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Cruciferous Vegetable Feeding Alters UGT1A1 Activity: Diet- and Genotype-Dependent Changes in Serum Bilirubin in a Controlled Feeding Trial

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Abstract Chemoprevention by isothiocyanates from cruciferous vegetables occurs partly through up-regulation of phase II conjugating enzymes, such as UDP-glucuronosyltransferases (UGT). UGT1A1 glucuronidates bilirubin, estrogens, and several dietary carcinogens. The *UGT1A1*28* polymorphism reduces transcription compared with the wild-type, resulting in decreased enzyme activity. Isothiocyanates are metabolized by glutathione *S*-transferases (GST); variants may alter isothiocyanate clearance such that response to crucifers may vary by genotype. We evaluated, in a randomized, controlled, crossover feeding trial in humans ($n = 70$), three test diets (single- and double-"dose" cruciferous and cruciferous plus apiaceous) compared with a fruit and vegetable–free basal diet. We measured serum bilirubin concentrations on days 0, 7, 11, and 14 of each 2-week feeding period to monitor UGT1A1 activity and determined effects of *UGT1A1*28* and *GSTM1/GSTT1-null* variants on response. Aggregate bilirubin response to all vegetable-containing diets was statistically significantly lower compared with the basal diet ($P < 0.03$ for all). Within each *UGT1A1* genotype, lower bilirubin concentrations were seen in **1/*1* in both single- and double-dose cruciferous diets compared with basal ($P < 0.03$ for both); **1/*28* in double-dose cruciferous and cruciferous plus apiaceous compared with basal, and cruciferous plus apiaceous compared with single-dose cruciferous ($P < 0.02$ for all); and **28/*28* in all vegetable-containing diets compared with basal ($P < 0.02$ for all). Evaluation of the effects of diet stratified by *GST* genotype revealed some statistically significant genotypic differences; however, the magnitude was similar and not statistically significant between genotypes. These results may have implications for altering carcinogen metabolism through dietary intervention, particularly among *UGT1A1*28/*28* individuals.

Consumption of cruciferous vegetables (from the Brassicaceae plant family) is associated with a reduced risk of several cancers, particularly cancers of the stomach and lung (1–4). Cruciferous vegetables contain high amounts of sulfur-containing compounds called glucosinolates (5), which, on hydrolysis by the enzyme myrosinase, results in the formation of biologically active compounds, such as indoles and isothiocyanates. These compounds are hypothesized to play a role in chemoprevention, in part, through regulation of phase II conjugating enzymes, including UDP-glucuronosyltransferase (UGT; refs. 3, 6–11).

UGTs are a superfamily of enzymes that catalyze the transfer of the glucuronyl group from uridine 5′-disphosphoglucuronic acid to endogenous molecules, as well as exogenous compounds, including therapeutic drugs and dietary carcinogens, to produce less toxic, more polar molecules that are more easily excreted (12). UGT1A1, one of nine enzymes in the UGT1A family, conjugates bilirubin and estrogens (17β-estradiol and estriol), as well as heterocyclic amines and polycyclic aromatic hydrocarbons (13). UGT1A1 is also the only UGT that preferentially conjugates bilirubin (14).

Polymorphisms in the upstream promoter region of *UGT1A1*, characterized by variation in the number of thymine-adenine (TA) repeats, alter *UGT1A1* transcriptional activity (14, 15). Compared with six TA repeats (*UGT1A1*1*) found in the wild-type allele, the presence of seven (*UGT1A1*28*) TA repeats has been shown to down-regulate transcription and is the genetic basis for mild unconjugated hyperbilirubinemia associated with reduced hepatic UGT conjugation of bilirubin (i.e., Gilbert syndrome; ref. 16).

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Glutathione *S*-transferases (GST) are also involved in the detoxification of a broad range of electrophiles by conjugation with glutathione (17). Isothiocyanates in cruciferous vegetables are substrates for GST, particularly GSTM1 (18). Null genotypes for *GSTM1* and *GSTT1* result in the absence of the respective enzymes; thus, among *GSTM1-null* and *GSTT1-null* individuals, isothiocyanates have been hypothesized to be metabolized more slowly, leading to greater accumulation of isothiocyanates at the tissue level and, therefore, possibly a greater chemoprotective effect (19).

Previously, in an observational study, we found a positive association between intake of Cruciferae and UGT1A1 activity among individuals with the **28/*28* variant, but not **1/*28* or **1/*1* genotypes (12). In a subsequent controlled feeding study, a mixed diet of crucifers, soy and citrus (all sources of *UGT* inducers; refs. 7, 8, 20–24), compared with a fruit and vegetable–free diet resulted in increased UGT1A1 activity but only in women with the **28/*28* genotype (25). Our objectives in the present human feeding trial were to examine the effects of cruciferous vegetables specifically, on conjugation of bilirubin, as a measure of UGT1A1 activity and to compare two different amounts to determine if response was dose dependent. In addition, we investigated whether *GSTM1-null* and *GSTT1-null* variants or the *UGT1A1*28* polymorphism affects this response. A secondary aim of the study was to evaluate whether the addition of apiaceous vegetables to cruciferous feeding further alters this response.

