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*UGT1A6 and UGT2B15* polymorphisms and acetaminophen conjugation in response to a randomized, controlled diet of select fruits and vegetables†

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Running title: UGT polymorphisms and acetaminophen conjugation

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Abbreviations:
- APAP, acetaminophen; APAPG, acetaminophen glucuronide; APAPS, acetaminophen sulfate;
- APAPG ratio, APAPG/(APAP+APAPG+APAPS); BMI, body mass index; F&V, fruit and vegetable supplemented diet
ABSTRACT

Acetaminophen (APAP) glucuronidation is thought to occur mainly by UDP-glucuronosyltransferases (UGT) in the UGT1A family. Interindividual variation in APAP glucuronidation is attributed, partly, to polymorphisms in UGT1A. However, evidence suggests that UGT2B15 may also be important. We evaluated, in a controlled feeding trial, whether APAP conjugation differed by UGT1A6 and UGT2B15 genotypes, and whether supplementation of known dietary inducers of UGT (crucifers, soy, citrus), modulated APAP glucuronidation compared to a diet devoid of fruits and vegetables (F&V). Healthy adults (n=66) received 1000 mg APAP orally on days 7 and 14 of each two-week feeding period, and collected saliva and urine over 12 h. Urinary recovery of % APAP dose as free APAP was higher (P=0.02), and %APAP-glucuronide (APAPG) lower (P=0.004) in women. %APAP was higher among UGT1A6*1/*1 genotypes, relative to *1/*2 and *2/*2 (P=0.045). For UGT2B15, %APAPG decreased (P<0.0001) and %APAP-sulfate increased (P=0.002) in an allelic dose-dependent manner across genotypes from *1/*1 to *2/*2. There was a significant diet-by-UGT2B15-genotype interaction for APAPG-ratio (APAPG/total metabolites*100) (P=0.03), with *1/*1s having an approximately 2-fold higher F&V-to-basal-diet difference in response compared to *1/*2s and *2/*2s. Salivary APAP Cmax was significantly higher in women (P=0.0003), with F&V (P=0.003), and among UGT1A6*2/*2s and UGT2B15*1/*2s (P=0.02 and 0.002, respectively). APAP half-life was longer in UGT2B15*2/*2s with F&V (P=0.009). APAP glucuronidation was significantly influenced by UGT2B15*2 polymorphism, supporting a role in vivo for UGT2B15 in APAP glucuronidation, whereas the contribution of UGT1A6*2 was modest. Selected F&V known to affect UGT activity led to greater glucuronidation and less sulfation.
INTRODUCTION

Acetaminophen (APAP; paracetamol) is extensively conjugated with glucuronic acid and sulfate prior to renal excretion. APAP glucuronidation is thought to occur mainly by UDP-glucuronosyltransferases (UGT) in the UGT 1A family, especially UGTs 1A1, 1A6, and 1A9 in the liver (Court, Duan et al. 2001) and 1A10 in the gut (Tukey and Strassburg 2001). However, despite the lack of 1A activity in the Gunn rat, residual APAP glucuronidation occurs in the hepatocytes from these animals, suggesting that there is APAP-glucuronidating capacity among UGT2B enzymes also (Kessler, Kessler et al. 2002). UGT2B7 and 2B15 have been shown to have some APAP-conjugating activity (Court, Duan et al. 2001; Kostrubsky, Sinclair et al. 2005), and additional data suggest that UGT2B15 may be a more important player in APAP conjugation than previously thought (Mutlib, Goosen et al. 2006).

The reported human interindividual variation in APAP glucuronidation is attributed, in part, to polymorphisms in several UGT1As, although the studies to date are not conclusive. The UGT1A1*28 polymorphism is the genetic basis for benign unconjugated hyperbilirubinemia associated with reduced hepatic UGT conjugation of bilirubin (Gilbert syndrome) (Burchell 2003). Individuals with Gilbert syndrome are reported to have altered APAP metabolism in some studies (De Morais, Uetrecht et al. 1992; Esteban and Perez-Mateo 1993), but not others (Ullrich, Sieg et al. 1987). Rauchschwalbe, et al (Rauchschwalbe, Zuhlsdorf et al. 2004) also reported no difference in urinary APAP/APAP-glucuronide ratios by UGT1A1*28 genotype, but suggested that other polymorphisms in linkage disequilibrium with this variant allele (e.g., UGT1A6*2) may be important. Liver tissue samples homozygous for UGT1A6*2 – characterized by amino acid substitutions T181A, R184S, and S7A – have exhibited a higher rate
of α-naphthol and ρ-nitrophenol glucuronidation relative to tissue samples with the other genotypes, whereas recombinant coexpressed UGT1A6*1/*2 allozymes were associated with the lowest enzyme activity (Nagar, Zalatoris et al. 2004). These findings are limited though, as ρ-nitrophenol is fairly nonselective for UGT1A6, and results were not replicated in another study, either with ρ-nitrophenol or serotonin, a probe specific for UGT1A6 (Krishnaswamy, Hao et al. 2005a). However, the same group subsequently reported greater glucuronidation of acetaminophen by human liver microsomes expressing UGT1A6*2/*2 compared to *1/*1 (Krishnaswamy, Hao et al. 2005b). The UGT2B15 Asp85Tyr (UGT2B15*1/*2) polymorphism has also been shown to affect conjugation of several drugs, including that of S-oxazepam and lorazepam (Court, Duan et al. 2002; Chung, Cho et al. 2005), but the effect on APAP has not been evaluated.

