Brassica vegetable consumption reduces urinary F2-isoprostane levels independent of micronutrient intake

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Isothiocyanates and indoles (e.g. indole-3-carbinol) from Brassica vegetables (e.g. broccoli) induce Phase I and Phase II enzymes responsible for the oxidation, reduction and metabolism of endogenous and exogenous carcinogens. Brassica vegetables also contain micronutrients that may provide additional DNA protection from reactive oxygen species. This randomized crossover trial (n = 20) compares the effects of a Brassica Vegetable (BV) intervention against a Micronutrient and Fiber Supplementation (M+F) intervention on urinary F2-isoprostane levels (F2-iP), a stable biomarker of systemic oxidative stress. Brassica intake was monitored by repeated 24 h recalls, urinary ITC levels and questionnaire. Urinary F2-iP levels were measured by mass spectrometry from first-morning urine samples collected at Baseline and after each intervention, and change in natural log transformed urinary F2-iP levels were analyzed using repeated measures regression. Brassica consumption increased from 2 grams/day (g/d) during the Baseline or M+F intervention periods to 218 g/d during the BV intervention, whereas exposure to most antioxidant vitamins and minerals was greatest during the M+F intervention. F2-iP levels significantly decreased by 22.0 or 21.8% during the BV intervention compared with Baseline or the M+F intervention (P = 0.05, P = 0.05, respectively). Urinary F2-iP levels did not significantly differ between Baseline and the M+F intervention (difference = 0.2%; P = 0.98). Brassica intake has been associated with reduced risk of colon, lung, bladder, breast, prostate and other cancers. Our results suggest that Brassica consumption reduces systemic oxidative stress independent of the vitamin and mineral content of these vegetables.

Introduction

Oxidative stress is believed to have a complex and multifunctional role in the development of most age-related chronic diseases (1,2). In the context of carcinogenesis, increased oxidative stress may damage DNA beyond DNA repair capacity, leading to the clonal expansion of initiated cells (3,4). Indeed, environmental factors that enhance oxidative stress, such as tobacco or a low fruit and vegetable (FV) intake, are associated with the risk of colon, lung, bladder and other cancers (5,6). These observations provide the foundation for public health campaigns promoting FV intake (7), as increased antioxidant potential through FV intake may support a response to challenge by reactive oxygen species (8–13).

Furthermore advancement of the diet-oxidative stress hypothesis has been possible through development of reliable biomarkers of systemic oxidative stress. These biomarkers include the F2-isopostanes (F2-iP), compounds formed from the non-enzymatic peroxidation of arachidonic acid (14,15). However, interventions promoting a healthy diet rich in FV intake have reported inconsistent results. For example, lower lipid peroxidation biomarker levels have been reported following interventions administering a FV-rich diet (16,17), lycopene-rich foods (9), fruit juice (18), or antioxidant supplements following strenuous exercise (19). However, other interventions administering an FV-rich diet (12,20,21), carrot-juice (9), spinach (9), processed vegetable burgers (10), or antioxidant supplements (22,23) reported no effect on biomarkers of lipid or DNA oxidation [i.e. 8-oxodG (24)].

Beyond FV intake, reduced cancer risk is fairly consistently associated with increased consumption of broccoli and other Brassica vegetables (25–30). Interestingly, a recent intervention administering 600 grams/day (g/d) FV as 100 g/d broccoli, 100 g/d other vegetables and 400 g/d fruit increased blood glutathione GSH levels, whereas Verhagen et al. reported 300 g/d Brussels sprouts added to the usual diet for 7 days significantly reduced urinary 8-oxodG levels among non-smoking males (31,32), suggesting a Brassica-rich diet induces a response to oxidative challenge (12,20). Aside from providing antioxidants such as vitamin C, Brassica is also the primary source of a class of compounds called the glucosinolates. Following consumption, glucosinolates may be enzymatically cleaved to produce a wide range of biologically active non-nutrient compounds. These compounds include the isothiocyanates (ITC), well-known inducers of glutathione-S transferases (GSTs) and other Phase II enzymes (33,34), and various indoles (e.g. diindolyl-methane) with potential to induce Phase I [i.e. cytochrome P-450s (CYPs)] and Phase II enzymes (35–37). However, whether Brassica intake and glucosinolate exposure affects oxidative stress beyond any effect of the antioxidant capacity derived from increased vitamin and mineral exposure remains unclear.

