Original Communication

Urinary 2- to 16α-hydroxyestrone ratio did not change with cruciferous vegetable intake in premenopausal women

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Abstract: The mass ratio of urinary 2-hydroxyestrone to 16-α-hydroxyestrone (2:16) is hypothesized as a biomarker of breast cancer risk in premenopausal women, with higher ratios being theoretically protective. Cruciferous vegetable intake has been associated with higher urinary 2:16 in some studies. We investigated whether a whole-food supplement made from dried Brussels sprouts and kale would increase urinary 2:16 in comparison with placebo or cruciferous vegetables in women. This randomized, parallel arm, placebo-controlled, partly blinded study included 78 healthy premenopausal women (38–50 y) with screening urinary 2:16 ≤3.0. Subjects received either six capsules containing 550 mg dried Brussels sprouts and kale per capsule, 40 g daily alternating broccoli or Brussels sprouts, or placebo for eight weeks. Urinary 2:16 and creatinine were measured at baseline, four, and eight weeks. Intent-to-treat repeated measures-ANOVA with multiple imputation (n=100) for missing values identified no treatment effect (P=0.9) or treatment-by-time interaction (P=0.6); however, a significant time effect was noted (P=0.02). Per-protocol analyses including complete cases found no treatment effect (P=1) or treatment-by-time interaction (P=0.6); however, the significant time effect remained (P=0.03). Restricting analysis to subjects with >80% compliance maintained the time effect (P=0.02). Using Pearson correlations, android-pattern and android:gynoid fat were predictive of change ($P \leq 0.05$). In conclusion, neither cruciferous supplements nor an added vegetable serving altered urinary 2:16 in premenopausal women with eight weeks treatment. This ratio did vary with time, which is important for designing future trials.

Keywords: broccoli, brussels sprouts, cruciferous supplement, glucosinolates

Introduction

Higher vegetable consumption may protect against breast cancer. Two-thirds of 87 case-control studies in a review suggested a protective effect of cruciferous vegetable consumption on all-cancer risk [1]. Among European studies, investigators found a protective effect of consuming at least one cruciferous vegetable serving per week on fourteen different cancers (odds ratio: 0.83, CI: 0.74, 0.94) [2]. One hypothesis attributes the chemoprotective effect of cruciferous vegetables to glucosinolates. When plant tissues containing glucobrassicin are damaged, myrosinase cleaves a glucose moiety from glucobrassicin to form unstable intermediates [3, 4], one of which rearranges and loses a thiocyanate ion to form indole-3-carbinol (I3C). Cruciferous vegetables [5, 6] and supplemental I3C (400–800 mg) given for eight weeks [7] to humans induced CYP1A2 activity, which is positively associated with 2-hydroxylation of estradiol and estrone [8]. In prospective and case-control studies, endogenous estrogens have been positively associated with breast cancer risk in pre- and post-menopausal women [9, 10, 11, 12]. 2-Hydroxyestrone (2-OHE_1) has been termed the "good estrogen" [13] because it has low affinity for estrogen receptors and is associated with normal differentiation and apoptosis in human mammary explant tissue and an epithelial cell line (184-B5) [14]. Estradiol predominantly undergoes 2-hydroxylation in the liver [15], but estrogens can also be hydroxylated at carbons 4 and 16 by cytochrome P-450 enzymes [8]. 16-Hydroxylation may increase the risk of carcinogenesis because 16-hydroxylase activity was higher in women with breast cancer [16]. Moreover, 16α-hydroxyestrone (16α-OHE_1) can covalently bind

estrogen receptors [17], is higher in common sites of carcinogenesis [18], and is associated with greater numbers of potential carcinogenic sites [19].

The 2- and 16-hydroxylation pathways compete [20]; therefore, the ratio of 2- to 16-α-OHE1 (2:16) has been hypothesized as a biomarker of breast cancer risk that may represent overall 2- and 16-hydroxylation pathway metabolites [20], with higher ratios being protective. Urinary 2:16 is representative of breast tissue and serum ratios [21]. Case-control studies suggest that higher urinary 2:16 may be inversely associated with breast cancer risk [22, 23, 24]. In large prospective studies, greater 2-hydroxylation was protective against breast cancer in pre-, but primarily in post-menopausal women [25, 26, 27, 28].

While 16-hydroxylation is difficult to modulate with lifestyle [20], increases in urinary 2:16 have been associated with physical activity, body fat, smoking, and dietary intakes of soy, vegetables, cruciferous vegetables, caffeine, and fiber [20, 29, 30, 31, 32, 33]. While significant increases in 2:16 were observed with 10 g/d increases in cruciferous vegetables, studies examining cruciferous vegetables or supplemental I3C to increase urinary 2:16 or 2:estriol (another 16-hydroxylation metabolite) fed large amounts of vegetables (e.g. 500 g broccoli [31, 32] or an increase of ~185 g/d [29]) or gave large I3C doses (e.g. 400–800 mg/day [7, 20], 6–7 mg/kg [30]). A pilot study observed an increase in urinary 2:16 in 11 of 13 subjects consuming a dried whole-food cruciferous supplement [31]. We hypothesized that a small serving of cruciferous vegetables (40 g/day), either in vegetable or supplement form, would shift urinary 2:16 relative to placebo in healthy premenopausal women.