Interindividual variation in UGT activity may also derive from differences in dietary exposures. Bioactive food components in plants (e.g., phytochemicals such as isoflavones, flavonoids, coumarins, monoterpenes, allyl sulfides, isothiocyanates, and those found in green-tea extract) increase hepatic UGT activity in rodents (reviewed in Saracino and Lampe 2007) and several lines of evidence support an effect of diet on UGT activity in humans (Pantuck, Pantuck et al. 1984; Navarro, Peterson et al. 2009). Thus, both dietary habits and genetic polymorphisms in UGTs may affect the toxicity of xenobiotics and the potency of drugs and steroid hormones by altering the amounts and activities of UGTs in the liver and other tissues. To date, the combined effects of certain exposures (e.g., chemopreventive agents or diet) and UGT polymorphisms on glucuronidation have received little attention; however, several studies suggest that these interactions may play a role in disease risk and treatment response (Harvard 1973; Bigler, Whitton et al. 2001; Nowell, Ahn et al. 2005).
The aims of this study were to test: 1) whether APAP conjugation differed by UGT1A6 and UGT2B15 genotypes; 2) whether supplementation of a controlled diet with specific fruits and vegetables modulated APAP glucuronidation in humans; and 3) whether the response of APAP glucuronidation to fruit and vegetable supplementation differed by these UGT genotypes. To minimize interindividual variation, we carried out these aims in the context of a randomized, controlled, crossover feeding trial in healthy men and women and monitored UGT activities by measuring salivary acetaminophen pharmacokinetics and urinary glucuronyl- and sulfo-conjugates of APAP.
METHODS

Participants

Healthy, non-smoking men and women, aged 20 – 40 years, were recruited for the feeding study as described previously (Chang, Bigler et al. 2007). Exclusion criteria were: medical history of gastrointestinal, hepatic, or renal disorders; current or planned pregnancy; lactation; weight loss or gain greater than 4.5 kg within the past 2 months; major changes in eating habits within the past year; antibiotic use within the past 3 months; body mass index (BMI, kg/m²) >30 or <18; exercise regimens that require or result in significant short-term dietary changes; current use of prescription or over-the-counter medications (including oral contraceptives); known allergies to acetaminophen, aspirin, and any foods used in the feeding trial; 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 greater than 2 drinks/day (720 ml beer, 240 ml wine, or 90 ml hard liquor); no interest in participating in a controlled feeding trial.

Prospective participants were genotyped for *UGT1A1*28, *UGT1A6*2, and *UGT2B15*2 and those with desired UGT genotypes and normal serum alanine aminotransferase concentrations (5-42 U/L) were invited to participate in the feeding study. The Institutional Review Board at the Fred Hutchinson Cancer Research Center (FHCRC) approved the study and informed, written consent was obtained from all participants.

Study design

The feeding study was conducted between April 2002 and May 2005. We recruited participants so as to maintain ratios of *I/*I, *I/*2 or *I/*3, and *2/*2 of *UGT1A6* as 2:2:1, and the ratio of
*1/*1, *1/*2, and *2/*2 of UGT2B15 as 1:2:1. Participants were randomized, blocked on sex and UGT1A1 and UGT1A6 genotype, using a crossover study design, with each participant receiving two experimental diets – a basal diet and a basal diet supplemented with fruits and vegetables (F&V) – in an assigned, random order.

The basal, low-phytochemical diet was devoid of fruits, vegetables, whole grains, and herbs and spices. The F&V diet included components of the basal diet supplemented with cruciferous vegetables (broccoli, cabbage, daikon radish sprouts), soy foods (soy milk, veggies slices, tofu, roasted soy nuts), and citrus fruits (grapefruit and orange juices, orange/grapefruit segments, dried orange peel). We chose plant foods that were shown in previous animal and human studies to induce UGTs (Saracino and Lampe 2007). The amount of fruits and vegetables provided to the participants was at levels equivalent to approximately 10 servings daily; however, we dosed on the basis of participant’s body weight (i.e., per 5 kg body weight – BW); details are provided elsewhere (Chang, Bigler et al. 2007). The amount of foods in the basal diet was also adjusted in order to accommodate the added fruits and vegetables, such that both diets provided the same amount of energy and a similar percent of energy from carbohydrate (56%), protein (16%), and fat (28%). Each diet was consumed for 14 days with at least a two-week wash-out period between the diet periods. Participants were instructed to consume only the food and beverages provided to them during both diet periods, maintain their usual physical activity, and not use any type of medication. Dinner was served at the FHCRC Prevention Center Human Nutrition Lab dining room under staff supervision, and food for the following day’s morning, midday meals and snacks was distributed at that time. Based on 24-h urinary analysis of total ITC and isoflavone excretion, and daily food check-off forms, participant compliance to the study diet
was excellent with consumption of non-study food items on fewer than 1% of the study days (Chang, Bigler et al. 2007).