We conducted a randomized crossover intervention to investigate the effects of Brassica consumption on biomarkers of colorectal cancer risk. Here, we compared the effects of a Brassica-rich diet with the effects of administering vitamin, mineral and fiber supplements on urinary F2-iP levels measured by mass spectrometry.

Abbreviations: F2-iP, F2-isoprostane levels; FV, fruit and vegetable; ITC isothiocyanates; GSTs, glutathione-S transferases; CYPs, cytochrome P-450s; VFQ, Vegetable and Fruit Questionnaire.

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Materials and methods

Overview and study design
This intervention investigated the effects of Brassica vegetable consumption on biomarkers of colorectal cancer risk and progression. The protective association between Brassica and colorectal cancer is one of the most consistent (6). A priori, colon adenoma patients were targeted because this clinical population is a probable target population for future colorectal cancer prevention trials and has shown to be motivated to participate and adhere to a colorectal cancer prevention protocols (38). Indeed, the crossover design employed for this intervention requires subjects to maintain a higher than usual level of attention and involvement than perhaps other intervention study designs. The Vanderbilt IRB approved all research protocols, and subjects provided written informed consent prior to participation.

Prior to data collection, we assessed subject motivation and ability to adhere to protocol with a 7 day Run-In trial. During Run-In, subjects were instructed to take one study micronutrient supplement per day, and adherence was measured by pill-counting. Baseline data were collected from subjects who successfully completed the Run-In trial. After Baseline, subjects participated in a 4 week Brassica vegetable (BV) intervention and a 4 week micronutrient and fiber (M+F) intervention. Randomization was used to determine the order in which each intervention was administered to each participant. A 2 week ‘wash-out’ period preceded Baseline data collection and separated each intervention. During wash-out, subjects were instructed not to consume Brassica, mustard or horseradish, or use any vitamin or fiber supplements, aspirin or other non-steroidal anti-inflammatory drugs, or corticosteroids. A 2-week wash-out period was determined to be adequate for this study, as ITC persists in the body for less than 72 h (39).

Subjects
Subjects included patients undergoing colonoscopy at the Vanderbilt University Medical Center (Nashville, TN) within the past year and diagnosed with a benign adenomatous polyp (≥0.5 cm). Exclusion criteria included current use of tobacco, antibiotics, hormone replacement therapy, non-prescription hormones, non-steroidal anti-inflammatory drugs, diabetes medication, tamoxifen, or Synthroid or other thyroid hormone-based medications. Women reporting a menstrual cycle within the past 12 months were excluded to prevent cyclical biomarker variations. Twenty-two subjects (15 male, 7 female) successfully completed the Run-In trial and were eligible for the intervention; however, two subjects were removed prior to Baseline data collection owing to an onset of cardiovascular or infectious disease symptoms. Thus 20 subjects from Nashville, TN or the surrounding communities completed the intervention (14 male, 6 female). These participants were generally healthy and were also motivated to participate in a diet and colorectal cancer prevention intervention.

BV intervention
The goal of the BV intervention was to increase and maintain Brassica consumption at two cups or more per day (>160 g/d). Subjects were provided diet counseling and weekly counseling telephone calls to incorporate these vegetables into their diet. Counseling emphasized raw vegetable consumption to retain phytochemical content (40,41). Subjects also received a weekly supply of raw Brassica vegetables, written goals and instructions, portion size guides, and a study cookbook containing recipes that included raw or lightly cooked Brassica vegetables. Subjects self-monitored their Brassica intake with 3 day diet diaries completed twice during the first 2 weeks of the intervention. Subjects reviewed each diary with a staff member at the end of each week to monitor adherence. Subjects also participated in a weekly group session led by the study dietitian. This group session provided the opportunity to discuss barriers to diet change with other study subjects and to develop food preparation skills in a teaching kitchen.

M+F intervention
The M+F intervention served as a control intervention to compensate for increased micronutrient and fiber intake during the BV intervention. The daily vitamin and mineral supplement (Centrum®) contained vitamins C (60 mg), E (30 mg), K (25 µg), A (5000 IU), D (10 µg), B6 (1.5 mg), B2 (1.7 mg), B3 (20 mg), B6 (2 mg), panthothenic acid (10 mg), B12 (6 mg), iron (0.5 mg), calcium (162 mg), phosphorus (109 mg), magnesium (100 mg), iron (18 mg), zinc (15 mg), copper (2 mg), selenium (20 µg), potassium (80 mg) and folic acid (400 µg). The amount of fiber supplement (Metamucil® in water or juice) was 1.5 g/d, estimated to be equal to that from increased Brassica consumption. Adherence to supplementation protocols during the M+F intervention was monitored during weekly telephone calls.

