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Consumption of baby kale increased cytochrome P450 1A2 (CYP1A2) activity and influenced bilirubin metabolism in a randomized clinical trial☆

Craig S. Charron[a,*], Janet A. Novotny[a], Elizabeth H. Jeffery[b], Matthew Kramer[c], Sharon A. Ross[d], Harold E. Seifried[d]

[a] U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Beltsville, MD 20705, USA
[b] Department of Food Science and Human Nutrition, University of Illinois, Urbana, IL 61801, USA
[c] U.S. Department of Agriculture, Agricultural Research Service, Statistics Group, Beltsville, MD 20705, USA
[d] National Institutes of Health, National Cancer Institute, Division of Cancer Protection, Rockville, MD 20852, USA

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ABSTRACT

Brassica vegetables may modulate cancer risk by regulation of xenobiotic metabolizing enzymes (XMEs). In a randomized crossover study, the effect of kale consumption on CYP1A2, CYP2A6, XO, and NAT2 activity was determined by urinary caffeine metabolite ratios, UGT1A1 activity by serum bilirubin concentrations, and GSTA protein and GST activity in blood by ELISA. Adults (n = 25) consumed a basal diet supplemented with kale and radish for 14 days or control vegetables. The kale diet increased CYP1A2 activity by 16.4% on day 8 and 15.2% on day 15 compared to control. Conjugated bilirubin was reduced by the kale diet, decreasing from 19.4 to 14.3 to 9.5% of total bilirubin on days 1, 8, and 15, respectively, which may be explained by induction of MRP2. Other XMEs were not affected by diet. The implications of these results for cancer risk will be clarified as the functions of these XMEs become better understood.

1. Introduction

Consumption of brassica vegetables has been inversely associated with the risk of breast, colorectal, prostate, and lung cancers (Liu, Mao, Cao, & Xie, 2012; Liu & Lv, 2013; Mori et al., 2017; Wu, Yang, Wang, Han, & Xiang, 2013), a result that has considerable support in preclinical studies (Bhattacharya et al., 2010; Chen, Wallig, & Jeffery, 2016; Melchini et al., 2013; Tang & Zhang, 2004; Zhang, Talalay, Cho, & Posner, 1992). The anti-cancer activity of brassica vegetables is generally associated with glucosinolates, which in the presence of plant or bacterial myrosinase, are converted to bioactive isothiocyanates, indoles, and other products. *In vitro* and *in vivo* studies have shown that isothiocyanates can modulate the activity of xenobiotic-metabolizing enzymes (XMEs), particularly phase II enzymes such as uridine diphosphate glucuronosyl (UGT) transferases and glutathione *S*-transferases (GSTs) (Conaway, Yang, & Chung, 2002; Hecht, 1999; Talalay & Fahey, 2001; Zhang et al., 2006). Isothiocyanates trigger upregulation of phase II XMEs via the nuclear factor-erythroid 2 related factor 2 (Nrf2), which also upregulates a few cytochromes P450 (CYP), including CYP2A6 (Yokota, Higashi, Fukami, Yokoi, & Nakajima, 2011), but with no impact on CYP1A2 (Umemura et al., 2006). Indole-3-carbinol from glucobrassicin upregulates both phase I CYPs such as CYP1A1 and 1A2, as well as phase II enzymes as has been shown in preclinical studies (Horn, Reichert, Bliss, & Malejka-Giganti, 2002).

CYP1A2 is involved in the metabolism of numerous pharmaceutical drugs (Faber, Jetter, & Fuhr, 2005) and in the bioactivation of procarcinogens such as aromatic and heterocyclic amines (Boobis et al., 1994; Turesky et al., 1998), nitrosamines (Faber et al., 2005), and

Abbreviations: BHNRC, Beltsville Human Nutrition Research Center; CYP1A2, cytochrome P450 family 1 subfamily A member 2; CYP2A6, cytochrome P450 family 2 subfamily A member 6; CV, control vegetables; GST, glutathione S-transferase; GSTA, glutathione S-transferase alpha; GSTM1, glutathione S-transferase μ 1; GSTT1, glutathione S-transferase θ 1; KR, kale and daikon radish; MRP2, multidrug resistance protein 2; MRP3, multidrug resistance protein 3; NAT2, N-acetyltransferase 2; NRF2, nuclear factor (erythroid-derived 2)–like 2; OATP1B1, organic anion transporter family member 1B1; OATP1B3, organic anion transporter family member 1B3; UGT1A1, uridine diphosphate glucuronosyl transferase 1A1; XME, xenobiotic metabolizing enzyme; XO, xanthine oxidase

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* Corresponding author at: U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, 10300 Baltimore Ave., BARC-EAST, Bldg. 307-B, Beltsville, MD 20705, USA.
E-mail address: craig.charron@ars.usda.gov (C.S. Charron).

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aflatoxins (Eom et al., 2013). The only major endogenous substrates are 17β-estradiol and estrone (2-hydroxylation) (Guengerich, 2015). The overall impact of CYP1A2 activity on potential carcinogenicity is controversial in that some *in vivo* studies indicate that CYP1A2 may play a protective role by detoxifying and clearing carcinogens (Reed, Arlt, & Phillips, 2018; Wang, Bott, Tung, Sugamori, & Grant, 2015). CYP1A2 activity varies among individuals by sex and genetic polymorphisms (Zanger & Schwab, 2013). Higher enzyme activity has been reported in individuals homozygous or heterozygous for the −163C > A polymorphism (rs762551) compared to the wild-type (Koonrungsesomboon, Khatsri, Wongchompoo, & Teekachunhatean, 2018) although CYP1A2 inducibility was not differentially affected by genotype in individuals consuming a mixed brassica diet (Peterson et al., 2009). Exogenous factors such as smoking (Butler et al., 1992), hormonal contraceptives (Matthaei et al., 2016), consumption of charbroiled meat (Pelkonen et al., 2008), and consumption of broccoli (Kall, Vang, & Clausen, 1996) or a mixture of broccoli, cabbage, cauliflower, and radish (Lampe, King, et al., 2000) are known to up-regulate CYP1A2 activity.

CYP2A6 is a member of the cytochrome P450 family that has an important role in nicotine and coumarin metabolism as well as the metabolic activation of nitrosamines and aflatoxin B1 (Di, Chow, Yang, & Zhou, 2009; Rendic & Di Carlo, 1997; Tiano et al., 1993). Elevated CYP2A6 activity has been associated with pancreatic (Kadlubar et al., 2009) and colorectal cancer risk (Nowell, Sweeney, Hammons, Kadlubar, & Lang, 2002). CYP2A6 was induced 1.4- to 5.5-fold by consumption of 500 g/day of broccoli for 6 days (Hakooz & Hamdan, 2007).