Materials and Methods

Research design

The project activities were conducted in the context of an existing study, "Enzyme Activation Trial 2" and carried out at Fred Hutchinson Cancer Research Center (FHCRC; Seattle, WA). The overall goal of the parent study was to evaluate the effect of plant foods from the Brassicaceae and Apiaceae families on activities of phase I (specifically CYP1A2) and phase II (specifically GST) conjugating enzymes and the effect of genetic polymorphisms in biotransformation enzymes on the response to diet. The focus of the work presented here builds on our previous findings of alterations in bilirubin concentrations (used as a surrogate measure of UGT1A1 enzyme activity) in response to cruciferous vegetable feeding and *UGT1A1* genotype (12, 25). Recruitment, enrollment, feeding, sample collection, and laboratory analysis for this project took place from March 2003 to July 2007. The Enzyme Activation Trial 2 study was a randomized, controlled, crossover feeding trial of four diets: basal (devoid of fruit and vegetables), two doses of cruciferous vegetables, and cruciferous plus apiaceous (carrot family) vegetables. Participants were blocked on *GSTM1/GSTT1* genotype by sex and assigned to the four controlled diets in a computer-generated random sequence.

Study participants

Participants were healthy nonsmokers, ages 20 to 40 y, and were recruited based on sex and *CYP1A2, GSTM1,* and *GSTT1* genotypes. Methods of recruitment included paid advertisements in campus newspapers and flyers posted on university campuses in Seattle, informational Web site, and four targeted, bulk mailings using lists obtained from the State of Washington, Department of Licensing. Screening was conducted in two phases. In the first phase, prospective participants were screened for eligibility using a self-administered questionnaire and were excluded if they reported any of the following: medical history of gastrointestinal, hepatic, or renal disorders; pregnancy or lactation; known allergies/intolerances to any foods used in the feeding trial; weight change >4.5 kg within the past year; major changes in eating habits within the past year (e.g., adoption of a faddish diet); antibiotic use within the past 3 mo; body weight >150% of desirable; exercise patterns that require or result in major changes in diet; current use of prescription medication (including oral contraceptives); current use of over-the-counter medications; regular exposure to passive smoke; occupational exposure to smoke or organic solvents; food dislikes that would preclude participation in the feeding trial; alcohol intake of more than two drinks per day (two drinks being equivalent to 720 mL beer, 240 mL wine, or 90 mL spirits); and no interest in participating in a controlled feeding trial.

In the second screening phase, eligible individuals were selected according to the genotypes of interest. The goal of the parent study was to recruit 72 participants according to three groups of *GST* genotypes (*GSTM1+/GSTT1+, GSTM1-null/GSTT1+,* and *GSTM1-null/GSTT1-null*) and three *CYP1A2*(C^{734} *A*) genotypes (*C/C, C/A,* and *A/A*), with a ratio of 2:2:1 and an equal number of men and women in each group. The *CYP1A2*(C^{734} *A*) genotypes were of interest in relation to other objectives, which were to investigate the genotypic effect on induction or inhibition of CYP1A2 activity in response to vegetable supplementation. These findings will be reported elsewhere. In all, we randomized 73 individuals: 14 *GSTM1+/GSTT1+* men, 12 *GSTM1+/GSTT1+* women, 16 *GSTM1-null/GSTT1+* men, 15 *GSTM1-null/GSTT1+* women, 5 *GSTM1-null/GSTT1-null* men, 9 *GSTM1-null/GSTT1-null* women, and 2 *GSTM1+/GSTT1-null* women. The two women with the *GSTM1+/GSTT1-null* genotypes, recruited for their *CYP1A2* genotype, and one woman who completed only the basal diet were not included in this analysis. Twelve participants dropped out of the study: 4 after the first feeding period, 5 after the second, and 3 after the third. Data for all completed diet periods were included in the analysis even if the participants did not complete all four diet periods. Reasons for dropping out included work schedules that prevented travel to the Center, intolerance of study foods, illness, and a move out of the area. As these individuals dropped out, new recruits were selected and randomized into the appropriate treatment orders to maintain the blocks. The Institutional Review Board at the Fred Hutchinson Cancer Research Center approved the study activities and informed written consent was obtained from all participants before the start of the study.