Several studies suggest that drug glucuronidation varies over the menstrual cycle (Cordaro, Morse et al. 1993; Tanaka 1999). Although women did not all start the study at the same time in their menstrual cycles, the study was designed to facilitate collecting samples during each feeding period at approximately the same point in the cycle; i.e., we used a 14-day study period followed by a 14-day washout, such that the second feeding period started after 28 days. Women were also asked to keep menstrual cycle diaries; these data were used to determine whether sample collection during the two treatments differed by phase of menstrual cycle.

**Acetaminophen test**

On the evenings of Day 7 and Day 14 of each feeding period, participants completed the APAP test, which consisted of consuming 1000 mg acetaminophen (two 500 mg Tylenol caplets; McNeil-PPC, Inc., Fort Washington, PA), collecting all urine for the subsequent 12 h and collecting saliva samples at prescribed times. On the days of the APAP test, participants were instructed to consume their dinner meal no later than 1700 h and not to eat or drink any study food, except water, until 3 hours after taking the APAP. At approximately 1900 h (or at least 2 h after the dinner meal), participants collected their first saliva sample and emptied their bladders. Then they took the APAP and collected all urine for the next 12 h and collected saliva over the next 4 h (every 15 minutes in Hour 1; every 30 minutes in Hour 2 and 3, and at the end of Hour 4) and at Hour 12. Participants recorded the test activities (e.g., times they stopped eating and drinking, took the APAP, collected saliva, and the last urine void) and reported any deviations
from the protocol. Urine and saliva samples were stored at 4°C until delivery the following morning. In the lab, total urine volume and pH were measured, and the sample was aliquotted into cryovials and stored at -80°C. Saliva samples were centrifuged and filtered, and stored at -80°C.

**Salivary acetaminophen pharmacokinetics**

Salivary APAP was measured on the Cobas Mira Analyzer (Roche Diagnostics, Indianapolis, IN) using Stanbio Acetaminophen LiquiColor reagent kit (Stanbio Laboratory, Boerne, TX). The assay was standardized using APAP standard supplied with the reagents. Prior to analysis, the frozen saliva was thawed, mixed well and filtered through a 0.45 micron filter to remove particulates. The intra-assay CV was measured at 3 different concentrations of salivary APAP and was 15.9%, 5.4% and 4.3% at concentrations of 3.3, 10 and 16µg/ml respectively. The inter-assay CV was 6% at 15.7µg/ml. Recovery of APAP added to filtered saliva was 98%, 100% and 110% at concentrations of 100µg/ml, 30µg/ml and 10µg/ml respectively. Linearity of APAP spiked into saliva was verified over a range of 2 to 100µg/ml ($r^2 = 0.99997$). Aqueous standards were linear over a range of 2 to 300µg/ml ($r^2 = 0.9999$). A subset of saliva samples were also run by HPLC with excellent correlation between assay methods ($r=0.988$; data not shown).

The pharmacokinetic parameters of salivary APAP were determined using STATA software (v7.0, Stata Corporation, TX). The parameters calculated included maximum concentration (Cmax; mg/l), time of maximum concentration (Tmax, min), elimination rate, half life (min), the standard area under the curve from 0 to the maximum observed time (AUC$_{0-Tmax}$; mg•min/l).
extension of the standard time-versus-concentration curve from the maximum observed time to infinity using linear regression of log concentration (AUC\(_{0-\infty}\); mg•min/l), and clearance (l/h).

The fitpoints used in the model to calculate the AUC\(_{0-\infty}\) were individually determined. For participants with two peaks in the pharmacokinetic plot, the later peak was chosen to determine the fitpoint. The data handling strategy during modeling included the following: missing time points were deleted; if the last time point collected was higher than the previous one, the higher data point was deleted; data values that were zero were deleted; if the last 4 data points leveled off, the last three time points were deleted; if the last time point was ≤ the baseline value, it was deleted as the modeling would produce a negative value; if the baseline value was larger than mean values + 2 SD, the value was replaced with that of the last collection time point.