Xanthine oxidase (XO) is a cytoplasmic enzyme interconvertible with xanthine oxidase reductase (XOR) and is involved in metabolism of purines and the bioreduction of anticancer agents to their active forms (Pritsos, 2000). XO activity has been reported to be higher after smoking or vigorous exercise (Vistisen, Loft, & Poulsen, 1991) and in females (Relling, Lin, Ayers, & Evans, 1992). In contrast, *in vitro*, flavonoids inhibited XO activity with strongest inhibition occurring with taxifolin, catechin, epichatechin, and epigallocatechin (Cos et al., 1998).

N-acetyl transferase-2 (NAT2) is a phase II enzyme that acetylates arylamine and hydrazine xenobiotics (Guilhen et al., 2009). Slow acetylator phenotypes have increased risk of bladder cancer when exposed to aromatic amine carcinogens such as 4-aminobiphenyl and β-naphthylamine (Hein, 2006) whereas rapid acetylators are more susceptible to colon cancer (Hengstler, Arand, Herrero, & Oesch, 1998). Among dietary influences, twice-daily intake of peppermint tea over six days decreased NAT2 activity (Begas et al., 2017) whereas consumption of heavily browned fish has been reported to induce NAT2 activity (Le Marchand et al., 1996). It has not been established whether these influences modulate cancer risk.

UGT1A1 is a phase II enzyme that glucuronidates bilirubin, hormones, pharmaceuticals, and dietary carcinogens for excretion from the body. UGT1A1 is most abundantly expressed in the liver, but is also found in the kidneys, small intestine, colon, stomach, lungs, epithelium, ovaries, testis, mammary glands and prostate (Rowland, Miners, & Mackenzie, 2013). In a controlled feeding trial, a mixture of cruciferous vegetables as well as a combination of cruciferous and apiaceous vegetables lowered serum bilirubin, which inversely reflects UGT1A1 activity (Navarro, Peterson et al., 2009). Resveratrol taken daily for 4 weeks likewise induced UGT1A1 activity in those with low baseline enzyme activity (Chow et al., 2010). An *in vitro* study showed that UGT1A1 activity was inhibited by flavonoids, with aglycone forms more inhibitory than glycosylated forms (X. Y. Liu et al., 2019). Genotype and sex may modulate the response to diet, as demonstrated in a controlled feeding study showing that a diet of brassica vegetables, soy, and citrus increased UTG1A1 activity compared to a fruit- and vegetable-free diet, but only in women with the *UGT1A1*28* polymorphism (7/7) (Chang et al., 2007). A subsequent observational study confirmed that UGT1A1 activity increased in women with the *UGT1A1*28* polymorphism (7/7) in response to citrus consumption, an effect that was not significant for men or other genotypes (Saracino et al., 2009).

GSTs are a family of isozymes that conjugate glutathione to electrophilic xenobiotic metabolites prior to excretion. A few reports indicate that consumption of brassica vegetables modulates GSTs. Consumption of 300 g/day of Brussels sprouts increased rectal GST-α and GST-π levels measured in biopsied rectal samples from humans (Nijhoff, Grubben, et al., 1995). Levels of plasma GST-α increased in response to Brussels sprouts intake in men, but not women (Nijhoff, Mulder, Verhagen, van Poppel, & Peters, 1995). In a comparison of various brassica vegetables, plasma GST activity was increased following 5 days of consumption of either Brussels sprouts or two cultivars of red cabbage, but not in response to broccoli or white cabbage; none of the brassica vegetables altered plasma GST-α concentrations (Steinkellner et al., 2001).

Kale is consumed throughout the world and is becoming an increasingly popular brassica vegetable. For example, per capita consumption increased by 47% from 1997 to 2017 in the United States (Parr & Daugherty, 2018). Nevertheless, to our knowledge, there have been no investigations of the effects of kale consumption on XMEs in humans. Therefore, our objective was to investigate in a controlled clinical trial the effects of daily kale consumption on CYP1A2, CYP2A6, XO, NAT2 as measured by urinary caffeine metabolite ratios, UGT1A1 as indicated by serum bilirubin, and GSTA and overall GST activity in blood as determined by ELISA. We selected these XMEs because of their importance to human health and because they can be measured relatively non-invasively.

2. Methods

2.1. Subjects

Healthy adults were recruited from the Washington, DC area to participate in this study at the Beltsville Human Nutrition Research Center (BHNRC) in Beltsville, MD, USA. Potential subjects were screened for general health by routine blood and urine screening and health history questionnaire. Applicants were excluded if they met any of the following criteria: (1) pregnant or lactating; (2) use of oral contraceptives; (3) had given birth during the previous 12 months; (4) presence of kidney disease, liver disease, gout, hyperthyroidism, hypothyroidism, certain cancers, gastrointestinal disease, pancreatic disease, other metabolic diseases, or malabsorption syndromes requiring special diets; (4) allergy or intolerance to brassica vegetables; (5) colonoscopy during three weeks prior to start of study; (6) use of tobacco products; (7) occupational or home exposure to organic solvents or passive smoke; (8) type 2 diabetes requiring the use of diabetes pills, insulin, or non-insulin shots; (9) fasting glucose ≥ 126 mg/dL; (10) use of blood-thinning medications such as warfarin or anisinidione; (11) self-report of alcohol or substance abuse within the past 12 months and/or current acute treatment or rehabilitation program for these problems. Subjects were recruited in two cohorts from January 2018 to May 2018. The recruitment and enrollment data of the combined cohorts are presented in Fig. 1. Characteristics of subjects who completed the study are reported in Table 1. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by Chesapeake IRB (Columbia, MD, USA). Written informed consent was obtained from all subjects. This trial was registered at clinicaltrials.gov (NCT03449849).

2.2. Kale

For ease of consumption, we used baby kale (*Brassica oleracea* L. var *acephala*), which is harvested at an early developmental stage (4–6 true-leaf stage) and which is considerably more tender than mature kale