Study diets

We measured usual dietary intake using 3-d records to assess habitual dietary intake at baseline and to determine energy needs. Nutrient data were collected by the staff of the Nutrition Assessment Shared Resource of FHCRC using collection tools they developed. Nutrient calculations were done using the Nutrient Data System for Research software version 4.05-33, developed by the Nutrition Coordinating Center, University of Minnesota (Minneapolis, MN), Food and Nutrient Database. As part of the intervention, participants consumed four different controlled diets: (*a*) a basal diet devoid of fruit and vegetables, (*b*) the basal diet supplemented with a prescribed amount of cruciferous vegetables (single-dose cruciferous diet), (*c*) the basal diet supplemented with twice this amount of cruciferous vegetables (double-dose cruciferous diet), and (*d*) and the basal diet supplemented with cruciferous and apiaceous vegetables (single-dose cruciferous plus apiaceous vegetable diet; Table 1). For a 70-kg individual, the amount of vegetables provided was equivalent to ~5 servings a day for the single-dose cruciferous diet, ~10 servings for the double-dose cruciferous diet, and ~7 for the cruciferous plus apiaceous diet, although the exact amounts were based on a per-kilogram body weight basis. The single dose of cruciferous vegetables was ~7 g/kg body weight, the double dose was ~14 g/kg, and the mixed diet included ~7 g/kg crucifers plus ~4 g/kg apiaceous vegetables. Diets were first standardized according to servings for a 70-kg individual, determining g food/kg body weight for each plant food item. Vegetable amounts were then increased or decreased to the nearest 5 kg increment in body weight for individuals who weighed more or less than

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Table 1. Amounts of vegetables fed on each vegetable-containing test diet calculated for an individual at the reference weight of 70 kg body weight

Dietary constituents	g/70 kg body weight	g/5 kg body weight*
Single-dose cruciferous diet		
Frozen broccoli†	203	15
Frozen cauliflower†	152	11
Fresh red cabbage‡	36	3
Fresh green cabbage‡	36	3
Fresh radish sprouts§	16	1
Double-dose cruciferous diet		
Frozen broccoli	406	29
Frozen cauliflower	305	22
Fresh red cabbage	71	5
Fresh green cabbage	71	5
Fresh radish sprouts	32	2
Cruciferous + apiaceous diet		
Frozen broccoli	203	15
Frozen cauliflower	152	11
Fresh red cabbage	36	3
Fresh green cabbage	36	3
Fresh radish sprouts	16	1
Frozen carrots†	111	8
Fresh parsnips‡	102	8
Fresh celery‡	50	4
Fresh parsley‡	5	0.4
Fresh dill weed‡	0.7	0.05

*Vegetable amounts were increased or decreased to the nearest 5 kg increment in body weight for individuals who weighed more or less than 70 kg. These foods supplemented a basal diet that was otherwise devoid of fruits and vegetables.
†Norpac Foods (Lake Oswego, OR).
‡Food Service of America (Seattle, WA).
§Uwajimaya's (Seattle, WA).

70 kg. We provided mixtures of vegetables within the botanical families rather than a single vegetable representing the family to better capture real-life exposure to a variety of vegetables. Each of the four diet periods lasted 14 d with a 21-d washout period between diet periods. These feeding period lengths were chosen based on our previous study that showed that serum GST-α protein concentrations were still increasing from day 6 to day 7 in response to cruciferous vegetable supplementation (26), despite reports that biotransformation enzymes are rapidly induced (27).

Participants were instructed to consume only the food and beverages provided for them during the diet periods and were requested not to take any type of medication, alcoholic beverages, or nutritional supplements and to maintain their usual physical activity during each feeding period. Dinner was served at the FHCRC Human Nutrition Lab dining room Sunday through Friday evening, and food for the following day's morning, midday meal, and snacks was distributed at that time. On Friday evening, participants picked up food for all of Saturday and for Sunday daytime meals. The major portion of the test vegetables was provided as part of the dinner under the supervision of the study staff. Any food not consumed was noted on the participant's chart. Compliance with the study diet was also monitored using daily food check-off forms with space provided so that participants could record any deviations from the diet.

Specimen collection

Biological samples were collected prospectively from participants and during each 2-wk feeding period. Buccal cells, collected before randomization, were isolated and DNA extracted for determination of *GSTM1* and *GSTT1* genotypes and participant eligibility. Prospective participants were asked to collect their buccal cells by vigorously swishing 10 mL of undiluted, commercial mouthwash in their mouth for 60 s. They then expelled the solution into a collection container, sealed, and returned the sample via U.S. mail to the FHCRC Specimen Processing Laboratory, where DNA was extracted using the QIAamp DNA Blood Midi kit (Qiagen).

At days 0, 7, 11, and 14 of each feeding period, blood was drawn in the morning after a 12-h overnight fast. The multiple draws were selected to allow for monitoring changes in response to diet over the course of each feeding period. Tubes without additive were allowed to clot at room temperature for 30 min before they were centrifuged to separate the serum. Serum was aliquoted and stored at −80°C. On day 0 of the first feeding period, blood was also drawn into a tube containing EDTA for collection of WBC DNA (buffy coat). On day 13 of each feeding period, participants collected urine for 24 h. All urine samples were refrigerated at 4°C until delivery in the morning (day 14) to FHCRC. The total volume and pH value were recorded and the sample was aliquoted and stored at −80°C.