Subjects and methods

Participants and screening

The Health Sciences Institutional Review Board at University of Wisconsin-Madison (UW) approved the protocol (2011-0872) registered at Clinicaltrials.gov (NCT01726127). Subjects were recruited from April 2014–May 2015 through flyers, advertisements, and emails to women on UW campus. Potential subjects were phone screened (Figure 1). Inclusion criteria included premenopausal woman (40–50 y) with regular menstrual cycles and screening urinary 2:16 $\leq$3.0. Exclusion criteria included current kidney or liver disease; tobacco use; vigorous exercise for >1 h/d; use of antibiotics, cimetidine, or black cohosh; systemic administration of estrogen or use of non-prescription hormones, tamoxifen, or diabetes medication; strong dislike of Brassica vegetables; and history of cancer in previous 5 years.

Following written informed consent, a medical history, Harvard Grid 2011 Food Frequency Questionnaire (FFQ) [34], and Baecke physical activity questionnaire (PAQ) [35] were completed. The FFQ included intake questions on multivitamins, nine single vitamin supplements, and 19 other supplements including a spot for a write-in. The dairy section includes intake of milk split by fat level, cream, yogurt, ice cream, margarine, butter, and cheeses, which includes cream, cottage, ricotta, and other cheeses split by fat level. Also included in this section is soy milk and non-dairy creamer. The fruit section includes 14 distinct fruits and whether they are fresh, dried, frozen, or canned, and five questions on juice intake. The vegetable section includes 25 distinct vegetables and different preparations including raw or cooked, sauce, soup, juice, or mixtures. This section also includes tofu and soy products. The egg question includes whether or not they are omega-3 fortified. The meat questions include beef, pork, chicken, turkey, and lamb and how they are processed or consumed as sandwiches, main course, or in mixed dishes. The fish section includes canned tuna, shellfish, dark meat fish, white fish, and other processed fish products. The bread, cereal, and starch section includes cold and hot cereals, bread, crackers, bagels, muffins, biscuits, pancakes, waffles, brown or white rice, pasta, tortillas, potatoes and how prepared, chips, and pizza. The beverage section includes low-calorie and sugared beverages, beer, wine, liquor, water, tea, and coffee; and whether or not they contain caffeine. The sweets, baked goods, and miscellaneous section includes candy, cookies, brownies, doughnuts, cakes, pie, breakfast and energy bars, popcorn, peanut butter, nuts, bran, soup, condiments, artificial sweeteners, olive oil, and mayonnaise and salad dressing including the level of fat. In addition, the FFQ specifically includes questions on added sugar to beverages, cold breakfast cereals, liver intake, and types of fats used at home for baking and frying, including the form of margarine. The final question is open-ended and asks whether any other important foods not included in the survey are consumed at least once per week. FFQ were analyzed at Harvard University (Cambridge, MA).

Screening blood and urine were collected. Blood tubes were placed in the dark for 30 min and centrifuged at 2800 x g for 15 min. Plasma was stored at 4°C before a comprehensive metabolic chemistry panel that included 20 measurements to evaluate overall health (Meriter Laboratories; Madison, WI). If subjects were enrolled and attended the baseline appointment, screening serum was analyzed for progesterone, estradiol, estrone, luteinizing hormone, and follicle-stimulating hormone by the UW Assay Services and Core Lab using radioimmunoassay (RIA). Serum was stored at –80°C. Screening urine 2:16 was run in duplicate by Estramet^{TM} EIA (Immuna Care, PA).