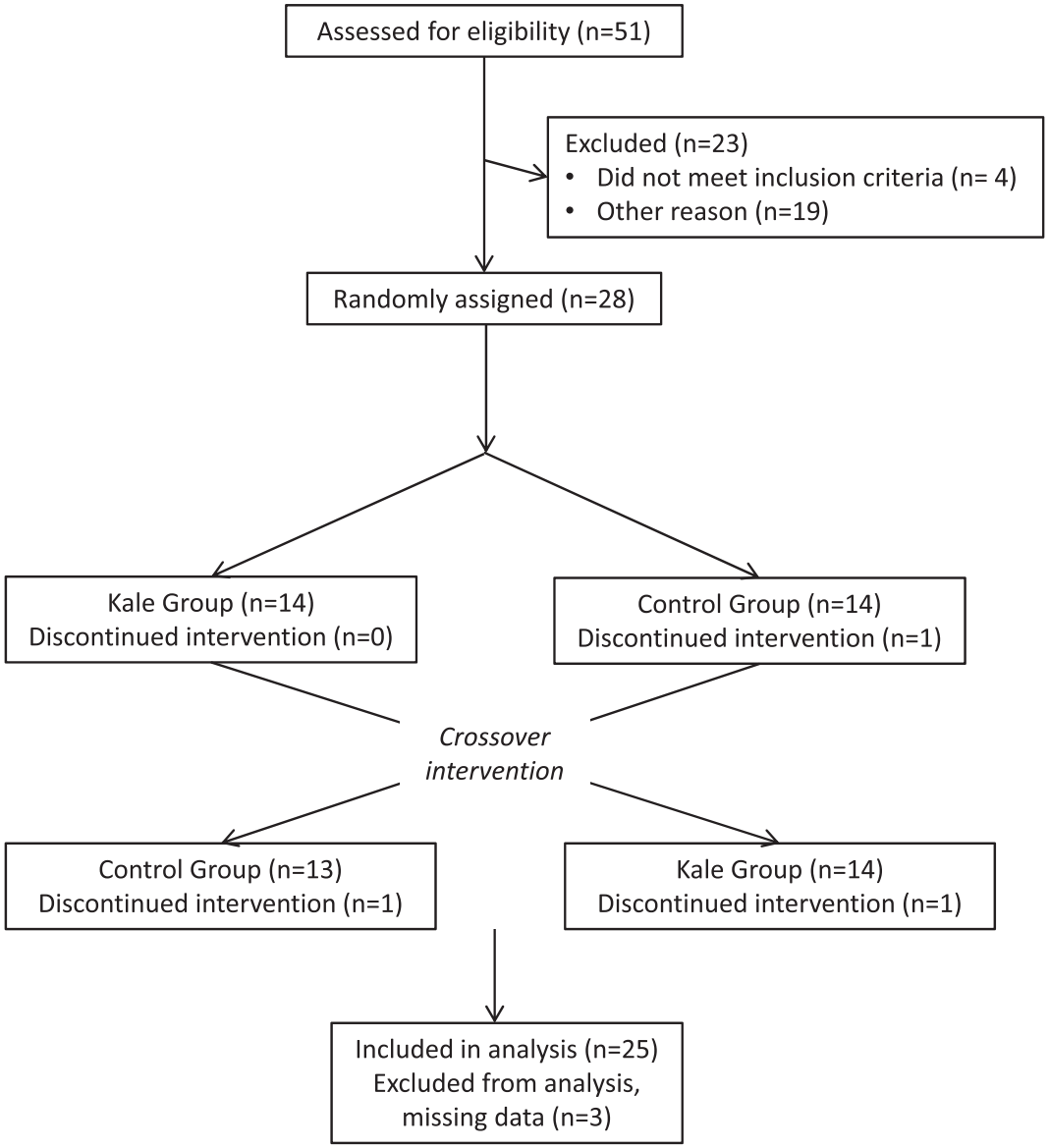

Fig. 1. Recruitment and enrollment of subjects.

Table 1
Characteristics of study participants.[a]

Cohort	*n*	Age (years)	BMI (kg/m^2)
Cohort 1	20	57.6 ± 14.2	30.0 ± 7.0
Male	5	56.0 ± 15.4	24.8 ± 3.2
Female	15	57.4 ± 14.3	31.7 ± 7.6
Cohort 2	5	51.4 ± 14.3	30.1 ± 4.8
Male	2	55.5 ± 13.4	33.1 ± 2.9
Female	3	48.7 ± 17.0	28.1 ± 5.2
Combined cohorts	25	55.9 ± 14.1	30.0 ± 6.9
Male	7	55.9 ± 13.7	27.1 ± 5.0
Female	18	55.9 ± 14.6	31.1 ± 7.3

[a] Mean ± SD.

(Saini, Ko, & Keum, 2017; Waterland et al., 2017). Raw kale to be used for the study was procured in 8 shipments prior to the study. Seven shipments corresponded to each day of a 7-day meal rotation and one shipment was prepared if needed as backup. Upon receipt, the kale was steamed for 8 min., vacuum-packed, and frozen at −80 °C. The steaming process increased kale weight by 5.2%. Because myrosinase is significantly deactivated by high temperatures as found in steaming (Dosz & Jeffery, 2013), the kale was served with fresh frozen daikon radish as a source of plant myrosinase. All daikon radish was purchased prior to the study and stored at −80 °C until used. Glucosinolates were measured according to a previously published method (Saracino et al., 2009) in 3 subsamples of each of the 8 kale shipments after the kale had been steamed and frozen and the subsamples had been lyophilized, and in raw daikon radish that had been frozen and lyophilized (Table 2).

2.3. Experimental design and treatments

This study was a randomized crossover design consisting of two 15-day periods separated by a 20-day washout. Power was calculated based on CYP1A2 activity as reflected by caffeine metabolite ratios. Power was determined by simulation, based on results from previous reports (Matthaei et al., 2016; Peterson et al., 2009). Essentially, 1000 simulated data sets were created, using between- and within-subject variance parameters given or estimated from each study and the two differences desired to detect, for each candidate number of subjects (5–30). Each simulated data set was then tested using the nlme package (Pinheiro, Bates, Debroy, & Sarkar, 2017) in R (R Development Core

Table 2
Mean daily macronutrients, fiber, and glucosinolates provided by the kale/daikon radish diet[a] and the control vegetables diet (based on 2000 kcal).

		Kale/radish		Control vegetables			
	Base Diet	Kale[b] (400 g)	Radish (40 g)	Peas (174 g)	Green beans (238 g)	White corn (68 g)	Weighted mean[c], control vegetables (121 g)
Protein (g)	79.6	11.4	0.2	9.1	7.1	4.5	7.2
Carbohydrate (g)	238.6	20.9	1.6	23.7	30.7	32.0	28.1
Fat (g)	66.9	2.0	0.0	0.7	0.8	1.06	0.8
Fiber (g)	13.3	8.0	0.6	7.8	7.1	3.8	6.5
Total glucosinolates (μmol)	0	235.0[d]	4.7[e]	0	0	0	0

[a] The control diet consisted of the base diet (7-day rotation) plus control vegetables consisting of peas (3 days/week), green beans (2 days/week), and white corn (2 days/week). The kale diet consisted of the base diet plus kale and daikon radish (7 days/week).
[b] Uncooked weight. Percent dry matter = 8.6%. Weight of steamed kale was 5.2% higher.
[c] Calculated as (3 × macronutrient weight from peas + 2 × macronutrient weight from green beans + 2 × macronutrient weight from white corn)/7.
[d] Mean ± SD (μmol): total glucosinolates, 235.0 ± 80.2; sinigrin, 177.9 ± 84.4; glucobrassicin, 39.0 ± 22.0; 4-hydroxyglucobrassicin, 8.4 ± 2.9; 4-methoxyglucobrassicin, 5.8 ± 3.8; neoglucobrassicin, 3.9 ± 2.6.
[e] Mean ± SD (μmol): total glucosinolates, 4.7 ± 0.5; glucoraphasatin, 2.6 ± 0.4; glucoraphenin, 1.2 ± 0.1; 4-methoxyglucobrassicin, 0.6 ± 0.1; 4-hydroxyglucobrassicin, 0.2 ± 0.0; neoglucobrassicin, 0.2 ± 0.