GSTM1 and *GSTT1* genotyping

GSTM1 and *GSTT1* genotyping was conducted on buccal cell DNA using primers described by Arand et al. (28). β-Globin was coamplified to ensure that the *GSTM1-null* and *GSTT1-null* genotypes were due to the deletion of the *GSTM1* or *GSTT1* alleles and not to failure of the PCR. Thermal cycling included an initial denaturation at 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 1 cycle of 72°C for 5 min. Individuals homozygous or heterozygous for *GSTM1* phenotypes gave a 215-bp PCR fragment; *GSTM1-null* individuals did not. A 480-bp fragment was detected for individuals who carried at least one *GSTT1* allele.

UGT1A1 genotyping

The *UGT1A1* polymorphism (*UGT1A1*28*) consists of seven TA repeats in the promoter region, six TA repeats being the wild-type (*UGT1A1*1*). Genotyping of the WBC DNA for the *UGT1A1* polymorphism was conducted as described previously (29).

Bilirubin analysis

Serum total and direct (conjugated) bilirubin were measured using a Cobas MIRA Plus centrifugal analyzer (Roche Diagnostic Systems), as described (29). Indirect (unconjugated) bilirubin is calculated by difference (total bilirubin − direct bilirubin). Mean intraassay and interassay coefficient of variations were, respectively, 2.1% and 3.2% for total bilirubin and 2.6% and 2.9% for direct bilirubin.

Isothiocyanate excretion

Twenty-four–hour urines collected on day 13 were used to measure isothiocyanates for compliance purposes (25). The assay for urinary total isothiocyanates was based on the high-performance liquid chromatography–based method developed by Chung et al. (30) and measures dithiocarbamates (isothiocyanates plus glutathione-derived conjugates) as well as the synthesis of phenethyl isothiocyanates-*N*-acetyl conjugate used for standard calibrations.

Statistical analysis

To make distributions more normal, a natural logarithmic transformation was done on total, direct, and indirect bilirubin concentrations before analysis. A linear mixed model was used, including sex, *UGT1A1*

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genotype, feeding periods, diet treatments, feeding order, sampling day, and interaction terms as fixed effects and participants as a random effect. The observations at day 0 and habitual diet were covariates adjusted in the model. Participants were blocked by *GST* genotype for diet-order randomization. All statistical analyses were done using the Statistical Analysis System Program (version 8.2; SAS Institute). Data are presented as back-transformed least squares (LS)—means ± LS SEs, unless otherwise indicated. Two-sided *P* value for statistical significance was set at <0.05.

To prevent confounding by body weight between the sexes, the amount of vegetables provided was based on a per-kilogram body weight calculation and ranged from 284 to 662 g for the single-dose cruciferous diet, 568 to 1,324 g for the double-dose cruciferous diet, and 458 to 1,065 g for the single-dose plus apiaceous diet. Analyses were conducted both with and without adjusting for vegetable amount; because there were no statistically significant differences between the analyses with and without adjustment, the data are presented without adjustment.

Results

Of the 70 participants in the study, 58 completed all four diet periods, 8 completed two or more, and 4 completed one. The distribution of *UGT1A1* genotypes in the study population was 41% ($n = 29$) and 51% ($n = 36$) for **1/*1* and **1/*28*, respectively, and 7% ($n = 5$) for **28/*28*. There were no statistically significant differences in the demographic and baseline characteristics of the 70 individuals by *UGT1A1* genotype (Table 2). As expected, serum total, direct, and indirect bilirubin concentrations for participants consuming their habitual diets were statistically significantly higher among those with the *UGT1A1*28/*28* genotype ($P < 0.02$ for all three bilirubin measures).

Aggregate (all individuals, irrespective of genotype, and days 7, 11, and 14 combined) LS mean ± LS SE total bilirubin concentrations for individuals on basal, single-dose cruciferous, double-dose cruciferous, and cruciferous plus apiaceous diets were 16.07 ± 1.27, 15.39 ± 1.37, 14.71 ± 1.20, and 14.88 ± 1.20 μmol/L, respectively. Response to all three vegetable-containing diets differed statistically significantly from the basal diet, with lower mean total bilirubin concentrations associated with consumption of the vegetable test diets ($P < 0.02$ for all three), suggesting greater UGT1A1 enzyme activity. Individual diet contrasts showed statistically significantly lower mean bilirubin concentrations on the double-dose cruciferous diet compared with the single-dose cruciferous diet, suggesting a dose response ($P = 0.03$) and a tendency toward lower bilirubin concentrations on the cruciferous plus apiaceous diet compared with the single-dose cruciferous diet ($P = 0.08$). Response to double-dose cruciferous and cruciferous plus apiaceous diets was similar ($P = 0.7$). Results for unconjugated bilirubin concentrations paralleled those of total bilirubin; however, the low values for conjugated bilirubin concentrations did not differ by diet (data not shown). The interaction term of diet by sex was not statistically significant, and although men tended to have higher total bilirubin concentrations on all diets, this also was not significant ($P = 0.75$).