Data collection
Demographics, health history, and alcohol and medication use were measured by structured questionnaire at study entry. Follow-up questionnaires were administered after each intervention to identify changes in medication use. Height was measured at Baseline, and weight was measured at Baseline and after each intervention using a calibrated scale without shoes and only light clothing. Adverse events and symptoms during each intervention were assessed by questionnaire and actively assessed by telephone calls. Also, subjects were instructed to call study staff if experiencing any adverse events potentially associated with the intervention.

Urine collection
Subjects were provided written and oral instructions for collection of a first morning urine sample. Urine was collected during the last week of each intervention in a standard sterile collection cup containing 100 mg ascorbic acid. The sealed collection cup was placed in an insulated bag with an ice-pack for transport, and the subject delivered the specimen to our research center that day. Urine specimens were aliquoted and frozen at −80°C upon delivery.

Diet assessment
Three highly complementary diet assessment methods were utilized. The primary diet assessment protocol used repeated 24 h diet recall interviews (24HR), a criterion assessment method best suited for interventions (42). A trained dietician called subjects on three randomly selected days (2 weekday, 1 weekend day) during the 7 day period prior to collection of the urine sample. Nutrient scores were calculated using the Nutritional Data System, and the scores from the three calls within each week were averaged to provide a stable estimate of diet during that study phase. In addition, we created a SAS program to read NDS files and to calculate the amount (g/d) and preparation (cooked, raw) for each Brassica vegetable reported.

The Vegetable and Fruit Questionnaire (VFQ) is a 57 item instrument developed to measure vegetable and fruit intakes during an intervention. The VFQ lists common Brassica and other vegetables, and subjects indicate the number of servings and the usual portion size during the prior 7 days. The VFQ was administered on the day of urine collection, thereby, providing a summary diet measure that includes the 4 days not included in the 24HR protocol. Our prior analysis found the 24HR and VFQ provide consistent, but not identical, Brassica intake scores (r = 0.67) (43). Urinary ITC levels were measured as an estimate of Brassica consumption independent of potential reporting errors. Details of the conjugation assay and spectral detection have been reported previously (25,44). Assays were performed in a single batch and blind to intervention status. Individual urinary ITC concentrations were calculated as the average of triplicate runs on each urine specimen. The average %CV was 0.23%, and the ITC assay was repeated for any sample with a %CV above 0.3, 0.2 or 0.1% for ITC concentrations of <1.0, 1.0–2.0 or >2.0 µM, respectively. Final ITC concentrations were standardized to urinary creatinine concentration for each subject.

F2-isoprostanes (F2-ip)
F2-isoprostanes are becoming widely recognized as a highly reliable index of systemic oxidative stress, and indeed these compounds have been described as a ‘gold standard’ measure of lipid peroxidation in vivo (15,45,46). These F2-ip s are eicosanoids formed in vivo from the free radical-catalyzed peroxidation of arachidonic acid (14,47). Urinary F2-ip levels are not altered by cyclooxygenase inhibitors and have provided a biomarker of oxidative stress in research involving cancer, heart disease and diabetes (18). Urinary F2-ip levels were assessed using our previously published methods (14). Briefly, F2-ip in urine is quantified by selected ion monitoring GC/negative ion chemical ionization mass spectrometry using [1H8]8-iso-prostaglandin F2α as an internal standard. Compounds are analyzed as pentafluorobenzyl ester and trimethylsilyl ether derivatives by monitoring the M-181 ions (m/z 2097). Assays were performed in a single batch and blind to intervention status. Assay precision is ±5% and accuracy is 96%. Urine F2-ip levels were expressed as µg/ml/mg creatinine.
evidence that the decrease in F2-iP was associated with group randomization status \((P = 0.68)\). The key contrasts compare F2-iP levels between the BV intervention and the M+F intervention or Baseline, whereas controlling for randomization group and the treatment \times randomization interaction. Also, we compared Baseline and M+F intervention F2-iP levels to characterize the stability of F2-iP levels over time. F2-iP levels were reverse transformed, and the geometric mean values are reported. Vitamin C was added to the model to control for an observed increase in vitamin C intake during the BV intervention beyond the M+F intervention. A cross-sectional analysis during the BV intervention used Student’s \(t\)-tests and Spearman correlation coefficients to explore the associations between raw or cooked \textit{Brassica} vegetable intake and urinary F2-iP levels.