Team, 2017), and the proportion of significant tests were output. We determined that 22 subjects were required to show a 20% change in CYP1A2 activity (on the original scale) with over 90% power (power was calculated assuming CYP1A2 activity was transformed to a log scale). Due to potential subjects becoming unavailable due to schedule conflicts and one subject dropping out during the study due to jury duty, 20 subjects completed the study initially, we reran the study and 5 more subjects completed the protocol. Twenty-five subjects in total completed the study.

All subjects consumed a controlled basal diet that excluded brassica and apiaceous vegetables, alcohol, apples, cherries, chocolate, grapefruit, oranges, charbroiled meat, fried foods, and smoked foods. Breakfast and dinner were consumed at the BHNRC dining facility on weekdays, and lunches and weekend meals were packed in coolers for carry-out. Subjects were instructed to eat all foods and only foods provided to them. Coffee and tea intake were limited to 2 cups per day and diet soda was limited to 12 oz per day. Foods were scaled in 200-kcal increments to meet individual energy requirements and to maintain subject weights. In addition to the basal diet, subjects consumed either kale with daikon radish (KR; radish was 10% of kale uncooked weight) or control vegetables (CV) consisting of peas, green beans, or white corn. 421 g of steamed kale (1 ¾ c; uncooked weight was 400 g) with 40 g of daikon radish were provided for the calorie range 1800–2200 kcal daily and 547 g of steamed kale (2 ¼ c; uncooked weight was 520 g) and 52 g of daikon radish were provided for the range 2400–2800 kcal daily. The daily macronutrient and fiber content of the foods were determined using Food Processor software, version 11.3.285 (ESHA Research, Salem, OR) and are reported in Table 2.

Kale and radish or control vegetables were consumed daily with half at breakfast and half at dinner, with the following exceptions: On the first day of the first period, subjects on the KR treatment consumed kale and radish at breakfast and had control vegetables at dinner in order to conduct a 24-h pharmacokinetic study to be reported separately. Kale that was consumed at BHNRC was heated in a 1200-W microwave oven (NE-1258R; Panasonic). At each breakfast and dinner, frozen (previously steamed) 210.5-g portions were microwaved for 110 sec and 273.5-g portions were microwaved for 175 sec. For kale consumed outside of BHNRC (weekends), subjects were instructed to heat the kale in a microwave oven until warm. On days on which the caffeine metabolite assay was conducted (days 8 and 15), the morning breakfast for all subjects consisted of a 100-g banana muffin, 13 g of margarine, 390 mL of water, and a 200-mg caffeine pill (NoDoz, GlaxoSmithKline Consumer Healthcare, Warren, NJ, USA).

2.4. Caffeine metabolite assay

Ratios of caffeine metabolites were determined to assess the effects of the kale intervention on CYP1A2, CYP2A6, NAT2, and XO on days 8 and 15 of each period. In order to minimize subject burden, we chose not to conduct the caffeine metabolite assay on day 1 of each period. We believed this approach to be satisfactory because in the crossover design, each subject serves as his/her own control. Following a 12-h fast and after abstaining from coffee for 24 h, subjects were asked to void and then consume 200 mg of caffeine with breakfast as described above. For the next 5 h no additional food or liquids were consumed. Four h after consuming the caffeine, subjects were asked to void, and then at 5 h after consuming the caffeine, subjects collected all their urine. Urine volumes were measured, and the pH was adjusted to 3.5 ± 0.1 with 1 M HCl. 1.5-mL aliquots were stored at −80 °C until analysis. Samples were extracted based on the procedure of Peterson et al. (Peterson et al., 2009) with some modifications. After centrifugation of urine at 16000*g* for 2 min (4 °C), a saturating amount of ammonium sulfate was added to 450 μL of urine combined with 50 μL of 4-acetamidophenol (APAP; Sigma-Aldrich, St. Louis, MO) as an internal standard. Samples were vortex-mixed and sat at room temperature for 1 h. Then samples were extracted twice with 3 mL of a 9:1 mixture of chloroform and isopropanol, and after mixing for 1 min., centrifuged at 540*g* for 15 min. (4 °C). The organic layers from both extractions were combined and dried under nitrogen in a 45 °C water bath. The residue was reconstituted with 500 μL of 0.05% acetic acid, sonicated for 30 s, and filtered with a 0.22-μm spin filter at 10,000*g* for 5 min (4 °C).

We analyzed the extracted samples according to a previously published method (Begas, Kouvaras, Tsakalof, Papakosta, & Asprodini, 2007) with slight modifications of the solvents and gradients to optimize separation of caffeine metabolites. An Agilent Model 1100 HPLC (Agilent Technologies, Santa Clara, CA) system was used with an Agilent Eclipse Plus C18 column (4.6 mm × 250 mm, 5 μm). Standards of caffeine metabolites were procured as follows: 1-methyluric acid (1U), 1-methylxanthine (1X), 7-methylxanthine (7X), 3-methylxanthine (3X), 1,3-dimethylxanthine (13X), 1,7-dimethyluric acid (17U), 1,7-dimethylxanthine (17X), 3,7-dimethyluric acid (37U), 3,7-dimethylxanthine (37X), and caffeine (137X) were purchased from Sigma; 7-methyluric acid (7U) and 1,3,7-trimethyluric acid (137U) were purchased from Cayman Chemical (Ann Arbor, MI); 3-methyluric acid (3U), 1,3-dimethyluric acid (13U), 5-acetylamino-6-amino-3-methyluracil (AAMU), and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) were purchased from Toronto Research Chemicals (Ontario, Canada). These standards were used for quantitation and identification (by comparing retention times and UV–vis spectra) and to establish that caffeine metabolites did not coelute in study samples. Solvent A was 0.1% acetic acid -methanol – acetonitrile (91:4.5:4.5 v/v); solvent B was 0.1% acetic acid – methanol (60:40 v/v), and solvent C was 0.1% acetic acid. The gradients and flow rates were as follows: 0 to 2 min

50% A, 50% C at 0.7 mL/min; 2 to 2.1 min ramp to 100% A at 0.7 mL/min; 2.1 to 14 min 100% A at 0.7 mL/min; 14 to 14.1 min 100% A and flow rate increased to 1.1 mL/min; 14.1 to 17 min hold at 100% A and 1.1 mL/min; 17 to 22 min ramp to 100% B at 1.1 mL/min; 22 to 26 min hold at 100% B and 1.1 mL/min; 26 to 28 min ramp to 50% A, 50% C at 0.7 mL/min; 28 to 30 min hold at 50% A, 50% C at 0.7 mL/min. Detection was at 280 nm. Stock solutions of AFMU, 1U, 1X, 17U, and 17X were diluted in 0.05% acetic acid for a standard curve of 6 concentrations ranging from 10 to 200 μM. The standard curves were generated on each day that samples were analyzed by HPLC. R-square values for the standard curves were typically > 0.99. Each subject's samples were run in a single block which was followed by a quality control sample consisting of 75 μM each of AFMU, 1U, 1X, 17U, and 17X. Interday coefficients of variation for AFMU, 1U, 1X, 17U, and 17X were 3.6, 4.1, 4.2, 4.9, and 6.8%, respectively.