*UGT1A1*28* genotype effects

Overall, total mean bilirubin concentrations differed by *UGT1A1* genotype ($P = 0.049$). Individuals with the **28/*28* variant had ~27% overall higher bilirubin concentrations compared with those with **1/*1* ($P = 0.02$) and 19% higher concentrations compared with those with **1/*28* ($P = 0.07$). The

Table 2. Characteristics of study participants by *UGT1A1* genotype

	*UGT1A1 *1/*1, n* = 29 (41%)	*UGT1A1 *1/*28, n* = 36 (51%)	*UGT1A1 *28/*28, n* = 5 (7%)	*P**
Age (y)	32 ± 7.16[†]	33 ± 5.50	31 ± 6.02	0.74
Height (m)	1.7 ± 0.11	1.7 ± 0.09	1.7 ± 0.14	0.79
Weight (kg)	71.0 ± 15.96	68.4 ± 13.86	72.74 ± 12.37	0.93
Body mass index (kg/m^2)	20.7 ± 3.8	20.1 ± 3.3	24.4 ± 2.4	0.98
Female (%)	45% (13)	55% (20)	60% (3)	0.64
Race (%)				
Caucasian	48% (14)	72% (26)	60% (3)	0.08
Asian	48% (14)	17% (6)	40% (2)	
Other[‡]	0.03% (1)	0.11% (4)	0	
GSTT1/GSTM1 genotype				0.96
(+/+), 46% female	12	12	2	
(–/+), 50% female	11	17	2	
(–/–), 64% female	6	7	1	
Serum bilirubin[§] (μmol/L)				
Total	14.02 ± 4.96	14.02 ± 4.79	25.31 ± 9.75	0.01
Direct	1.88 ± 0.68	1.88 ± 0.51	3.25 ± 1.03	0.01
Indirect	12.14 ± 4.45	12.14 ± 4.45	22.06 ± 8.89	0.02

*Tests for a trend across *UGT1A1* genotypes (χ^2).
[†]Mean ± SD.
[‡]Other includes African-Americans and Pacific Islanders.
[§]When consuming habitual diets.

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Table 3. Serum total bilirubin concentrations in participants stratified by *UGT1A1* genotype, diet period, and day of sampling

	Total bilirubin (μmol/L)			
	Basal	**Single-dose cruciferous**	**Double-dose cruciferous**	**Cruciferous plus apiaceous**
*UGT1A1 *1/*1* (*n* = 29)				
Day 7	14.88 ± 1.54*[a]	13.85 ± 1.37[a]	14.88 ± 1.37[a]	15.05 ± 1.37[a]
Day 11	15.05 ± 1.54[a]	14.19 ± 1.37[ab]	13.34 ± 1.20[b]	14.02 ± 1.37[ab]
Day 14	15.73 ± 1.54[a]	14.36 ± 1.37[b]	14.02 ± 1.37[b]	14.02 ± 1.37[b]
Mean†	15.22 ± 1.37[a]	14.19 ± 1.37[b]	14.19 ± 1.20[b]	14.36 ± 1.37[ab]
*UGT1A1 *1/*28* (*n* = 36)				
Day 7	16.59 ± 1.54[a]	16.59 ± 1.54[a]	15.90 ± 1.54[a]	15.73 ± 1.54[a]
Day 11	15.90 ± 1.54[a]	15.56 ± 1.54[ab]	14.36 ± 1.54[bc]	14.19 ± 1.37[c]
Day 14	15.90 ± 1.54[a]	15.56 ± 1.54[ab]	15.05 ± 1.37[ab]	14.31 ± 1.37[b]
Mean	16.07 ± 1.54[a]	15.90 ± 1.54[ac]	15.05 ± 1.37[bc]	14.71 ± 1.37[b]
*UGT1A1 *28/*28* (*n* = 5)				
Day 7	21.72 ± 3.25[a]	19.32 ± 2.91[ab]	16.59 ± 2.57[b]	18.13 ± 2.74[ab]
Day 11	20.35 ± 3.08[a]	18.81 ± 2.74[ab]	17.78 ± 2.74[ab]	15.39 ± 2.22[b]
Day 14	22.06 ± 3.25[a]	16.07 ± 2.39[b]	17.44 ± 2.74[ab]	17.27 ± 2.57[b]
Mean	21.38 ± 2.91[a]	17.96 ± 2.39[b]	17.27 ± 2.39[b]	16.93 ± 2.39[b]

NOTE: a, b, and c: diet means within a row not sharing a common superscript are significantly different as tested by ratio of means ($P < 0.05$).

*LS back-transformed means ± LS SE, adjusted for sex, baseline, and day 0 bilirubin concentrations.

†Mean of three sampling days (7, 11, and 14).

difference between individuals with **1/*1* and **28/*28* was particularly apparent on the basal and single-dose cruciferous vegetable diets, where overall difference in values was 40% and 27% higher, respectively ($P < 0.01$ for basal and 0.03 for single-dose cruciferous; Table 3). Although this difference was still evident within the double-dose cruciferous (22%) and cruciferous plus apiaceous diets (18%), it was no longer statistically significant ($P = 0.07$ and 0.15, respectively). The differences between **1/*1* and **1/*28* were smaller and statistically significant only on the single-dose cruciferous diet, with **1/*28* having 10% higher bilirubin concentrations than **1/*1* ($P = 0.02$).