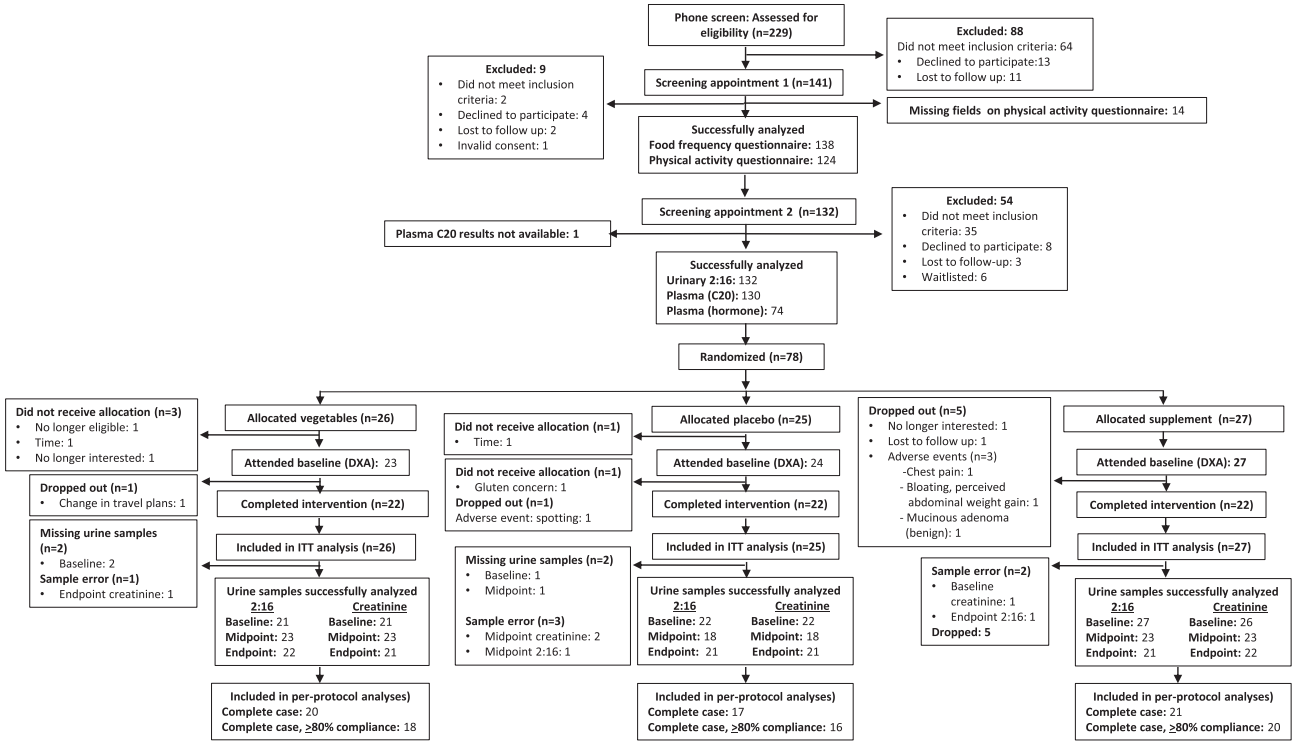

Figure 1. CONSORT diagram of recruitment, randomization, and reasons for discontinuation of 38–50-year-old premenopausal women (n=78) randomized to placebo, supplement or alternating 40 g frozen broccoli and Brussels sprouts for eight weeks.

Study design

This was a randomized, parallel-arm, placebo-controlled study in which subjects were randomized to: 1) 40 g/d broccoli or Brussels sprouts (previously frozen); 2) cruciferous supplements, or 3) matched opaque placebo capsules. Staff and subjects were blinded to capsule allocation, but not vegetable treatment. During washout (2 weeks) and the intervention (8 weeks), subjects avoided cruciferous vegetables, soy products, caffeine pills, and energy drinks, and limited caffeinated beverage (e.g. coffee, tea, soda) intake to <500 ml/d. Subjects used food diaries to assess compliance to prohibited foods or beverages. Subjects returned unused pills or vegetables.

Subjects with screening urinary 2:16 $\leq$3.0 were invited to a baseline appointment, which was scheduled to fall within three days of day 10 of the menstrual cycle (day 1 period commencement) in order to minimize differences in urinary 2:16 due to menstrual phase. A first-void, up-for-the-day urine sample was collected before the appointment. Subjects collected urine at baseline, four, and eight weeks. After urine was aliquoted for creatinine, ascorbic acid (100 mg/ml) was added to prevent metabolite degradation [36]. All samples were stored at –80°C. Creatinine was analyzed by EIA (Sigma-Aldrich; St. Louis, MO). Urinary 2:16 was analyzed by EIA (Immuna Care, PA) in triplicate.

Dual-energy x-ray absorptiometry

Regional total body and android-pattern and gynoid-pattern body fat percentage were determined by dual-energy x-ray absorptiometry (DXA) on 74 women eligible for the study using a GE Healthcare Lunar-iDXA with enCORE software v13.6. Spine, hip, and total body scans were acquired and analyzed per standard operating procedures.

Treatments

The supplement consisted of 550 mg dried Brussels sprouts and kale, calcium stearate, and water in a gelatin capsule (Standard Process, Inc., Palmyra, WI). Placebos appeared identical and placed in generic packaging. Subjects were asked to take two capsules, three times a day, six hours apart, for eight weeks. Frozen broccoli or Brussels sprouts were purchased from UW Meat Market (Middleton, WI) and stored at −30°C. Frozen vegetables (40 g) were portioned into zip-top bags. Subjects steamed the vegetables in stainless steel baskets each day for 3–4 min to minimize the loss of myrosinase activity and consumed them immediately. They were instructed not to allow water to touch the vegetables to avoid leaching of glucosinolates.

Glucosinolates, isoflavones, and tofu equivalents

Glucosinolates and isothiocyanates (ITC) were analyzed by HPLC [37, 38, 39]. Total glucosinolates were higher in the vegetables than the supplement and changes in content during the trial were published [40]. Total ITC content was higher in the supplements on a dry weight basis [40]. To estimate a theoretical I3C exposure per day for supplement and vegetable groups, conversion efficiencies were estimated and applied to the glucobrassicin content.

Dietary glucosinolates were estimated. Total isoflavones were calculated from the Harvard food composition database by multiplying total isoflavone content per g by portion estimate in g per day. "Tofu" included "soft tofu" and boiled, unsalted green soybeans, which were averaged for isoflavone content. "Soy foods" were summed from the FFQ variables "tofu" and soymilk and converted to g tofu equivalents [41, 42].

Portion estimates were compiled using frequency weights suggested by Harvard and converted to g per day using reference weights. Total, cruciferous, and Apiaceous vegetables were estimated. Ethanol (g/d) was converted to standard drinks per week by multiplying by 7 days/week and dividing by 14 g ethanol/standard drink [43].