## Results

Subjects ranged in age from 36 to 80 years, averaging 57 years, and included 14 males (70%) and one female African American (Table I). Most subjects were married and had attended college. Randomization groups were equally balanced across demographic characteristics. No subjects dropped out or experienced a serious adverse event, although one male subject reported a short period of constipation during the M+F intervention.

Consistent with wash-out instructions, \textit{Brassica} intake during the Baseline and M+F intervention phases averaged 2.5 or 1.5 g/d, respectively (Table II). \textit{Brassica} consumption significantly increased during the BV intervention, as measured by 24HR, VFQ and urinary ITC levels. Broccoli (77g/d), cauliflower (69 g/d), Brussels sprouts (26 g/d) and cabbage (38 g/d) were the most commonly consumed \textit{Brassica}, and 69% of \textit{Brassica} was reportedly consumed raw.

Urinary F2-iP levels did not significantly differ between Baseline and the M+F intervention (%Change = 0.2%; \(P = 0.98\)) (Figures 1 and 2). However, urinary F2-iP levels significantly decreased by 21.8% \((P = 0.05\) and 22.0% \((P = 0.05\) during the BV intervention compared with the M+F intervention or Baseline, respectively. The decrease in urinary F2-iP levels with the BV intervention from Baseline was statistically significant in an analysis restricted to men (%Change = −25.1%, \(P = 0.05\)). F2-iP levels also decreased among women, although this effect was not significant (%Change = −15.9%, \(P = 0.69\)). No outlier or highly leveraging value was identified.

We conducted a detailed evaluation of diet intake and weight to explore alternative explanations for the decrease in F2-iP levels associated with the BV intervention (Table III). Only vitamin C intake was higher during the BV intervention; however, controlling for vitamin C in our repeated measures regression analysis had only a small impact on the precision of the analysis (Figure 1). Measured weight and intakes of energy, fat or protein did not change. As designed, exposure to most vitamins and minerals was highest during the M+F intervention. Fiber intake during the BV intervention was higher than that at Baseline; however, fiber supplementation was adequate to stabilize fiber intake during the M+F intervention.

In a cross-sectional analysis during the BV intervention, F2-iP levels were not significantly correlated with total \textit{Brassica} consumption, raw \textit{Brassica} consumption, cooked \textit{Brassica} consumption or urinary ITC levels. However, raw \textit{Brassica} consumption was marginally and inversely correlated with F2-iP levels after removing one subject with a highly influential F2-iP level (raw \textit{Brassica}: \(r = −0.41, P = 0.08\); cooked \textit{Brassica}: \(r = −0.09, P = 0.72\)). Subjects consuming >129 g/d raw \textit{Brassica} (median raw \textit{Brassica} intake) had significantly lower mean F2-iP levels than those consuming <129 g/d (1.37 versus 0.82, \(P = 0.02\), respectively). Urinary F2-iP levels did not significantly differ across median categories of cooked \textit{Brassica} intake.

## Discussion

In this randomized crossover intervention, urinary F2-iP levels provided a measure of \textit{in vivo} systemic oxidative stress relevant to most chronic diseases, including cancer (49). Other than a prior colon adenoma diagnosis, study participants were generally healthy. Current smokers or persons with diet restrictions were excluded, and few participants had a family history of cancer, diabetes or heart disease. Our primary observation that \textit{Brassica} intake, but not micronutrient supplementation, decreased F2-iP levels suggests that \textit{Brassica} intake reduces estimated oxidative stress independent of the antioxidant potential of the vitamins and minerals within \textit{Brassica}. Indeed, prior studies found vitamin or antioxidant supplementation had little impact on oxidative stress markers in healthy subjects (8,16,19,20,22,23,50), and there is limited evidence that micronutrient supplements prevent cancer (51–55).

### Table I. Study population characteristics (\(n = 20\))

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>19</td>
</tr>
<tr>
<td>Black</td>
<td>1</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
</tr>
<tr>
<td>Prior smoker</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>11</td>
</tr>
<tr>
<td>Single</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>High school or less</td>
<td>3</td>
</tr>
<tr>
<td>Any college</td>
<td>10</td>
</tr>
<tr>
<td>Any graduate school</td>
<td>7</td>
</tr>
</tbody>
</table>