Response to diet also tended to differ by genotype, although the formal interaction term of diet by *UGT* genotype on total bilirubin concentrations was not statistically significant ($P = 0.13$). Within **28/*28* individuals, serum bilirubin concentrations were 16% to 21% lower on all three vegetable-supplemented diets than on the basal diet ($P < 0.02$ for all three). In contrast, within **1/*1* individuals, bilirubin concentrations were only ~7% lower for both single- and double-dose cruciferous diets compared with the basal diet ($P < 0.03$ for both) and there was no effect of the cruciferous plus apiaceous diet compared with basal. Within **1/*28* individuals, bilirubin concentrations were ~6% lower for double-dose cruciferous diet ($P = 0.02$) and 8% lower for the cruciferous plus apiaceous diet ($P = 0.002$). There was no effect of the single-dose cruciferous diet within **1/*28*. When comparing differences in response to diets between groups, **28/*28* individuals responded marginally greater to the single-dose cruciferous diet compared with **1/*28* individuals ($P = 0.049$) and statistically significantly greater to the cruciferous plus apiaceous diet ($P = 0.03$).

GSTM1/GSTT1 genotype effects

GST enzymes metabolize isothiocyanates. Because variation in GST activity may influence isothiocyanate exposure, we also tested the effect of *GSTM1* and *GSTT1* genotypes on response to diet. There was not a statistically significant main effect of the combined *GSTM1* and *GSTT1* genotypes ($P = 0.48$) or the interaction of genotype by diet ($P = 0.54$). Although evaluation of the effects of diet stratified by genotype revealed some statistically significant genotypic differences, the magnitude was similar and not statistically significant between genotypes (Table 4). For example, individuals who were *GSTM1-null/GSTT1+* had ~7% lower bilirubin concentrations on both the single- and double-dose cruciferous vegetable diets compared with the basal diet ($P < 0.01$ for both) and a 10% decrease on the cruciferous plus apiaceous vegetable diet ($P < 0.01$). *GSTM1-null/GSTT1-null* individuals had 5% lower bilirubin concentrations on the single-dose cruciferous diet ($P = 0.19$), a 12% decrease on the double-dose cruciferous diet ($P = 0.01$), and a 10% decrease on the cruciferous plus apiaceous vegetable diet ($P = 0.07$) compared with the basal diet. *GSTM1+/GSTT1+* individuals did not respond to the single-dose cruciferous vegetable diet but had ~7% lower bilirubin concentrations on the double-dose cruciferous and cruciferous plus apiaceous diets ($P = 0.01$ and 0.02, respectively).

Twenty-four–hour urinary analysis of isothiocyanate metabolite excretion, collected on day 13, was measured for compliance. Mean ± SD total isothiocyanate concentrations for the basal, single-dose cruciferous, double-dose cruciferous, and cruciferous plus apiaceous diets were 2.14 ± 2.53, 112.51 ± 63.08, 257.76 ± 172.17, and 105.90 ± 66.26 μmol/24 hours,

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respectively, indicating a steep and dose-dependent increase in isothiocyanate excretion over the basal diet period. Ninety percent of the study vegetables were consumed on the single-dose cruciferous vegetable diet, 87% on the double-dose cruciferous diet, and 89% on the cruciferous plus apiaceous diet. Based on the daily food check-off forms, participants consumed nonstudy food items <3% of the study days.

To evaluate the effect of the length of intervention on serum bilirubin, we measured serum bilirubin on days 0, 7, 11, and 14 of each feeding period. There was a statistically significant effect of day of sampling ($P < 0.001$), but the interaction of day by diet was not statistically significant ($P = 0.28$). With vegetable supplementation, serum bilirubin concentrations were lower on days 11 and 14 compared with day 7 ($P < 0.01$) but bilirubin concentrations increased slightly from days 11 to 14 ($P = 0.04$).

Because participants were selected to enrich for the *CYP1A2*1F* genotype in the parent study, analyses were carried out to evaluate whether this genotype was associated with changes in bilirubin. The relationship between *CYP1A2*1F* and bilirubin was not statistically significant (data not shown).

Discussion

In this study, serum bilirubin concentrations decreased in response to all three vegetable-containing diets compared with the fruit and vegetable–free basal diet, underscoring the capacity of cruciferous vegetable consumption to modulate UGT1A1 enzyme activity *in vivo*. This response was particularly prominent among individuals with the **28/*28* genotype. Mean total bilirubin concentrations for the single-dose cruciferous diet fell between the values for the basal diet and the double-dose cruciferous vegetable diet, suggesting a dose response.