Statistical analysis

A previous study investigating supplemental I3C to modulate urinary 2-OHE_1 to estriol (2-OHE_1:estriol) in 60 premenopausal women reported a standard deviation of ~0.5 [20]. A study feeding 500 g broccoli/day for 12 days to 16 adults saw an increase of 0.46 in urinary 2:16 [32]. A four-week educational intervention to increase Brassica vegetable consumption in 34 women found a dose-response of urinary 2:16 (0.08 increase per 10-g consumption) [29]. At α=0.05, 20/group gives 90% power to detect a 0.5 difference in 2:16 at eight weeks between the supplement or vegetable and placebo arm. To account for dropouts, the goal was 22/group.

Values are mean±SD unless otherwise stated. Normality was assessed by examination of quantile-quantile plots and the Shapiro Wilk test (univariate procedure). Variance constancy was assessed by examination of residual plots (RM-ANOVA) or Levene's test. If assumptions of normality were not met, data were transformed or a non-parametric or ranked test was used. Statistical analyses were carried out in SAS 9.4 (Cary, NC). Blinding of treatment groups was maintained until after statistical analyses for urinary 2:16 by treatment and time. Intent-to-treat (ITT) analysis was performed. Multiple imputations (MI) using the Markov chain Monte Carlo method for missing data were used to generate values using 100 imputations for baseline, midpoint, and endpoint 2:16. MIANALYZE generated regression parameters [44] separately for each time point and missing values were replaced with values estimated by the resulting regression parameters. Results were analyzed by repeated-measures (RM)-ANOVA with the mixed procedure. If the overall F-test was significant, groups were compared with Fisher's LSD. Sensitivity analyses included comparisons of the number of drops per arm (Fisher's exact test; freq procedure), comparisons of imputed and observed values (T-test), and variables associated with subjects with all and missing data were compared with a non-parametric test (Kruskal-Wallis).

Two RM-ANOVA were run as per-protocol analyses to determine the treatment effect under idealized conditions: complete cases and cases with 80% minimum compliance. RM-ANOVA, with MI and MIANALYE to estimate missing values, was used to determine if creatinine was different by treatment and time. An additional exploratory analysis compared the percentage change from baseline to endpoint in 2-OHE_1 and 16α-OHE_1 (both creatinine-corrected) in subjects whose baseline 2:16 was <2.0 using the MIXED procedure.

Dietary comparisons

Pearson's correlation coefficients to urinary 2:16 were determined with partial correlations for PAQ scores and relevant body size or fat-related variables. Energy-adjusted [45] dietary variables included known and hypothesized modulators of urinary 2:16 or CYP1A2 activity: fiber (g/d) [20], caffeine (mg/d) [33], ethanol (g/d) [33], total vegetables (g/d) [33], cruciferous vegetables (g/d) [20, 32], apiaceous vegetables [5], total glucosinolates (mg/d), soy foods (g tofu equivalents) [41, 42], and calculated total soy isoflavones (mg/d). Because the distribution of soy foods revealed clustering of residuals, urinary 2:16 was compared between soy food consumers and non-consumers (ttest procedure, Satterthwaite approximation for unequal variances). Regressions were carried out with the GLM procedure.

Results

Analysis of interventions

Total glucosinolate content of cooked Brussels sprouts (146±13.5 μmol/serving) was higher (P=0.0004) than cooked broccoli (65.9±9.0 μmol/serving) and the supplement (33.6±0.1 μmol glucosinolates/dose), which were not different from one another. During the intervention, the vegetables contained more glucosinolates than the

Table 1. Screening plasma chemistry panel (C20) for women (n=132). Serum estrone, estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone for women (n=73)[a] who were enrolled and attended the baseline appointment

Characteristic	Value	Reference range[b]
Fasting, %	85%	–
Glucose,[c] *mg/dL*	91 (87, 97)[d]	70–99
Blood urea nitrogen, *mg/dL*	13 (11, 14)	6–21
Creatinine, *mg/dL*	0.8 (0.7, 0.8)	0.5–1.1
Uric acid, *mg/dL*	4.2 (3.7, 4.9)	2.5–6.4
Cholesterol, *mg/dL*	185 (167, 207)	0–199
Triglycerides, *mg/dL*	72 (54, 111)	0–149
Total bilirubin, *mg/dL*	0.4 (0.3, 0.6)	0.3–1.1
Total protein, *g/dL*	7.1 (6.9, 7.4)	5.8–8.3
Albumin, *g/dL*	4.3 (4.2, 4.5)	3.6–5.4
Calcium, *mg/dL*	9.2 (9.1, 9.4)	8.5–10.4
Phosphorus, *mg/dL*	3.3 (3.1, 3.6)	2.1–5.1
Sodium, *mmol/L*	140 (138, 141)	135–145
Potassium, *mmol/L*	4.0 (3.8, 4.1)	3.4–4.8
Chloride, *mmol/L*	101 (100, 103)	97–108
Iron, *μg/L*	880 (630, 1120)	59–158
Estimated glomerular filtration rate, *mL/min × 1.73*	82 (75, 89)	>60
Aspartate aminotransferase, *units/L*	16 (14, 20)	0–32
Alanine aminotransferase, *units/L*	14 (11, 17)	0–33
Lactate dehydrogenase, *units/L*	140 (130, 154)	104–211
Alkaline phosphatase, *units/L*	53 (46, 63)	35–104
Gamma-glutamyl transferase, *units/L*	11.5 (9.0, 15.0)	5–36
Serum hormones	Mean±SD	
Estrone, *ng/mL*	0.09±0.03	
Estradiol, *pg/mL*	254±1039	
Progesterone, *ng/mL*	8.35±9.46	
Luteinizing hormone, *IU/L*	0.65±0.28	
Follicle-stimulating hormone, *mIU/L*	0.48±0.19	