CYP1A2 activity was estimated by the ratio (1U + 1X + AFMU)/17U. This ratio is least influenced by urine flow rate (Perera, Gross, & McLachlan, 2012). CYP2A6, NAT2, and XO were determined by the ratios 17U/(17U + 17X), AFMU/(1X + 1U + AFMU), and 1U/(1X + 1U), respectively (Begas et al., 2017).

2.5. Serum bilirubin

Fasting blood samples from days 1, 8, and 15 of each diet period were analyzed for serum bilirubin using the Vitros 5,1 FS Chemistry System (Ortho Clinical Diagnostics, Raritan, NJ) according to the manufacturer's protocol. Conjugated bilirubin was calculated as the difference of total bilirubin and unconjugated bilirubin.

2.6. GSTA concentration and GST activity

Fasting blood samples were collected on days 1, 8, and 15 of each period, with the day 1 samples serving as a baseline measure. A DYNEX Technologies (Chantilly, VA) ELISA Processing System was used to measure serum GSTA concentration using an assay from Oxford Biomedical Research (Rochester Hills, MI) and to measure plasma GST activity using an assay from Cayman Chemical according to the manufacturers' instructions. For GSTA concentration, the average %CV was 5.5% and the inter-assay CV was 8.6%. For GST activity, the average %CV was 12.1% and mean inter-assay %CV was 16.0%.

2.7. Statistical analysis

Analyses of variance (ANOVA) of the data were performed using the MIXED procedure in SAS (ver. 9.4, SAS Institute Inc., Cary, NC, USA). Data were tested for normality with the Shapiro–Wilk statistic, and by inspection of stem-leaf plots and normal probability plots of residuals. CYP1A2, total bilirubin, conjugated bilirubin, unconjugated bilirubin, GSTA concentration, and GST activity were skewed and log-transformed (natural log) before analysis. NAT2 was bimodally distributed, corresponding to slow (n = 13) and rapid acetylators (n = 12) as determined by the antimode at 0.25 (Begas et al., 2017). ANOVA was performed on the total group of subjects as well as each of the groups of slow and rapid acetylators. ANOVA models for CYP1A2, CYP2A6, NAT2, and XO included period, sequence, day, and diet as fixed effects, and subject was treated as a random effect. Models for total bilirubin, conjugated bilirubin, unconjugated bilirubin, GSTA and GST activity included a term for baseline corresponding to the sample collected on day 1 of each period. Age, BMI, and cohort were examined in preliminary analyses, were found to be nonsignificant, and thus were not included in the final analyses. LS means were back-transformed to their original scales. Model effects are reported as LS means with 95% confidence intervals. Adjusted *P*-values were calculated using false discovery rates (FDR) to control for experiment-wise error rate.

3. Results

Twenty-five subjects completed the study with no report of adverse effects or difficulty consuming either the CV or KR diet. The macronutrients, fiber and glucosinolates consumed by subjects on the CV and KR diets are shown in Table 2. Based on the 2000 kcal level, the KR portion of the diet provided 11.6, 22.5, 2.0, and 8.6 g of protein, carbohydrates, fat, and fiber, respectively. In comparison, the CV portion of the diet contributed 7.2, 28.1, 0.8, and 6.5 g of protein, carbohydrates, fat, and fiber, respectively. Based on the 2000 kcal level, the KR diet provided 239.7 μmol of glucosinolates daily, 98% of which were glucosinolates from kale. Sinigrin was the predominant glucosinolate and contributed 177.9 μmol/day. Glucobrassicin, an indole glucosinolate, was the next most abundant and contributed an additional 39.0 μmol/day. The other indole glucosinolates, which included 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin, together provided 19.1 μmol/day. The control vegetables do not contain glucosinolate metabolic pathways and thus did not contribute glucosinolates to the CV diet.

Urinary metabolic ratios representing CYP1A2 activity were significantly higher in subjects on the KR diet compared to the CV diet ($P = 0.0067$; Table 3). On days 8 and 15, CYP1A2 activity was 16.4% and 15.2% higher, respectively, in subjects on the KR diet compared to the CV diet, and did not differ by day ($P < 0.05$). CYP1A2 was generally higher in males than in females ($P = 0.0067$) regardless of diet or day of measurement, ranging from 31.7% higher on day 8 (CV diet) to 22.5% higher on day 15 (KR diet). The percent induction in females by the KR diet was 17.1% and 15.7% on days 8 and 15, respectively, and in males was 15.7% and 15.2% on days 8 and 15, respectively. The activities of CYP2A6 and XO were not influenced by diet, sex, or day of measurement. NAT2 activity was not affected by diet or day for either rapid or slow acetylators, or the total group of subjects.

Serum concentrations of total, unconjugated, and conjugated bilirubin are reported in Fig. 2. Conjugated bilirubin concentrations were essentially constant with time in subjects on the CV diet, ranging from 2.0 (1.5, 2.5; LS mean with 95% CI) μmol/L on day 1 (baseline) to 2.2 (1.7, 2.8) μmol/L on day 15. The KR diet significantly decreased conjugated bilirubin concentrations ($P = 0.0034$) and the effect appeared to be more pronounced with time. The baseline day 1 values did not differ between dietary groups (2.0 μmol/L) whereas on day 8, conjugated bilirubin for those on the KR diet was 25.5% lower than for those on the CV diet ($P = 0.0556$), and by day 15, conjugated bilirubin was 54.5% lower for those on the KR diet than for those on the CV diet ($P = 0.0020$). Conjugated bilirubin was 19.1, 18.7, and 18.4% of total bilirubin on days 1, 8, and 15 respectively, in subjects on the CV diet, and was 19.4, 14.3, and 9.5% of total bilirubin on days 1, 8, and 15, respectively, in subjects on the KR diet. The effect of diet was not significant for total bilirubin ($P = 0.4373$) or unconjugated bilirubin ($P = 0.8286$). The sex of the subject did not influence any of the bilirubin measures (data not shown).

GSTA concentration in serum and GST activity were not influenced by the KR diet in comparison to the control, and there was no effect of day or the sex of the subject (Table 4).

