by −131 ± 845 mL·d^{-1}. However, this assessment was based only on one week of data, the Bland Altman statistical analysis utilized did not account for within or between subject variation, and water from food was not included in this analysis. TWI was also assessed between the Liq.in^7 and a 24-h dietary recall in Indonesian adolescents and adults using a Bland Altman analysis (12). An overestimation of 382 mL·d^{-1} was observed compared to the 24-h dietary recall with limits of agreement 1,600 and −2,300 mL·d^{-1}. Although the limits of agreement were narrower than those in the current study, the difference was determined to be significant as 11% of values fell outside of these limits. The mean difference also increased with greater TWI, with underestimation of 139 mL·d^{-1} for the lowest quartile of TWI and overestimation of 1,265 mL·d^{-1} for the highest quartile of TWI. Thus, it appears individuals are less able to recall fluid intake accurately with greater consumption.

Our approach does not come without limitations. Metabolic water production was determined through self-reported data in 24-h diet records. Self-report dietary assessments are subject to error (e.g., difficulty interpreting handwriting, day-to-day variation in consumption, or misreporting of consumption) and can be burdensome to participants. However, metabolic water is a small component of water turnover (250–350 mL·d^{-1}) (4) and over- or underestimation would not substantially impact the outcomes of this investigation. Furthermore, a prominent study in this field that determined water turnover in 458 adults (40–79 y) estimated metabolic water from the average macronutrient content of the diet based on a one-time 24-h recall in the general population in the US (26). In contrast, participants in the present investigation completed multiple 24-h diet recalls for metabolic water estimates.

The accuracy of the TWI-FQ may vary day-to-day, with TWI estimates that are more representative of days closer to the day of questionnaire completion. However, we were not able to evaluate this as participants are asked to recall consumption for the entire week rather than for each day of the week. Similarly, the D_2O method utilizes three urine samples to determine an average daily TWI for the 7-day period and does not allow for estimation for each specific day. Furthermore, we were not able to evaluate potential differences in validity or reliability of the TWI-FQ by age or sex as this study was not powered for these comparisons. Finally, we were not able to validate whether the TWI-FQ is sensitive to change in TWI. Therefore, this tool may not be suitable for use in intervention studies

designed to change TWI, particularly if detection of small changes is desired.

In conclusion, the TWI-FQ may be a useful tool to assess population-level TWI behaviors. Due to the large variances observed, the TWI-FQ should not be utilized to assess individual-level TWI behaviors in which greater accuracy may be needed. Utilization of the TWI-FQ to assess population-level TWI may allow investigators to better determine relationships between liquid intake, hydration, and health. Moreover, the TWI-FQ could be utilized in conjunction with multiple 24-h diet recalls/records to better reflect water from food and subsequently TWI. Several studies have successfully improved accuracy of self-report dietary data through combining 24-h diet recall/records with food frequency questionnaires (36). The findings of this study can only be generalized to individuals 19–65 y. Further investigation is needed to assess application of the TWI-FQ in different geographical regions, climates, cultures, activity levels, and age groups.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Arkansas, IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows–SK, FP, IG, and EP designed research (project conception, development of overall research plan, and study oversight). EJ, LJ, CC-J, and JA conducted research (hands-on conduct of the experiments and data collection). AC, EJ, FP, SK, and AM analyzed data or performed statistical analysis. AC and SK wrote paper. SK had primary responsibility for final content. All authors have read and approved the final manuscript.

FUNDING

This study was funded by Danone Research (grant number: 30000273).

ACKNOWLEDGMENTS

The authors would like to thank Costas N. Bardis, LynnDee Summers, Yasuki Sekiguchi, Mickey Hammer, Thomas Vidal, Ainsley Hoffman, Berkley Vaughan, Laurent Smith, Rebecca Mishler, and Weldon Murry, for their help during data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.676697/full#supplementary-material

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Conflict of Interest: EJ had received grants from Danone Research and FP had served as member of the scientific advisory board of Danone Research. IG and EP were Danone Research employees. SK had served as scientific consultant for Quest Diagnostics and Standard Process and had served as a member of the scientific advisory board of Danone Research, and had active grants with Danone Research and Standard Process.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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