[a]n = 73 instead of 74 because a tube broke during analysis. [b]Clinical reference range used by the contract laboratory. [c]n = 131. [d]Data are median (25th percentile, 75th percentile).

supplement. Glucobrassicin was higher in Brussels sprouts (33.8±9.9 μmol/dose) than broccoli (9.5±0.1 μmol/dose) and the supplement (3.6±0.2 μmol/dose; P=0.001) and the mean vegetable serving had more glucobrassicin than the supplement (P=0.003). The supplement contained more sulforaphane equivalents than the vegetables. The theoretical I3C dose of the supplement and a mid-point for the vegetables were 0.22 and 0.13 mg/day, respectively.

Screening, compliance, and adverse events

Two hundred twenty-nine women were phone-screened and 151 were excluded (Figure 1). Common reasons were not meeting inclusion criteria (n=101), choosing not to participate (n=26), or loss to follow-up (n=24). Plasma chemistry and screening dietary and PAQ characteristics appear in Tables 1 and 2, respectively. Screening 2:16 was 2.46±1.36. From the 78 women randomized after screening (Table 3), 71, 65, and 65 provided urine at baseline, mid-point, and endpoint, respectively. Although screening urine 2:16 was <3.0 indicating inclusion, five subjects had urinary 2:16 >3.0 at baseline measurements (placebo: 2, supplement: 1, vegetables: 2). Baseline 2:16 for enrolled subjects was 1.73±0.74. Screening serum estrone, progesterone, luteinizing hormone, and follicle-stimulating hormone were not related to screening urinary 2:16 in the women who were enrolled and attended baseline appointments (Table 1).

Median BMI for enrolled women was 24.4 kg/m^2 with 45% overweight or obese. Compliance with dietary restrictions was 95% with no difference among groups. Treatment compliance was 87%. Neither PAQ scores nor FFQ-estimated dietary variables were different among treatment groups (Table 4).

Table 2. Dietary intake of premenopausal women (*n*=141) estimated by Harvard Grid 2011 Food Frequency Questionnaire [34]. Physical activity scores were estimated by Baecke physical activity questionnaire [35]. Urinary 2:16 was assessed by EIA

Characteristic	Mean±SD [*n*]
Age at screening appointment,[a] *y*	45.3 (43.2, 47.6) [141]
Urinary 2:16	2.46±1.36 [132]
Questionnaires	
Physical activity questionnaire score	8.10±1.24 [124]
Dietary analysis (n = 138)	**Median (25th, 75th percentile)**
Energy[b], *kcal/day*	1960 (1540, 2430)
Fiber, *g/day*	26.6 (19.5, 31.1)
Vegetables, *g/day*	284 (205, 421)
Cruciferous vegetables, *g/day*	37.3 (17.3, 66.4)
Total glucosinolates, *mg/day*	13 (5, 24)
Glucobrassicin, *mg/day*	4 (2, 8)
Soy foods, *g tofu equivalents/day*	0.0 (0.0, 0.4)
Total soy isoflavones, *mg/day*	0 (0, 2)
Caffeine, *mg/day*	112 (24, 248)
Ethanol, *g/day*	4.1 (0.0, 9.9)
Ethanol, *standard drinks/week*	2.0 (0.0, 5.0)

[a]Results are reported as mean±SD [*n*]. [b]Data for dietary variables are reported as median (25th percentile, 75th percentile) [*n*].

Adverse events were reported by 22, 11, and 11 subjects in the supplement, vegetable, and placebo groups, respectively (Figure 1), but were judged to be unrelated to treatments.

Urinary 2:16 by treatment over time

ITT analysis of urinary 2:16 by treatment and time identified no treatment effect (P=0.9) or treatment-by-time interaction (P=0.6; Figure 2A), but a significant time effect existed (P=0.02; Figure 2B). Similar results were obtained when the analysis was restricted to complete cases where time was significant (P=0.03) or when the analysis was restricted to complete cases with minimum treatment compliance of 80% (time: P=0.02). Mean within-subject standard deviation for urinary 2:16 across time points was 0.55 (range: 0.05, 3.55). Urinary 2:16 was associated among time points (range: P=0.01 – <0.0001) and the R^2 ranged from 0.1 to 0.4. We hypothesized that the percentage change in creatinine-corrected 2-OHE_1 or 16α-OHE_1 may be greater in subjects whose baseline 2:16 was <2.0, as their 2:16 may be more easily affected by dietary or supplementary intervention. When the percentage change (end-line compared to baseline) of 2-OHE_1 and 16α-OHE_1 were compared among treatment groups in women whose baseline 2:16 was <2.0 (n=35), neither were significant. Potential confounding variables of interest, such as body composition, fat distribution, physical activity, and dietary intake, were not different among intervention groups at baseline (Tables 3 and 4) and therefore the main outcome analysis was not adjusted for these variables.