**HPLC analysis of acetaminophen in urine**

Urine samples were analyzed for APAP, acetaminophen-glucuronide (APAPG), and acetaminophen-sulfate (APAPS) by HPLC using an Agilent Model 1100 series HPLC with an Agilent Zorbax Extend C18 column, 3.5um, 4.6 x 100mm. (Agilent Technologies, Wilmington, DE). The mobile phase was 5% acetonitrile with 0.35%TFA (solvent A) and 95% 0.05M sodium acetate buffer (solvent B). An isocratic elution at a flow rate of 0.8 ml per minute was used with a column temperature of 30\(^\circ\) C and a run time of 15 min per 20µl injection. The signal was recorded at 250nm. Urine samples were thawed, centrifuged and diluted with water prior to analysis. The dilution depended on the original volume of the collected urine and ranged from a 1:5 to 1:40 dilution. 1,7 dimethyl xanthine at a concentration of 100µg/ml was added as an internal standard to each sample and to the standards. Diluted urine samples were run in
duplicate and a standard curve was run with each set of samples. APAP and APAPG were obtained from Sigma-Aldrich Inc and APAPS was a gift from McNeil Consumer Healthcare (Fort Washington, PA). Quality-control urines were run at the beginning and end of each run. On-column limits of quantification (LOQ) for the various analytes were as follows: APAP 1 μg/ml; APAPS 5 μg/ml; APAPG 20 μg/ml. Results in the diluted sample that fell below this limit were assigned a concentration half way between zero and the LOQ. Intra-assay CVs were 0.6, 1.7 and 1.0% for APAP, APAPS and APAPG respectively. Inter-assay CVs were 8.9, 10.1 and 7.8% for APAP, APAPS and APAPG respectively at concentrations of 2.14 μg/ml, 27.5 μg/ml, and 50.8 μg/ml.

Measurement of urinary isoflavones and isothiocyanates

Twenty-four hour urines collected on Day 14 were analyzed for isoflavones (genistein, daidzein, equol, and O-desmethylandangolensin) and total isothiocyanates (ITC) as dietary compliance markers of soy-food and cruciferous-vegetable intake, respectively (Lampe, Skor et al. 2001; Chang, Bigler et al. 2007). Intra- and inter-assay CVs were <10% for all analytes.

Determination of UGT1A1, UGT1A6, and UGT2B15 Genotypes

The UGT1A1*28 polymorphism consists of 5-8 TA repeats in the promoter region, 6 TA repeats being the wildtype; the others being variants. Genotyping of the UGT1A1 polymorphism (rs8175347) was completed as described previously (Lampe, Bigler et al. 1999) with the exception of the use of a fluorescently tagged forward primer and analysis of amplified fragments on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The two UGT1A6 polymorphisms are missense mutations in exon I, causing a Thr to Ala substitution at
amino acid 181 (T181A; rs2070959) and an Arg to Ser substitution at amino acid 184 (R184S; rs1105879) (Ciotti, Marrone et al. 1997; Lampe, Bigler et al. 1999). They were determined by sequencing, using PCR conditions described previously (Lampe, Bigler et al. 1999) and BigDye version 3.2 on an ABI 3100 genetic analyzer. The UGT2B15 polymorphism (rs1902023) consists of a G-to-T point mutation, causing an Asp (*1) to Tyr (*2) substitution at amino acid 85. The polymorphism was determined by sequencing, using PCR conditions described previously (Lampe, Bigler et al. 2000).

**Statistical Analysis**

Based on the available data at the time we designed this study, our *a priori* hypothesis was that UGT1A6 genotypic differences would be most relevant to APAP metabolism. Thus, our primary analyses are focused on UGT1A6 genotypes. However, additional data later suggested an important role of UGT2B15 (Mutlib, Goosen et al. 2006), thus, we also present the analyses in the context of the 2B15 genotypes, as we had also recruited on this basis. We evaluated the distribution of demographic variables such as age, height, weight, and BMI by UGT1A6 genotype and sex. Using ANOVA, we tested whether there were any differences in these demographic variables, dietary intakes, and urinary dietary biomarkers among the three UGT1A6 genotypes after adjusting for sex. Applying a linear mixed model, we tested the effect of diet, UGT1A6 genotype, sex, and the interaction between diet and UGT genotype on APAP pharmacokinetic parameters and urinary APAP metabolites. We adjusted for diet order in the model and accounted for the correlation of APAP metabolite outcomes between the two feeding periods within participants. UGT2B15 genotypes were evaluated separately using the same model. To determine the effect of phase of menstrual cycle on response to diet, the differences
in APAP variables between the F&V diet vs. basal diet were calculated and were compared between those women who were in the same phase of cycle vs. those who were in different phases when APAP metabolism was measured. Statistical analyses were conducted using SAS (V8.2; SAS Institute) and two-sided $P$-value for significance was set at <0.05.
RESULTS

A total of 72 individuals were randomized into the study. Of these, five individuals withdrew from the study within the first five days of the initial diet, two withdrew after the initial diet period, one withdrew during the second diet period, and one was noncompliant. A total of 66 participants were included in the analysis including partial data for the three individuals among the eight described above who completed only the first diet period. Characteristics of study participants by UGT1A6 genotype are presented in Table 1.

Urinary Acetaminophen Metabolites

We measured the amount of free APAP, APAPG, and APAPS excreted in urine over 12 hours after APAP administration on days 7 and 14 of the two controlled diet periods. Data were analyzed as the molar percentage of oral APAP dose: % as free APAP (%APAP), APAPG (%APAPG), and APAPS (%APAPS), and as % molar ratios of APAP metabolites excreted: APAPG/(APAP+APAPG+APAPS)*100 [APAPG ratio], APAPG/APAP, and APAPS/APAP.