Our results support the hypothesis that feeding cruciferous vegetables increases UGT1A1 activity. A variety of dietary components, including isothiocyanates, have been shown to induce UGT activity, in general, in animals (21, 31) and humans (32, 33); however, the substrates used to test for UGT activity have not been specific for UGT1A1. Recently, Gasper and colleagues (34) examined the expression of xenobiotic metabolizing enzymes in gastric mucosa in four individuals 6 hours after consumption of a single meal of 100 g of broccoli or high-glucosinolate broccoli. Induction of several metabolizing enzymes was observed with high-glucosinolate broccoli, but not the standard broccoli meal, and UGT was not among the enzymes that were induced. Most *in vitro* evidence suggests that up-regulation of phase II enzymes by isothiocyanates occurs through interaction of the isothiocyanates with the cytoplasmic-anchoring protein Kelch-like ECH-associated protein 1 and the nuclear transcription factor erythroid-derived 2–like 2 (official nomenclature for the more commonly used Nrf2) via the antioxidant response element (3, 6–11). *UGT1A1* has an antioxidant response element (9) as well as a xenobiotic response element (XRE) in the promoter region (35–37). Thus, the indoles in cruciferous vegetables, which induce phase I and II enzymes through binding to the aryl hydrocarbon receptor and interacting with the XRE, may also induce *UGT1A1* expression. Cruciferous vegetables contain a mixture of isothiocyanates and indoles; thus, it is possible that both contributed to increased UGT1A1 enzyme activity and lower serum bilirubin in our study.

The combination of single-dose cruciferous and apiaceous vegetables resulted in a reduction in bilirubin concentrations similar to that seen with the double-dose cruciferous vegetable diet. This suggests that constituents in apiaceous vegetables may also increase UGT1A1 activity. Apiaceous vegetables (i.e., carrots, dill, parsley, parsnips, and celery in our study) are rich sources of furanocoumarins (e.g., psoralens and methoxypsoralen; ref. 38). Effects of these specific apiaceous vegetable constituents on *UGT1A1* induction have not been evaluated; however, Diawara et al. (39) showed that psoralens induced mRNAs of hepatic enzymes that are typically induced by compounds that interact with the aryl hydrocarbon receptor and, subsequently, the XRE (e.g., indoles). In addition to furanocoumarins, some plants in the Apiaceae family contain the flavonoid apigenin. Apigenin has been shown to induce *UGT1A1* transcription *in vitro* (8, 40). These data support our current findings and offer a plausible explanation for our results. Finally, other enhancer element motifs have been identified in *UGT1A1*, including a PXR response element (41). Ruhl et al. (42) showed PXR-mediated induction activity in PXR-responsive *CYP450* genes by β-carotene in HepG2 cells. As carrots were included in our apiaceous diet protocol, we cannot rule out the possibility of modulation of *UGT1A1* by β-carotene as an additional factor.

The presence of an additional TA repeat in the *UGT1A1* promoter region has been shown to reduce transcription of this gene by as much as 50% among homozygous variants (14, 15). In our study, as expected, a statistically significant phenotypic difference was observed between **1/*1* and

Table 4. Mean serum total bilirubin concentrations in participants stratified by *GSTM1/T1* genotype and diet period

	Total bilirubin (μmol/L)			
	Basal	**Single-dose cruciferous**	**Double-dose cruciferous**	**Cruciferous plus apiaceous**
GSTM1+/GSTT1+ ($n = 26$)	15.39 ± 1.37*[ac]	15.05 ± 1.37[ac]	14.19 ± 1.20[b]	14.36 ± 1.20[bc]
GSTM1-null/GSTT1+ ($n = 30$)	15.90 ± 1.54[a]	14.88 ± 1.37[b]	14.88 ± 1.37[b]	14.36 ± 1.37[b]
GSTM1-null/GSTT1-null ($n = 14$)	17.10 ± 1.71[a]	16.07 ± 1.54[ab]	14.88 ± 1.54[b]	15.56 ± 1.54[ab]

NOTE: Means not sharing common superscript within a genotype are significantly different as tested by ratio of means ($P < 0.05$).
*Back-transformed LS means ± LS SE, adjusted for sex, diet order, and habitual and day 0 bilirubin concentrations.

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**28/*28 UGT1A1* genotypes, with **28/*28* individuals having ~27% overall higher total bilirubin concentrations than **1/*1* individuals. Moreover, there were differences in diet response among the *UGT1A1* genotypes: among **1/*1* and **1/*28* individuals, there was a modest reduction (1-8%) in bilirubin in response to the single- and double-dose cruciferous diets, whereas statistically significant decreases in bilirubin concentrations were achieved on both the single- and double-dose cruciferous vegetable diets among **28/*28* individuals, and the magnitude of change was much larger (16-21%). These results, along with our previous findings in an observational study and controlled fruit and vegetable feeding trial (12, 25), suggest that there may be a greater capacity for up-regulation of *UGT1A1* among individuals with the **28/*28* genotype.