### Table II. \textit{Brassica} vegetable consumption

<table>
<thead>
<tr>
<th></th>
<th>Baseline BV</th>
<th>BV Versus M+F</th>
<th>M+F</th>
<th>Base Versus M+F</th>
<th>BV Versus Base</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>(P)</td>
<td>Mean</td>
</tr>
<tr>
<td>\textit{Brassica}—24HR (g/d)</td>
<td>2.5</td>
<td>1.7</td>
<td>21.8</td>
<td>&lt;0.01</td>
<td>1.5</td>
</tr>
<tr>
<td>\textit{Brassica}—VFQ (g/d)</td>
<td>2.3</td>
<td>1.2</td>
<td>250.4</td>
<td>&lt;0.01</td>
<td>1.2</td>
</tr>
<tr>
<td>Urinary ITC</td>
<td>0.8</td>
<td>1.4</td>
<td>14.4</td>
<td>&lt;0.01</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\textit{Brassica} intake was estimated using repeated 24 h diet recall interviews (24HR), a Vegetable and Fruit questionnaire (VFQ) and urinary isothiocyanate excretion (ITC). Urinary ITC levels were log transformed prior to analysis, and geometric mean ITC levels are reported.
Alternatively, several recent interventions report that FV intake decreases biomarker levels used to estimate oxidative stress (12,17,18). The protective associations between *Brassica* consumption across cancers range across colon (56), lung (28), bladder (57), prostate (26), breast (29,58), and the head and neck (29,59), suggesting *Brassica* affects pathways common to many cancers and beyond what might be provided by the limited antioxidant potential of these vegetables (60). Verhagen et al. reported that 300 g/d cooked Brussels sprouts for 3 weeks significantly decreased urinary 8-oxodG levels by 28% (n = 10 males) (31). This result was reproduced in a later study among men but not among women (32). Our results that F2-ip levels are lower with greater *Brassica* intake are consistent with these prior studies. Although *Brassica* consumption levels were lower in our study than in these prior interventions, glucosinolate cleavage and enhanced ITC and indole exposure might have been facilitated through greater raw vegetable intake in our study. Although statistical power within women was limited, F2-ip levels decreased by only 15% among women during the BV intervention, and, thus, it remains possible that the *Brassica* and F2-ip association varies by sex as observed previously by Verhagen et al.

Studies of cultured cancer cells and animal models of cancer provide the most compelling evidence that the non-nutrient phytochemical composition of *Brassica* reduces tumor incidence (61–64). *Brassica* glucosinolates are a diverse set of phytochemicals with an S–C = N backbone. The human gut microflora also has a level of myrosinase-like activity (39). Glucosinolate degradation products include the ITCs (e.g. sulforaphane) and the indoles (e.g. indole-3-carbinol). *Brassica* glucosinolates were also found to prevent DNA oxidative damage and lipid peroxidation in vitro (65,66), and indeed both the ITCs and indole analogs have well-known effects on oxidative metabolism.

The ITCs induce several Phase II enzymes, including GSTs M1, T1 and P1 (GSTM1, GSTT1, GSTP1) (34,67). These enzymes conjugate ROS with glutathione (GSH), stabilizing the compound and improving water-solubility prior to excretion (67,68). For example, Hecht et al. reported that

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**Table III.** Comparison of diet intake from repeated 24-h diet recall interviews and weight across intervention phases