Several of the urinary APAP excretion measures differed by sex. In women, %APAP was higher (P=0.02), %APAPG was lower (P=0.004), and thus, APAPG ratio and APAPG/APAP were lower (P=0.026 and P=0.002, respectively; data not shown). In contrast, %APAPS and ratio of APAPS/APAP did not differ by sex. There was also a strong sex-by-diet interaction for %APAPG (P=0.0045) reflecting statistically significantly higher %APAPG among women (P=0.004), but not men (P=0.3) on the F&V diet compared to the basal diet.
Overall, for the UGT1A6 genotype, %APAP was higher ($P=0.045$) and APAPG/APAP ratio was lower ($P=0.02$) among *1/*1 relative to *1/*2 and *2/*2 individuals. For UGT2B15, %APAPG, APAPG ratio, and APAPG/APAP decreased ($P<0.0001$), and %APAPS increased ($P=0.002$) in an allelic dose-dependent manner across the UGT2B15 genotypes from *1/*1 to *2/*2.

There were no overall main effects of diet on %APAP and %APAPG or APAPG/APAP ratio; however, on the F&V diet relative to the basal diet, the APAPG ratio was higher ($P<0.0001$), and the %APAPS and APAPS/APAP were lower ($P<0.0001$).

The effect of diet on urinary APAP measures, stratified by UGT1A6 and UGT2B15 genotypes, is presented in Tables 2 and 3, respectively. The formal interaction term for diet-by-genotype for UGT1A6 was not statistically significant. However, there was a statistically significant diet-by-UGT2B15 genotype interaction for APAPG ratio ($P=0.03$). Despite having a higher APAPG ratio overall, UGT2B15 *1/*1 individuals also had approximately a 2-fold higher F&V-to-basal diet % difference in APAPG ratio response ($4.6 \pm 0.8$) compared to the *1/*2 ($2.1 \pm 0.5$) and *2/*2 individuals ($2.2 \pm 0.7$), suggesting that *1/*1 individuals were more responsive to the intervention.

Urinary APAP excretion was measured on days 7 and 14 (data not shown). Day-by-diet interactions were statistically significant for some measures. For %APAPG there was not a statistically significant difference by diet on day 7, but by day 14, statistically significantly higher %APAPG was observed on the F&V compared to the basal diet ($P=0.003$). Similarly, for APAPG/APAP, the ratio was lower on day 7 than day 14 on the F&V diet ($P=0.002$). In contrast, for %APAPS and APAPS/APAP ratio, the F&V diet effect was most pronounced
within the first week of the intervention ($P=0.049$ and 0.002, respectively), but by day 14 values were similar on both diets. The F&V effects on APAPG ratio were detected by day 7 and persisted to day 14.

We also tested whether diet effects were similar among women who were in the same menstrual cycle phase for both feeding periods (approximately 60-66% of women, depending whether day 7 or day 14 and urine or saliva data) compared to women who were not. For all outcomes, except %APAPS, no statistically significant relationships between menstrual phase difference and response to F&V supplementation were observed. The mean difference in %APAPS between the basal and F&V diets was greater among the women who were not in the same phase of cycle during both diets at day 7 ($P=0.04$), but was lower on F&V than basal in both groups of women as well as men (data not shown), and was no longer significant by day 14.

**Salivary Acetaminophen Pharmacokinetics**

Effects of diet on salivary APAP pharmacokinetics by *UGT1A6* and *UGT2B15* over the course of 12 h were evaluated. Overall, the maximum concentration of salivary APAP (Cmax) was statistically significantly higher in women than men ($P=0.0003$) and was higher when participants were fed the F&V diet compared to the basal diet ($P=0.003$; Figure 1). No sex or diet differences were observed for half-life, clearance or AUC. There were no statistically significant overall effects of *UGT1A6* genotype or genotype-by-diet interactions on Cmax; however, when stratified by *UGT1A6* genotypes, the higher Cmax on the F&V diet was due predominantly to a response among individuals with the *UGT1A6* *2/*2 genotype ($P=0.02$; Table 2). Similarly, when stratified by *UGT2B15*, the diet effect on Cmax was predominantly a
response among the *UGT2B15* *1/*2 individuals (*P*=0.002; Table 3) with smaller, non-significant differences in *1/*1 and *2/*2 individuals. Comparison of day 7 and 14 collections suggested that the differences in Cmax between diets were greater on day 7 than 14 (data not shown).