4. Discussion

Kale consumption has been increasing in popularity and due to its constituent glucosinolates, we conducted a study to determine the effects of kale intake on the XMEs CYP1A2, CYP2A6, XO, NAT2, UGT1A1, GSTA, and GST activity in humans. The glucosinolate profile of the baby kale used in this study was similar to that reported previously in that sinigrin and glucobrassicin were the two most predominant glucosinolates (Velasco, Cartea, González, Vilar, & Ordás, 2007). Like sulforaphane from glucoraphanin in broccoli, allyl isothiocyanate from sinigrin is an aliphatic isothiocyanate, shown to upregulate a number of phase II XMEs and protect against certain cancers, including breast

Table 3
CYP1A2, CYP2A6, XO, and NAT2 activities (least squares means with 95% confidence intervals) determined by urinary caffeine metabolic ratios from subjects consuming a diet including either control vegetables or kale with daikon radish.

	Day 8		Day 15	
	Control	Kale	Control	Kale
CYP1A2[a,b]				
Total (n = 25)	1.89 (1.74, 2.05)	2.20* (2.03, 2.38)	1.84 (1.69, 2.00)	2.12* (1.96, 2.29)
Female[c] (n = 18)	1.64 (1.49, 1.79)	1.92* (1.76, 2.09)	1.65 (1.51, 1.80)	1.91* (1.75, 2.08)
Male (n = 7)	2.16 (1.89, 2.46)	2.50* (2.20, 2.83)	2.04 (1.78, 2.33)	2.35* (2.06, 2.67)
CYP2A6[d]				
Total (n = 25)	0.59 (0.54, 0.64)	0.58 (0.53, 0.63)	0.62 (0.57, 0.66)	0.58 (0.53, 0.63)
Female (n = 18)	0.61 (0.56, 0.66)	0.59 (0.54, 0.64)	0.64 (0.59, 0.69)	0.60 (0.55, 0.66)
Male (n = 7)	0.57 (0.49, 0.66)	0.58 (0.50, 0.66)	0.59 (0.51, 0.68)	0.55 (0.47, 0.64)
XO[e]				
Total (n = 25)	0.42 (0.40, 0.45)	0.40 (0.37, 0.42)	0.42 (0.39, 0.44)	0.41 (0.39, 0.43)
Female (n = 18)	0.41 (0.38, 0.43)	0.40 (0.38, 0.43)	0.41 (0.39, 0.44)	0.40 (0.38, 0.43)
Male (n = 7)	0.44 (0.40, 0.48)	0.39 (0.35, 0.43)	0.42 (0.38, 0.46)	0.41 (0.37, 0.45)
NAT2[f]				
Total (n = 25)	0.24 (0.20, 0.29)	0.24 (0.20, 0.28)	0.24 (0.20, 0.28)	0.24 (0.20, 0.28)
Female (n = 18)	0.21 (0.16, 0.25)	0.21 (0.17, 0.25)	0.21 (0.17, 0.26)	0.21 (0.16, 0.25)
Male (n = 7)	0.28 (0.21, 0.35)	0.27 (0.19, 0.34)	0.27 (0.19, 0.34)	0.27 (0.20, 0.35)
Rapid acetylators, (values > 0.25, n = 12)	0.35 (0.33, 0.37)	0.35 (0.33, 0.37)	0.34 (0.32, 0.36)	0.34 (0.32, 0.37)
Slow acetylators, (values > 0.25, n = 13)	0.12 (0.09, 0.14)	0.11 (0.09, 0.13)	0.11 (0.09, 0.14)	0.12 (0.09, 0.14)

*Different from control on that day, $P < 0.05$ by Fisher's protected LSD test.
[a] LS means are back-transformed to the original scale.
[b] Effect of diet on CYP1A2 activity was significant, $P = 0.0067$.
[c] Overall significant effect of sex, $P = 0.0067$.
[d] Effect of diet on CYP2A6 activity was not significant, $P = 0.6997$.
[e] Effect of diet on XO activity was not significant, $P = 0.7638$.
[f] Effect of diet on NAT2 activity was not significant for all subjects ($P = 0.9383$), or for either rapid acetylators ($P = 0.9384$) or slow acetylators ($P = 0.9384$).

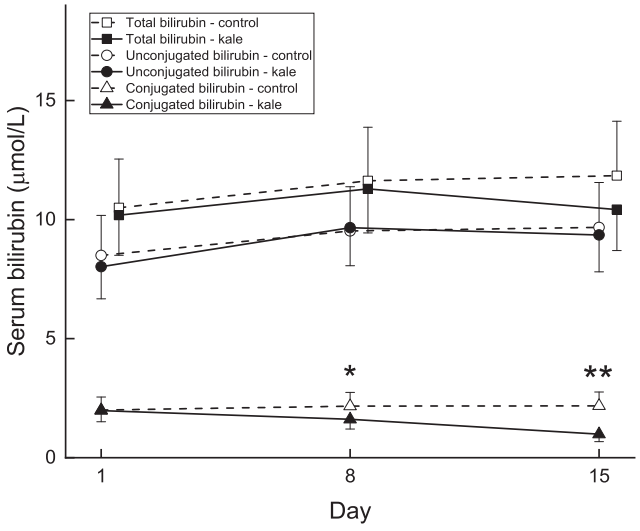

Fig. 2. Serum concentrations (µmol/L) of total bilirubin, unconjugated bilirubin, and conjugated bilirubin by diet (kale or control) and by day. Values are back-transformed least squares means with upper 95% confidence limits shown above largest mean for each type (total, unconjugated, or conjugated) of bilirubin measurement at a given time and lower 95% confidence limits shown below smallest mean at a given time. The values for total bilirubin are offset +0.5 day so that error bars can be distinguished from those of unconjugated bilirubin. Overall diet effect was significant for conjugated bilirubin, $P = 0.0034$, adjusted for baseline. *$P = 0.0556$ for diet effect on day 8 (Fisher's protected LSD test). **$P = 0.0020$ for diet effect on day 15 (Fisher's protected LSD test). Diet effect was not significant for total bilirubin ($P = 0.4373$) or unconjugated bilirubin ($P = 0.8256$).

cancer (Thangarasu, Pachaiappan, & Subbaiyan, in press). Glucosinolate concentrations in plant tissue vary with stage of plant development, and in kale, glucosinolate concentrations typically increase with plant maturation (Velasco et al., 2007). Thus the baby kale used in this study may have had as little as 20% of the levels of glucosinolates typical of kale that has developed to the more mature stage that is commonly consumed (Velasco et al., 2007). In this study, the daily portions of steamed kale (1 ¾ cups and 2 ¼ cups) were less than the USDA MyPlate dietary guidelines which recommend 2.5–3.0 cups of vegetables daily for adults, varying slightly depending on age and sex (U.S. Department of Agriculture). Therefore, the amounts of kale used in this study could reasonably be consumed as part of a routine diet.