Several factors were evaluated as predictors of 2:16. Baseline 2:16, BMI, and total fat percentage were not

Table 3. Baseline comparison of healthy 38–50-year-old premenopausal women (*n*=78) randomized to placebo, supplement or alternating 40 g frozen broccoli and Brussels sprouts (vegetables)

	Placebo	Supplement	Vegetables	Total
Randomized,[a] *n*	25	27	26	78
Attended baseline,[b] *n*	24	27	23	74
Age,[b] *y*	46.3 (42.6, 47.8)	45.5 (43.7, 47.8)	44.4 (41.6, 47.3)	45.5 (42.8, 47.7)
BMI,[b] kg/m^2	24.0 (20.8, 29.3)	23.3 (20.9, 28.3)	24.7 (21.9, 29.2)	24.4 (21.8, 29.1)
BMI category,[c] *n*				
Underweight (<18.5)	1	1	1	3
Normal (18.5 ≤ BMI < 25.0)	12	15	11	38
Overweight (25.0 ≤ BMI < 30.0)	6	8	8	22
Obese (BMI ≥ 30.0)	5	3	3	11
Total body fat,[2] %	34.8 (29.3, 42.0)	35.7 (30.8, 40.6)	34.2 (31.6, 42.7)	35.4 (31.1, 40.7)
Android-pattern body fat,[b] %	35.1 (23.1, 47.4)	35.4 (25.8, 42.7)	33.0 (25.6, 46.2)	35.0 (25.6, 45.0)
Gynoid-pattern body fat,[b] %	39.1 (35.8, 44.7)	37.8 (33.8, 43.1)	39.9 (36.5, 44.4)	39.2 (36.0, 44.0)
Ratio of android-pattern to gynoid-pattern body fat percentage[b,d]	0.86 (0.66, 1.0)	0.96 (0.74, 1.1)	0.89 (0.62, 1.0)	0.89 (0.68, 1.02)
Urine				
Creatinine,[b,d] *mg/mL*	1.94 (1.41, 2.42) [23]	1.63 (1.26, 1.99) [26]	1.65 (1.26, 1.88) [21]	1.69 (1.28, 2.18) [70]
2-OHE_1/creatinine,[b,d] *μmol/mg mL*	3.17 (1.76, 5.60) [23]	3.19 (2.57, 4.27) [26]	3.90 (2.69, 6.37) [21]	3.32 (2.26, 5.55) [70]
16α-OHE_1/creatinine,[b,d] *μmol/mg mL*	1.72 (1.13, 3.27) [23]	1.92 (1.40, 3.34) [26]	2.35 (1.61, 4.33) [21]	2.11 (1.40, 3.34) [70]
2-OHE_1/16-α-OHE_1[b,d]	1.71 (1.20, 2.35) [23]	1.79 (1.26, 2.30) [27]	1.74 (1.21, 2.41) [21]	1.74 (1.21, 2.34) [71]

[a]Data are median (25th, 75th percentiles) [*n*] because residuals were not normally distributed. None of the parameters differed at baseline. [b]Seventy-four women attended baseline. [c]The proportion of normal- and overweight subjects among groups were compared with chi-square tests (treatment×BMI category) and subsequent Bonferroni correction, which did not differ. [d]Three subjects had missing baseline samples. One subject had no urinary creatinine.

Table 4. Physical activity questionnaire scores[a] and selected dietary variables[b] compared among healthy 38–50-year-old premenopausal women (*n*=78) randomized to placebo, supplement or alternating 40 g frozen broccoli and Brussels sprouts (vegetables)

	Placebo	Supplement	Vegetables	Total	*P*-value
Physical activity questionnaire score[c,d]	8.38 (7.25, 8.75) [23]	8.00 (7.50, 8.50) [25]	8.25 (7.63, 8.88) [25]	8.13 (7.50, 8.75) [73]	0.6
Energy, *kcal/day*	1744 (1515, 2309) [25]	1832 (1505, 2271) [27]	1805 (1516, 2460) [26]	1805 (1515, 2363) [78]	0.9
Fiber, *g/day*	22.5 (18.8, 31.8) [25]	22.1 (18.1, 28.6) [27]	26.4 (16.8, 29.9) [26]	23.4 (17.8, 29.9) [78]	0.9
Vegetables,[e] *g/d*	265 (209, 356) [25]	247 (150, 389) [27]	272 (205, 423) [26]	261 (197, 383) [78]	0.9
Cruciferous vegetables,[f] *g/day*	32.7 (20.8, 66.4) [25]	26.9 (16.8, 57.9) [27]	46.5 (19.6, 63.7) [26]	35.2 (16.8, 62.4) [78]	0.4
Apiaceous vegetables,[g] *g/d*	14.1 (6.6, 26.4) [25]	14.1 (6.6, 29.2) [27]	15.5 (7.8, 38.0) [26]	15.5 (6.6, 33.5) [78]	0.9
Total glucosinolates,[h] *mg/day*	11.5 (5.5, 23.6) [25]	8.6 (4.2, 17.6) [27]	15.7 (5.1, 23.6) [26]	12.2 (4.9, 22.3) [78]	0.2
Glucobrassicin, *mg/d*	4 (2, 9) [25]	3 (2, 6) [27]	5 (2, 9) [26]	4 (2, 7) [78]	0.2
Soy foods,[i] *g tofu equiv/d*	0.4 (0.0, 0.4) [25]	0.0 (0.0, 0.6) [27]	0.0 (0.0, 0.6) [26]	0.0 (0.0, 0.6) [78]	0.7
Total soy isoflavones,[j] *mg/d*	1.6 (0.0, 1.6) [25]	0.0 (0.0, 2.3) [27]	0.0 (0.0, 2.8) [26]	0.0 (0.0, 2.3) [78]	0.7
Caffeine, *mg/d*	140 (35.9, 166) [25]	104 (17.2, 262) [27]	92.7 (22.2, 162) [26]	105 (22.6, 237) [78]	0.7
Ethanol, *g/d*	4.1 (0.0, 8.3) [25]	6.1 (0.0, 10.5) [27]	2.7 (0.0, 11.1) [26]	4.3 (0.0, 10.5) [78]	0.8
Ethanol,[k] *standard drinks/week*	2.0 (0.0, 4.2) [25]	3.1 (0.0, 5.3) [27]	1.4 (0.0, 5.5) [26]	2.1 (0.0, 5.3) [78]	0.8