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>BV intervention</th>
<th>M+F intervention</th>
<th>Baseline versus M+F</th>
<th>BV versus M+F</th>
<th>BV versus Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>86.4</td>
<td>86.2</td>
<td>86.2</td>
<td>0.70</td>
<td>0.75</td>
<td>0.05</td>
</tr>
<tr>
<td>Energy (kcal/d)</td>
<td>2031.5</td>
<td>1977.1</td>
<td>1977.1</td>
<td>0.09</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>77.7</td>
<td>6.2</td>
<td>86.3</td>
<td>0.64</td>
<td>0.50</td>
<td>0.06</td>
</tr>
<tr>
<td>Carbohydrate (g/d)</td>
<td>243.9</td>
<td>214.2</td>
<td>214.2</td>
<td>&lt;0.01</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>77.6</td>
<td>214.2</td>
<td>214.2</td>
<td>&lt;0.01</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>Fiber (g/d)</td>
<td>18.9</td>
<td>22.3</td>
<td>22.3</td>
<td>0.22</td>
<td>0.28</td>
<td>0.07</td>
</tr>
<tr>
<td>Soluble Fiber (g/d)</td>
<td>6.6</td>
<td>8.3</td>
<td>8.3</td>
<td>0.51</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Vit A (IU/d)</td>
<td>7561.3</td>
<td>10813.0</td>
<td>10813.0</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Vit D (µg/d)</td>
<td>4.3</td>
<td>4.2</td>
<td>4.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vit E (mg/d)</td>
<td>10.1</td>
<td>12.2</td>
<td>12.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vit C (mg/d)</td>
<td>100.1</td>
<td>227.5</td>
<td>227.5</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vit B1 (mg/d)</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.87</td>
</tr>
<tr>
<td>Vit B2 (mg/d)</td>
<td>1.8</td>
<td>1.9</td>
<td>1.9</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Vit B3 (mg/d)</td>
<td>23.4</td>
<td>23.6</td>
<td>23.6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vit B6 (mg/d)</td>
<td>1.9</td>
<td>2.4</td>
<td>2.4</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vit B12 (µg/d)</td>
<td>5.1</td>
<td>4.7</td>
<td>4.7</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Folate (µg/d)</td>
<td>364.8</td>
<td>481.0</td>
<td>481.0</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Selenium (µg/d)</td>
<td>109.8</td>
<td>98.9</td>
<td>98.9</td>
<td>0.05</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Calcium (mg/d)</td>
<td>715.7</td>
<td>841.5</td>
<td>841.5</td>
<td>0.10</td>
<td>&lt;0.01</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Includes folate and folic acid from supplements.*
ITC-rich watercress consumption increased urinary excretion of nitrosamine 4-(methylisoguanosino)-1-3-pyridyl-1-butane (NNK) (69,70). Also, the indoles from *Brassica* undergo self-condensation to form aryl hydrocarbon receptor agonists (71,72), leading to Phase I (CYP) enzyme induction (73,74). Although *Brassica* consumption may induce Phase I enzymes (75) to activate pro-carcinogens (76,77), Phase I oxidation also precedes Phase II conjugation. For example, I3C increased hepatic NNK metabolism, inhibited NNK-induced DNA methylation (78–80) and inhibited heterocyclic amine induced DNA adduct formation (81). Furthermore, the effects of ITCs and indoles on metabolism are not clearly singular, as ITCs have been reported to induce and inhibit Phase I enzymes and indoles may induce Phase II enzymes (66,73,82,83). Thus, the reduction in oxidative stress markers (both 8-oxodG and F2-iP) following *Brassica* consumption may result from the ITCs, the indoles or from the combined exposures following *Brassica* intake on Phase I and Phase II enzyme induction.

In addition, it is possible that *Brassica* consumption reduces oxidative stress markers through a reduction in GSH reserves. GSH is an endogenous antioxidant and an important cofactor for detoxification of reactive oxygen species. Interestingly, F2-iP synthesis is dependent on GSH reduction of prostaglandin H2 (PGH2) (47). Perhaps Phase I/II induction following *Brassica* consumption, or the sequestration of GSH through conjugation with ITC, depletes GSH reserves sufficiently to inhibit F2-iP synthesis (84). In rat liver microsomes, oral ITC significantly decreased GSH levels (85), and GSH depletion reduced F2-iP levels relative to D2-iP or E2-iP levels (47). However, ITC exposure also may increase GSH synthesis in cultured liver cells (86) or the rat liver and pancreas (87,88), and GSH levels in human lymphocytes, duodenum or rectum were not affected by Brussels sprouts’ consumption (89). Further work is needed to understand the role of GSH depletion and synthesis on human oxidative stress markers in response to *Brassica* consumption.

Strengths of this study include the randomized crossover design, a micronutrient and fiber control, repeated diet assessments including a urinary biomarker of *Brassica* intake, a high adherence level, F2-iP measurement by mass spectroscopy and exclusion of subjects using tobacco or with chronic or acute disease. Any effect of genetic variability between participants on our repeated-measures analytic approach would probably reduce the observed association between *Brassica* and F2-iP. We cannot exclude the possible effects of quercetin or other non-nutrient antioxidants on F2-iP, although flavonol exposure is not consistently associated with markers of oxidative stress (90). Furthermore, we could not control for potential differences in the absorption of antioxidants from *Brassica* and micronutrient supplements.

Urinary F2-iP levels provide a biomarker of systemic oxidative stress that is responsive to diet and other environmental factors believed to be highly relevant to cancer risk (2). We found *Brassica* vegetable consumption significantly reduced urinary F2-iP levels independent from many antioxidant micronutrients found in these vegetables. This estimated reduction in systemic oxidative stress may be attributable to the non-nutrient phytochemicals derived from *Brassica* intake.

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