We report here that CYP1A2 activity increased in response to daily kale consumption, and that the increase was similar on days 8 (16.4%) and 15 (15.2%) of the diet period showing that CYP1A2 induction was sustained from the measurement at one week to that at two weeks. This is the first investigation to our knowledge to determine CYP1A2 activity at two time points with daily consumption of a brassica vegetable. Other studies reported an increase of 19% in CYP1A2 activity following intake of 500 g of broccoli daily for 6 days (Kall et al., 1996), 83% following intake of 500 g of broccoli daily for 12 days (Hakooz & Hamdan, 2007), 18% following intake of 436 g of mixed brassica vegetables daily for 6 days (Lampe, King, et al., 2000), and 14–27% following intake of 7 or 14 g/kg body weight of mixed brassica vegetables daily for 14 days (Peterson et al., 2009). Induction of CYP isoforms likely occurs in response to 3,3′-diindolylmethane (DIM), a condensation product of indole-3-carbinol (I3C) which is a metabolite of the indole glucosinolate glucobrassicin. DIM has been shown to induce CYP1A, CYP2B, and CYP3A subfamily isoforms in rat liver slices (Renwick et al., 1999) and CYP1A2 in cultured human liver slices (Lake, Tredger, Renwick, Barton, & Price, 1998). A phase I trial in women demonstrated that 400 µg (2.7 µmol) of I3C ingested daily for 4 weeks followed by 800 µg (5.4 µmol) daily for another 4 weeks increased CYP1A2 activity four-fold (Reed et al., 2005). Our finding that the kale in this study provided 39.0 µmol of glucobrassicin daily (per 2000 kcal) establishes a baseline for future studies to determine minimum levels of glucobrassicin intake necessary for CYP1A2 induction when delivered as a whole food rather than via the purified

Table 4
Serum GSTA protein concentration (least squares means with 95% confidence intervals) and plasma GST activity (least squares means with 95% confidence intervals) in subjects consuming the kale and control diets.[a]

	Day 8		Day 15	
	Control	Kale	Control	Kale
GSTA concentration (ng/mL)[b]				
Total (n = 25)	2.09 (1.59, 2.68)	2.41 (1.86, 3.05)	2.00 (1.52, 2.58)	1.90 (1.44, 2.45)
Female (n = 18)	1.77 (1.30, 2.33)	2.15 (1.62, 2.80)	2.11 (1.58, 2.73)	1.86 (1.37, 2.44)
Male (n = 7)	2.44 (1.53, 3.68)	2.68 (1.73, 3.97)	1.90 (1.13, 2.94)	1.95 (1.19, 2.98)
GST activity[c] (nmol/min/mL)				
Total (n = 25)	1.08 (0.89, 1.29)	1.03 (0.85, 1.23)	1.09 (0.90, 1.30)	0.95 (0.77, 1.14)
Female (n = 18)	1.18 (0.97, 1.41)	1.12 (0.91, 1.34)	1.13 (0.93, 1.36)	1.01 (0.81, 1.23)
Male (n = 7)	0.99 (0.69, 1.34)	0.95 (0.66, 1.30)	1.04 (0.74, 1.40)	0.89 (0.61, 1.23)

[a] LS means are back-transformed to the original scales.
[b] Effect of diet on GSTA concentration was not significant, $P = 0.8839$.
[c] Effect of diet on GST activity was not significant, $P = 0.7098$.

degradation product I3C.

We considered whether the daikon radish contributed to the increase in CYP1A2 activity. Studies reporting induction of CYP1A2 in animals in response to radish or glucosinolates found therein have used levels far in excess of that used in this current study. We provided 40-g daily portions of daikon radish (fresh weight) containing 2.6 ± 0.4 μmol of glucoraphasatin and 1.2 ± 0.1 μmol of glucoraphenin (Table 2), which were the two most predominant glucosinolates. Based on the mean weight of the study subjects (83.1 kg, data not reported), subjects consumed glucoraphasatin and glucoraphenin at rates of 0.031 and 0.014 μmol/kg of body weight, respectively. CYP1A2 activity was induced in livers of male Sprague-Dawley rats given glucoraphasatin at a rate of 57.2 μmol/kg or glucoraphenin at 55.1 μmol/kg, which are, respectively, 1845 and 3936 times the dose that we provided via daikon radish (Barillari et al., 2007). Hepatic CYP1A2 mRNA increased 3-fold in BL/6j mice given Spanish black radish providing 1185 μmol of glucoraphasatin/kg of body weight, which is 38,226 times the dose we provided (Scholl, Eshelman, Barnes, & Hanlon, 2011). The threshold concentration in media at which CYP1A2 activity was induced in HepG2 cells by Spanish black radish isothiocyanates was ≥1.73 μM (Hanlon, Webber, & Barnes, 2007). Taking these results into account, and given that even a 200-μmol dose of the isothiocyanate sulforaphane resulted in a peak plasma concentration of < 2.0 μM in humans (Atwell et al., 2015), we therefore conclude that the dose of radish used in this current study (containing 4.7 ± 0.5 μmol of total glucosinolates) was too low to have a detectable influence on CYP1A2 activity.

Increased CYP1A2 activity is of interest because it supports estrogen 2-hydroxylation, which is considered as a factor supporting decreased estrogen carcinogenicity and subsequent decreased breast cancer risk (Ayari et al., 2013). As with CYP1A1, 1A2 is also associated with activation of polycyclic hydrocarbon (PAH) environmental carcinogens like benzpyrene, questioning the benefit (He & Feng, 2015). Yet, not only is CYP1A2 also associated with enhanced phase II clearance of PAH, but whole brassica also contains aliphatic isothiocyanates, able to positively impact clearance of PAH. Thus whereas coffee and drugs that induce CYP1A2 alone may have both positive and negative impact on breast cancer risk, dietary broccoli has been shown to protect against breast cancer (Ambrosone et al., 2004).

Previous population studies in the United States (n = 342) and China (n = 229) have reported that CYP1A2 activity was lower in women than in men, in line with the results of this study (Ou-Yang et al., 2000; Relling et al., 1992). However, a clinical study (n = 36) in the United States and a population study in Korea (n = 133) did not detect an effect of the sex of the subjects on CYP1A2 activity as measured by caffeine metabolite ratios (Chung, Kang, Park, Cho, & Cha, 2000; Lampe, King, et al., 2000), and a clinical study in a Jordanian population (n = 10) reported that CYP1A2 activity was higher in women than in men (Hakooz & Hamdan, 2007). The sex-specific expression of P450s has been shown to be regulated by the temporal pattern of plasma growth hormone release that differs in men and women (Waxman & Holloway, 2009).

CYP2A6, XO, and NAT2 activities were not affected by the KR diet. There are some reports that CYP2A6 can be influenced by diet. CYP2A6 has been reported to increase in response to broccoli consumption (Hakooz & Hamdan, 2007). Conversion of nicotine to cotinine decreased in response to grapefruit juice intake, suggesting a decrease in CYP2A6 activity (Hukkanen, Jacob, & Benowitz, 2006). We saw no changes in CYP2A6 activity with diet.