[a]Physical activity was assessed by Baecke Physical Activity Questionnaire, which gives principal components-identified indices of physical activity during work and leisure time [35]. [b]Habitual diet was assessed with 2011 Harvard Grid Food Frequency Questionnaire [34]. [c]Data appear as median (25^{th}, 75^{th} percentiles) [*n*] because residuals were not normally distributed. *P*-values reflect ranked analyses unless otherwise noted. [d]Because of missing data fields, physical activity questionnaire scores were available for 73 enrolled women. [e]"Vegetables" were summed from portion estimates (converted to g/d): tomato products, green beans, beans, peas, broccoli, cabbage, cauliflower, cabbage, Brussels sprouts, carrots, mixed vegetables, sweet potatoes, yellow squash, zucchini, kale, spinach, lettuces, celery, peppers, and onions. [f]"Cruciferous vegetables" were summed from portion estimates (converted to g/d) of kale, broccoli, cauliflower, cabbage, and Brussels sprouts. [g]"Apiaceous vegetables" include carrots (raw, cooked) and celery. [h]"Total glucosinolates" include 26 glucosinolates (see "Methods" for list). [i]"Tofu equivalents" were calculated by converting the portion estimates to dry weights and dividing those by the dry weight of tofu [41, 42]. [j]"Total isoflavones" include daidzein, genistein, glycitein, formononetin, and biochanin. [k]Ethanol in "standard drinks per day" was calculated from g ethanol/day, multiplied by 7, and divided by 14 g ethanol per standard drink [43].

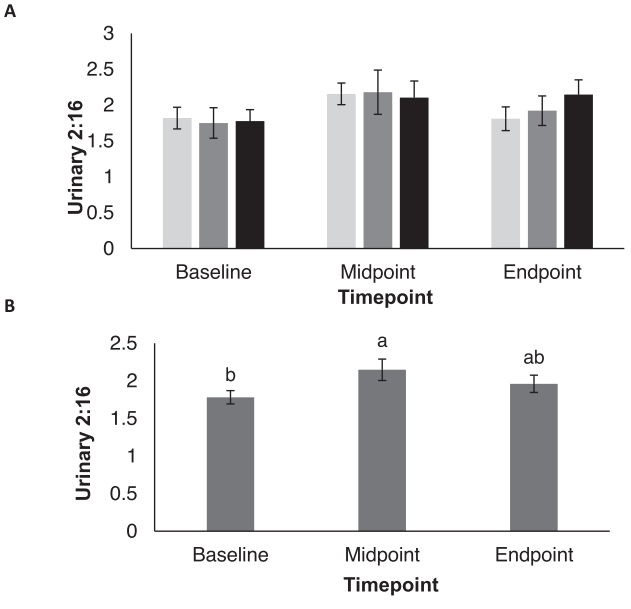

Figure 2. (A) Urinary ratio of 2-OHE_1:16-α-OHE_1 in (*n*=78) 38–50-year-old premenopausal women randomized to placebo (light grey bars), supplement (darker grey bars), or vegetables (black bars) for eight weeks. Values are means±SEM. There was no treatment effect (*P*=0.9) or treatment-by-time interaction (*P*=0.7). (B) There was a significant time effect (*P*=0.02).

significantly related. However, using Pearson correlations, the android-pattern body fat percentage (*P*=0.05) and android:gynoid fat (*P*=0.02) were predictors of change.

Discussion

Urinary 2:16 did not change with an 8-week cruciferous vegetable intervention. Subjects reported habitual cruciferous vegetable consumption similar to the treatment amount of vegetables at screening. Subjects' median baseline 2:16 was similar to that observed in other studies in premenopausal women [46], which found that within-person 2:16 variability is moderate and consistent within individuals [47]. Observed within-subject SD for the urinary 2:16 across time points (0.55) was similar to that (0.5) [20] reported in the study used in the power calculation. The baseline appointment was timed for day 10 (follicular phase) of the menstrual cycle by subject but the midpoint and endpoint samples were by month. While this would give samples in the same phase for a woman with a 28-day cycle, women with shorter or longer cycles (inclusion criterion was 23–35 days) may have ended up in a different phase, which would add to variation. Nonetheless, the results of the ITT and per-protocol analyses were consistent.