Stratified analyses suggest some differences in the other pharmacokinetic parameters by genotype, although there were no consistent patterns. Clearance was higher among *UGT1A6* *1/*2 relative to the *1/*1 and *2/*2 individuals on the basal diet (*P*=0.04). Further, among the *UGT2B15* *1/*1 relative to the *2/*2 and *1/*2 individuals, salivary APAP half-life on the basal diet was higher (*P*= 0.007). Response to diet also differed within genotype: APAP half-life was longer in *UGT2B15* *2/*2 individuals with F&V compared to the basal diet (*P*=0.009), an effect that was not present in the other two *UGT2B15* genotypes. As with Cmax, the diet differences were most prominent on day 7 (data not shown).
DISCUSSION

We hypothesized that APAP glucuronidation would differ by UGT1A6 genotype, such that there would be a trend toward increasing APAPG and an increasing ratio of APAPG to free APAP in urine from individuals with UGT1A6*1/*1 to *2/*2 genotypes. Individuals with the homozygous variant have been shown to have a slower metabolism of some phenolic substrates and a faster metabolism of others, including APAP, in vitro (Ciotti, Marrone et al. 1997). Increased susceptibility to APAP toxicity in cats and Gunn rats, which both lack UGT1A6 activity, lends support to the hypothesis that UGT1A6 may be important for APAP glucuronidation. In cats UGT1A6 is a pseudogene and APAP administration results in prolonged half-life of the drug and significant formation of oxidative metabolites (Court and Greenblatt 2000). In Gunn rats, the UGT1A6 gene is expressed but is not functional due to a frame shift mutation that inactivates all the UGT1A enzymes (Iyanagi, Watanabe et al. 1989). Court et al (Court, Duan et al. 2001) showed, using recombinant UGTs and human liver microsomes, that although most UGTs could glucuronidate APAP, UGT1A6 was most active at low concentrations (< 50 μM), whereas at higher, therapeutic concentrations to toxic concentrations (50 μM to 5 mM), UGT1A9 was more active, and at toxic concentrations, UGT1A1 contributed substantially. Our results do not support either faster or slower metabolism of APAP among individuals with a UGT1A6*2 genotype.

In the context of a controlled feeding study, we observed only a modest relationship between UGT1A6, diet, and APAP conjugation. In contrast, we detected strong associations between UGT2B15 genotype, diet, and APAP glucuronidation and sulfation. Two-weeks of a controlled diet supplemented with citrus fruits, cruciferous vegetables, and soy foods – dietary sources of
phytochemicals that increase UGT activity (Saracino and Lampe 2007) – had minor effects on salivary APAP pharmacokinetics, but significantly increased urinary excretion of APAPG as percent of excreted metabolites, and decreased excretion of APAPS, particularly among carriers of the \textit{UGT2B15}^{*1/*1} genotype.

\textit{UGT2B15} is expressed in liver, kidney, testis, mammary gland, prostate, and lung (Lévesque, Beaulieu et al. 1997). Although considered to be a major catalyst for C19-conjugation of androgens (e.g., testosterone and dihydrotestosterone), \textit{UGT2B15} also glucuronidates a wide range of other substrates, including simple phenolic compounds, coumarins, flavonoids, anthraquinones, and drugs such as oxazepam (Chen, Ritter et al. 1993; Green, Oturu et al. 1994). Court et al (Court, Duan et al. 2001) showed that \textit{UGT2B15} also has the capacity to conjugate APAP. The two polymorphic forms of the enzyme, expressed in HK293 cells, demonstrated similar substrate specificities and \( K_m \) values; however, \textit{UGT2B15}^{*2} had a higher \( V_{max} \) than \textit{UGTB15}^{*1} for 3-\( \alpha \)-diol and DHT (Lévesque, Beaulieu et al. 1997). Given the rather small differences in kinetics, Lévesque et al (Lévesque, Beaulieu et al. 1997) postulated that differences in steroid hormone metabolism are unlikely to be observed \textit{in vivo}; however, they suggested that this polymorphism may be contributing to individual variability in xenobiotic glucuronidation. In liver tissue, \textit{UGT2B15} D85Y(*2) was found to be a major determinant of S-oxazepam glucuronidation (Court, Hao et al. 2004) and also a major determinant of interindividual variability in pharmacokinetics and pharmacodynamics of lorazepam, a 2'-chloro-substituted oxazepam (Chung, Cho et al. 2005). As with our study, the \textit{UGT2B15}^{*2/*2} genotype was associated with lower glucuronidation in both studies.
Few studies have evaluated the effects of diet on glucuronidation in humans, and on APAP conjugation in particular. Pantuck et al (Pantuck, Pantuck et al. 1984) showed that 500 g cruciferous vegetables (cabbage and Brussels sprouts) consumed for 10 days stimulated formation of APAP glucuronyl conjugates and increased the APAPG/APAP ratio. Gwilt et al (Gwilt, Lear et al. 1994) found that supplementation of habitual diet with garlic extract (equivalent to 6-7 cloves/d) resulted in a small, but significant, increase in area under the plasma APAP glucuronide curve after 3 months, but no change in other glucuronide parameters. We recently reported that a controlled diet containing either 5 or 10 servings a day of cruciferous vegetables resulted in statistically significantly lower bilirubin concentrations, a surrogate marker for UTG1A1 activity (Navarro, Peterson et al. 2009) but APAP glucuronidation was not directly evaluated. In an observational study, ~30% lower bilirubin concentrations were observed among women with the highest intake of citrus consumption (Saracino, Bigler et al. 2009). Induction of UGT by a number of phytochemicals in rodents and cell culture systems has been well-documented (Saracino, Bigler et al. 2009). Most evidence suggests that up-regulation of phase 2 enzymes by these phytochemicals occurs through interaction with the cytoplasmic-anchoring protein Kelch-like ECH-associated protein 1 (Keap1) and the transcription factor Erythroid-derived related factor 2-like 2 (Nrf2), via the antioxidant response element (Dinkova-Kostova, Holtzclaw et al. 2005).