In this study, decreases in serum bilirubin were observed in response to cruciferous vegetable feeding in both men and women. In contrast, in our previous controlled feeding trial, which included a mixed diet of crucifers, soy and citrus, the intervention effects on UGT1A1 activity were limited to women within the **28/*28* genotype (25). Several differences between the two studies may contribute to these observations. The dose of crucifers in the previous study was 2.7 g/kg body weight compared with 7 and 14 g/kg provided in the present study. Additionally, we used a mix of foods from several botanical families in the previous study (i.e., crucifers, citrus, and soy), whereas the focus was on crucifers in the present study. It may be that the higher isothiocyanate doses are needed to elicit an effect in men and in individuals without the **28/*28* genotype or that the inclusion of other phytochemicals attenuated the response to crucifers.

Given that isothiocyanates are substrates for GST (18) and also induce UGT and other enzyme systems, we hypothesized that the greatest difference in serum bilirubin concentrations in response to diet would occur in *GSTM1-null/GSTT1-null* individuals followed by *GSTM1-null/GSTT1+* and *GSTM1+/GSTT1+*. Compared with *GSTM1+/GSTT1+* individuals, *GSTM1-null/GSTT1-null* individuals had slightly greater decreases in bilirubin with cruciferous vegetable supplementation; but this response was not statistically significant, and slight decreases in bilirubin concentrations were observed in *GSTM1-null* genotypes, regardless of *GSTT1* genotype. Few studies have evaluated the capacity of *GST* genotype to modulate the effect of cruciferous vegetable intake on biomarkers in intervention trials. We showed previously that the *GSTM1-null*, compared with *GSTM1+*, genotype resulted in greater increases in serum GST-α concentrations in response to a 7-day cruciferous vegetable feeding intervention (26). In both of these studies, the circulating biomarkers measured reflect hepatic enzyme activity. In contrast, Traka et al. (43) recently reported *GSTM1* genotype-related changes in transforming growth factor-β1 and epidermal growth factor signaling pathways in prostate tissue after men consumed 400 g broccoli per week for 6 months. *GSTM1+* individuals showed greater diet-induced changes in prostate tissue gene expression. To further characterize the modifying effects of *GST* genotypes, future cruciferous vegetable interventions should be designed to include testing of the effects of these genotypes on biological responses.

A strength of our study is the controlled feeding study design with the focus on a botanical family of vegetables tested in two different doses. Additional strengths include the 2-week duration of each study diet, the blood sampling at three time points during each feeding period, and dosing based on participant body weight. Overall, total bilirubin concentrations decreased until day 11 and then increased slightly from day 11 to day 14, suggesting that we achieved the maximal reduction in bilirubin with our vegetable treatments. Further, the stringent inclusion criteria minimized potential confounding due to other factors that may influence UGT activity (e.g., age, body mass index, and alcohol, tobacco, or medication use).

A primary limitation of the study is our use of serum bilirubin concentrations as an indirect measure of UGT1A1 activity. The actual change in hepatic enzyme activity in response to vegetable feeding may be greater than what we are able to measure indirectly using circulating bilirubin. Another limitation was that this was an ancillary study. Consequently, the number of individuals with the **28/*28* genotype was small and the study was not statistically powered to examine a priori effects of cruciferous vegetable consumption on UGT1A1 enzyme activity. Nonetheless, within the context of the parent study, we estimated that we would need a sample size of five individuals with the *UGT1A1*28* genotype to detect, with 80% power, a 15% difference in total bilirubin concentrations from the basal diet. Although we achieved this sample size, the small number of individuals with the **28/*28* genotype did not allow for formal statistical evaluation of the diet-by-sex-by-genotype interactions. Another potential limitation is generalizability. The average intake of cruciferous vegetables in the United States is about 25 to 30 g/d (2). Although the cruciferous vegetables used in our study (i.e., broccoli, cauliflower, and cabbage) are some of the most commonly consumed vegetables in the U.S. diet, they are not usually consumed in the relatively large amounts we gave in this study (e.g., 5-10 servings per day or ~300-1,300 g.) Additionally, whereas radishes are commonly consumed, radish sprouts, part of our diet protocol, are not.

In summary, cruciferous vegetable supplementation lowered bilirubin concentrations in a dose-dependant manner. Although *UGT1A1* activity, as measured by serum bilirubin, was greater in *GSTM1-null* individuals compared with *GSTM1+* individuals, this response was not statistically significant. Differences in bilirubin concentrations occurred with a lower cruciferous dose and to a greater extent within **28/*28* individuals compared with those with one or more **1* allele. Given the reduced transcriptional activity of *UGT1A1* in individuals with seven or more TA repeats (i.e., **28;* ref. 16) and the role of this enzyme in the conjugation of estrogens and carcinogens, such as heterocyclic amine metabolites (12, 44), improving glucuronidation may be beneficial in this subset of individuals. Finally, our results in humans agree with the *in vitro* and experimental animal model data that suggest that constituents of apiaceous vegetables may also modulate UGT1A1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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