While the vegetables had higher total glucosinolate and glucobrassicin content, they had lower total ITC content than the supplement. The estimated conversion efficiency of glucosinolates or glucobassicin alone to ITCs was higher for the supplement than the vegetables, resulting in a higher theoretical I3C exposure. However, the theoretical dose of I3C was lower than the 400–800 mg [7, 20] or 6–7 mg/kg body weight [30] given in studies that observed a change

in 2:16 or 2:estriol. Despite a lower theoretical I3C content per vegetable treatment, the change in urinary 2:16 in the vegetable group was three times higher than that of the supplement group. The vegetable group's change in 2:16 was 0.4, which is higher than predicted for a 4×10 g incremental change (4×0.08=0.32) [29]. Although not significant, the supplement group's change was similar to the 0.08 change per 10 g increment of cruciferous vegetable consumption observed [29]. Smaller I3C doses may have greater potential to shift the urinary 2:16 in subjects with lower 2:16, which may occur because of less healthy habits.

Baseline 2:16 was inversely associated with BMI and total and regional body fat percentage. Previous studies have observed lower urinary 2:16 with higher BMI at low physical activity levels [48] and an inverse association of serum ratios of 2- to 16-hydroxylation pathway metabolites with BMI [49]. Another study observed an inverse association of urinary 2:16 with total and trunk body fat percentage, which appeared to be driven by an increase in $2OHE_1$ with increasing lean body mass [50]. The ratio of android- to gynoid-pattern body fat percentage was negatively associated with the change in 2:16, suggesting that subjects with more gynoid-pattern (hips, thighs) than visceral body fat deposition may have seen greater urine 2:16 increases from baseline to endpoint. Future studies examining interventions to affect low 2:16 and estrogen metabolism may consider regional body fat distribution.

Screened subjects habitually consumed cruciferous vegetables (~37 g/d), indicating that the vegetable serving of 40 g was similar to reported intake. This suggests that the dose may not have been enough to induce CYP1A2 activity, although subjects did avoid cruciferous vegetables and other CYP1A2 inducers for two weeks before baseline. Screened subjects were consuming several servings of vegetables per day, suggesting that they may have been health-conscious. The study was advertised as the "Green Vegetable Study", and a commonly stated reason for declining participation was unwillingness to follow restrictions, particularly the restriction on cruciferous vegetables. It is possible that the study dose was too close to habitual intake.

The strengths of this study include examination of a moderate dietary or supplemental intervention using common vegetables versus a whole-food supplement. While we did not directly determine myrosinase activity, we determined vegetable glucosinolate and total ITC content using a proxy measure to estimate endogenous myrosinase. Using an estimate of activity may give a more realistic measure of how much ITC subjects would be exposed to on an as-consumed basis compared with methods that add exogenous myrosinase, which measure what is present in the vegetable but may not reflect bioaccessibility. We did not directly measure I3C content, but higher amounts of total ITCs in the supplement compared with the vegetable serving hint at preservation of myrosinase, which may indicate higher I3C exposure in the supplement.

Conclusion

Neither the supplement nor a small serving of vegetables substantially altered urinary 2:16 after two months. Variation may have obscured a treatment effect. The unanticipated change in 2:16 over time within subjects is an important finding that needs to be considered when designing future studies because it could affect statistical power. The fact that 5 subjects' 2:16 values changed between screening and baseline measurements was noteworthy. Android:gynoid pattern body fat percentage was inversely associated with the change in urinary 2:16. Future studies evaluating estrogen metabolism should account for regional body fat deposition. More research is needed to determine if moderate dietary interventions shift estrogen metabolism or other hypothesized biomarkers of breast cancer risk.

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History
Received October 27, 2022
Accepted May 1, 2023
Published online June 19, 2023

Acknowledgements
We thank Diane Krueger, Ellen Siglinsky, and Jessie Libber at the OCRP for blood draws and DXA scans. We thank Christopher Scholl and Joseph Dever for vegetable and supplement analysis, Peter Crump and Devika Suri for statistical consultation, and Kiersten Olsen for serving as backup coordinator.

Conflict of interest
SJD, SG, CM, NB, and SAT declare no conflict of interest. SAA worked at Standard Process, Inc., when the study was carried out but was not involved in data collection or analysis. She did have input into the study hypothesis based on current literature at the time the proposal was written.

Authors contributions
SM conducted the study and wrote the first draft; SG assisted in human subjects' procedures and supplies; CM assisted in screening potential subjects and allocating treatments; NB was the physician on record and oversaw sample collection. SAA and SAT designed study and edited the manuscript Dr. Davis was Ms. Mondloch during the orchestration of the study and manuscript preparation.

Funding
This work was supported by Standard Process Inc., and Global Health funds at UW-Madison during sample analysis and manuscript preparation. This manuscript is dedicated to the late David Barnes, former director of research at Standard Process, Inc., for his patience and kind support of this study.

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