Soy protein, and soy isoflavones have been shown to increase hepatic UGT activity in rats and mice (Staack and Jeffery 1994; Appelt and Reicks 1997). Like endogenous estrogens, the soy isoflavones, daidzein and genistein, and their metabolites are substrates for UGTs. In humans,
glucuronides are the major isoflavone conjugates accounting for approximately 95% of total isoflavones, with sulfates and free isoflavones accounting for the remaining 5% (Adlercreutz 1993). Furthermore, some UGTs are induced by estrogens (Mackenzie, Hu et al. 2010), and it is likely that isoflavones, as weak estrogens, induce various isoforms of these enzymes via a similar mechanism, although they also act through other signaling pathways (Saracino and Lampe 2007).

Consistent with other studies reporting sex differences (Miners, Attwood et al. 1983), we observed greater APAP gluronidation among men, whereas there were no sex-related differences in sulfation. Court, et al (Court, Duan et al. 2001) also reported higher APAP glucuronidation in male human liver microsomes, and later work showed higher S-oxazepam glucuronidation by UGT2B15 in male compared to female livers (Court, Hao et al. 2004). Differences in drug metabolism have been attributed to hormonal changes during the menstrual cycle, percent body fat, glomerular filtration rate and differences in biotransformation enzyme activity (Anderson 2008). Women were much more responsive to the F&V diet, excreting a greater percentage of glucuronides after consumption of a diet rich in soy, crucifers and citrus for two weeks. As fruit and vegetables were provided on the basis of body weight, greater consumption of dietary bioactive compounds by women relative to men are unlikely to be responsible for this difference. Although we designed the study to accommodate women’s menstrual cycles (i.e., we used a 14-day intervention and 14-day washout such that the second period would start 28 days after the first), logistics did not always allow for this level of control. In addition, both the controlled diet and intervening on women, often alters menstrual cycle length themselves. However, we did not see any significant differences in glucuronidation on the basis of menstrual phase. The
observation of greater responsiveness to diet among women may be explained by higher basal glucuronidation activity in men.

A major strength of this study is the design. We recruited on the basis of genotype, evaluated the relationship between genotype and phenotype on the background of a controlled diet, and tested the effects of adding 10 servings of plant foods to a basal diet devoid of phytochemicals (e.g., whole grains, fruits, vegetables, herbs, and spices). However, there are some possible limitations. Although larger than the previous controlled feeding studies designed to evaluate effects of diet on glucuronidation, the sample size is relatively small for detecting genotype-diet interactions. Our results suggest that certain UGT genotypes may be more responsive to a F&V intervention than others, but the data do not provide conclusive evidence regarding differences in these interactions. Additionally, we did not evaluate polymorphisms in all UGTs known to glucuronidate APAP, including UGT1A1 and UGT1A9. Separate analyses were not performed on UGT1A1*28, as this polymorphism has been shown to be in high linkage disequilibrium with UGT1A6*2, and would probably not be informative. UGT1A9 has the capacity to glucuronidate APAP (Court, Duan et al. 2001), and work by Ohno et al (Ohno and Nakajin 2009) in various tissues from healthy human donors showed higher abundance of UGT1A9 mRNA compared to UGT1A6, both in the liver and overall. Although it is not known whether these mRNA levels correlate with actual protein concentrations, these data suggest an important role for UGT1A9 in APAP conjugation. Two variants in the promoter region of the UGT1A9 gene have been shown to alter glucuronidation activity. T-275A and C-2152T are associated with increased enzyme activity, however given that they have an allelic prevalence of ~12 and 8%, respectively, (Sanchez-Fructuoso, Maestro et al. 2009) we were not sufficiently powered to
evaluate this polymorphism in our current sample. Studies enriching for these variants, or a larger sample size, are needed to evaluate their impact on APAP glucuronidation. Further, we cannot rule out the possibility that the observed effects are the result of dietary influence on phase 3 excretion of the various APAP metabolites.

Saliva APAP concentrations have been shown to correlate well with plasma concentrations of free APAP (Adithan and Thangam 1982; al-Obaidy, Li Wan Po et al. 1995; Hahn, Mogensen et al. 2000) making this a convenient, noninvasive method of conducting pharmacokinetic studies in larger-scale studies. Although there is fairly good agreement between these measures, there are several factors that may limit its utility, including differences in salivary flow rate, individual collection, and residual oral drug. In the present study, a gap in sampling between 4 and 12 hours after dosing, may have resulted in inaccurate elimination phase estimates. Our results suggest that testing genotype effects using more conventional pharmacokinetic approaches are warranted.