Regulation of XO is under genetic control with little clinical evidence to support susceptibility to dietary influence. XO expression did not change even in free-living subjects measured at three times during a 25-day period (Asprodini et al., 2019). Similarly, NAT2 activity is almost exclusively determined genetically (Matthaei et al., 2016). Interestingly, an impact of diet was reported in a clinical study measuring activities of CYP2A6, XO, and NAT2 in response to intake of quercetin, a flavonoid present in kale. Subjects who were given 500 mg of quercetin/day for 13 days had higher CYP2A6, XO, and NAT2 activities (Y. Chen et al., 2009). Given that we provided 400 g (uncooked weight) of kale/2000 kcal/day, and based on the USDA Flavonoids database indicating that kale contains a mean of 22.58 mg of quercetin/100 g of kale (Bhagwat, Haytowitz, & Holden, 2014), we estimate that subjects consumed 90 mg of quercetin/2000 kcal daily. This level of quercetin intake may have been too low to induce a measurable change in CYP2A6, XO, and NAT2.

Serum conjugated bilirubin (bilirubin glucuronide) concentrations decreased in response to the KR diet compared to the CV diet, and the magnitude of the decrease was greater on day 15 than on day 8. In contrast, unconjugated bilirubin was not influenced by the KR diet. In hepatocytes, unconjugated bilirubin is glucuronidated by UGT1A1 to two conjugates, monoglucuronosyl bilirubin and bisglucuronosyl bilirubin, followed by MRP2-mediated transport across the canalicular membrane into bile (Nies & Keppler, 2007). In addition, following glucuronidation, the bilirubin conjugates may be effluxed into sinusoidal blood via the basolaterally-localized MRP3 (Lee et al., 2004), leading either to urinary excretion or reuptake by OATP1B1 and OATP1B3 in downstream hepatocytes (Van De Steeg et al., 2012). A similar excretory process occurs in intestinal epithelial cells where UGT1A1 glucuronidates bilirubin (Tukey & Strassburg, 2000) and MRP3 mediates efflux into blood (Fujiwara, Chen, Karin, & Tukey, 2012). In this study, the decreasing fraction of conjugated bilirubin with time on the KR diet suggests a sustained perturbation of the relative pools of conjugated and unconjugated bilirubin, perhaps mediated by alterations in the activities of UGT1A1, MRP2, and/or MRP3.

However, any substantial induction of UGT1A1 likely would be reflected by significant reductions in unconjugated bilirubin, in addition to the decrease in conjugated bilirubin that we observed. Because unconjugated bilirubin did not respond to the KR diet, it is unlikely that UGT1A1 activity was affected by diet.

Our finding that kale consumption altered the relative pools of conjugated and unconjugated bilirubin may indicate differential expression of MRP2 and MRP3. The decrease in conjugated bilirubin relative to unconjugated bilirubin could be explained by induction of MRP2 resulting in enhanced removal of conjugated bilirubin from blood to bile. *In vitro* studies have shown that sulforaphane and erucin, isothiocyanates derived from brassica vegetables, increase MRP2 mRNA in primary hepatocytes (Payen, Courtois, Loewert, Guillouzo, & Fardel, 2001) and MRP2 mRNA and protein in Caco-2 cells in a dose-dependent manner (Harris & Jeffery, 2008; Jakubíková, Sedlák, Mithen, & Bao, 2005). Like GSTA, MRP2 is regulated by the NRF2/ARE signal transduction pathway in response to exposure to xenobiotics (Vollrath, Wielandt, Iruretagoyena, & Chianale, 2006). The kale used in this study contained both sinigrin and glucobrassicin, which metabolize respectively to allyl isothiocyanate and I3C/DIM, which have been shown to activate the NRF2/ARE pathway (Ernst, Schuemann, Wagner, & Rimbach, 2011; Ernst, Wagner, et al., 2011). Thus, it is plausible that these glucosinolate breakdown products induced MRP2 and redirected the conjugates toward biliary excretion. Alternatively, any down-regulation of MRP3 by the KR diet could similarly shift a greater proportion of product toward MRP2 and biliary excretion, causing a depletion of serum conjugated bilirubin. However, there is little reported evidence to support significant changes in MRP3 in response to diet in healthy humans.

GSTA protein concentration and GST activity in blood were not affected by the dietary intervention. It is possible that an influence of diet would have been detectable had the study been designed to detect the effects of *GSTM1* and *GSTTT1* polymorphisms, or had the quantity of kale consumed been greater. Highest induction of GSTA was reported in *GSTM1-null/GSTT1-null* men in a study in which subjects consumed 7 or 14 g of mixed brassica vegetables/kg of body weight (490 or 980 g for a 70-kg person) for 14 d (Navarro, Chang, et al., 2009). In addition, although measurement of GSTA concentration and GST activity in blood reflects normal hepatic cell turnover (Yukihiko et al., 1980) and has the advantage of being relatively noninvasive to human subjects, it may not be sufficiently sensitive to reliably detect low-level GST responses to diet (Lampe, Chen, et al., 2000).

The strengths of this study include the use of controlled feeding, the characterization of the glucosinolate content in kale and daikon radish, the sample collections at multiple time points, and the use of the crossover design. There were also limitations to the study. We did not use fresh kale in this study, a common manner of food preparation for kale. Subjects were not evaluated or selected for genetic polymorphisms, which in future studies, might provide additional insights into the effects of kale consumption on expression of XMEs. Finally, in order to minimize the amount of time subjects would be at the BHNRC, we chose not to conduct the caffeine metabolite assay on day 1 of each period, which also might have provided useful information.

In summary, daily kale consumption increased CYP1A2 activity as determined by caffeine metabolite ratios by 16.4% and 15.2% after one and two weeks of feeding, respectively, and was higher in men than women regardless of diet or day of measurement. Also, daily kale consumption modified bilirubin metabolism such that serum conjugated bilirubin decreased from 19.4% of total bilirubin on day 1 to 14.3% and 9.5% on days 8 and 15, respectively. This result might reflect induction of the drug transporter MRP2, although this has yet to be confirmed. There was no evidence for any influence of the kale diet on CYP2A6, XO, or NAT2 as measured by the caffeine metabolite assay, UGT1A1 as measured by serum bilirubin, or GSTA concentration or plasma GST activity as measured by ELISA. The overall health impacts of these results will become clearer as more understanding is brought to the roles of CYP1A2 and perhaps MRP2 in xenobiotic metabolism.

5. Ethics statement

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by Chesapeake IRB (Columbia, MD, USA). Written informed consent was obtained from all subjects. This trial was registered at clinicaltrials.gov (NCT03449849).

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Declaration of Competing Interest

None.

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