Finally, the differences in APAP glucuronidation between genotypes in response to diet are small. It is not clear whether these minor differences affect disease risk or response to therapy, but given the role of UGT2B15 in conjugation and subsequent elimination of potentially toxic xenobiotics, these results may have implications for altering carcinogen metabolism through dietary intervention. While most of the foods included in our study protocol are commonly consumed, soy foods, orange peel and radish sprouts are not. Further, these foods may not be consumed in the combined quantities given in this study.
In conclusion, we showed that APAP glucuronidation is significantly influenced by the 
UGT2B15*2 polymorphism, whereas UGT1A6*2 contributed only modestly. The genotype 
effects of UGT2B15*2 also support, in vivo, a role for UGT2B15 in APAP glucuronidation. In 
addition, we showed that a basal diet supplemented with selected fruits and vegetables known to 
affect UGT activity, can alter APAP metabolism in the direction of greater glucuronidation and 
less sulfate formation.
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AUTHORSHIP CONTRIBUTIONS:

*Participated in research design:* Lampe, Potter, King, S. Li and Bigler

*Conducted experiments:* Lampe, Schwarz, Bigler, King, Chang

*Contributed new reagents or analytic tools:* N/A

*Performed data analysis:* Chen, L. Li, S. Li

*Wrote or contributed to the writing of the manuscript:* Navarro, Lampe, Chang, Chen, Potter
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Cumulative salivary APAP concentrations over 12 h for 63 individuals by A) UGT1A6 and B) UGT2B15 genotypes on a phytochemical-free controlled diet (basal diet) and basal diet supplemented with soy foods, cruciferous vegetables, and citrus fruits (F&V diet).
TABLES

Table 1. Characteristics of 66 participants by UGT1A6 genotype

<table>
<thead>
<tr>
<th>UGT1A6*1/*1</th>
<th>UGT1A6*1/*2</th>
<th>UGT1A6*2/*2</th>
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<tr>
<td>Men</td>
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<td>Men</td>
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<tr>
<td>n=14</td>
<td>n=14</td>
<td>n=11</td>
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<tr>
<td>Age, y[^b]</td>
<td>31.3 ± 5.8</td>
<td>28 ± 6.0</td>
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<tr>
<td>Height, cm</td>
<td>178.8 ± 6.5</td>
<td>160.7 ± 8.6</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76.3 ± 10.5</td>
<td>58.1 ± 9.1</td>
</tr>
<tr>
<td>BMI, kg/m[^2]</td>
<td>23.8 ± 2.5</td>
<td>22.5 ± 2.6</td>
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</tbody>
</table>

Ethnicity

<table>
<thead>
<tr>
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<th>Men</th>
<th>Women</th>
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<th>Women</th>
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<tbody>
<tr>
<td>Caucasian, n (%)</td>
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<td>8 (57)</td>
<td>8</td>
<td>73</td>
<td>10</td>
<td>71.5</td>
</tr>
<tr>
<td>Asian, n (%)</td>
<td>4</td>
<td>6 (43)</td>
<td>1</td>
<td>8</td>
<td>3</td>
<td>21.5</td>
</tr>
<tr>
<td>Other[^c] n (%)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>17</td>
<td>1</td>
<td>7</td>
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</table>

Distribution of UGT1A1*28 genotypes

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
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<th>Women</th>
<th>Men</th>
<th>Women</th>
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</thead>
<tbody>
<tr>
<td>6/6, n (%)</td>
<td>12</td>
<td>11 (79)</td>
<td>2</td>
<td>18</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>6/7, n (%)</td>
<td>2</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>82</td>
</tr>
<tr>
<td>7/7, n (%)</td>
<td>0</td>
<td>3 (21)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
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</table>

Distribution of UGT2B15*2 genotypes
<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (%)</th>
<th>n (%)</th>
<th>n (%)</th>
<th>n (%)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1, n (%)</td>
<td>4 (29)</td>
<td>2 (14)</td>
<td>2 (18)</td>
<td>5 (36)</td>
<td>3 (37)</td>
</tr>
<tr>
<td>*1/*2, n (%)</td>
<td>5 (36)</td>
<td>7 (50)</td>
<td>7 (64)</td>
<td>5 (36)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>*2/*2, n (%)</td>
<td>5 (36)</td>
<td>5 (36)</td>
<td>2 (18)</td>
<td>4 (28)</td>
<td>0</td>
</tr>
</tbody>
</table>

*No significant differences between genotypes at \( P<0.05 \)

*Mean ±SD

*Other category includes 1 black, 1 mixed race, 1 Pacific Islander, and 1 unreported
### Table 2. Salivary APAP pharmacokinetics and urinary APAP metabolite excretion by diet and UGT1A6 genotypes within diet (LS means ± SE)

#### Salivary Pharmacokinetics

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>UGT1A6*1/*1</th>
<th>UGT1A6*1/*2</th>
<th>UGT1A6*2/*2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal F&amp;V</td>
<td>Basal F&amp;V</td>
<td>Basal F&amp;V</td>
<td>Basal F&amp;V</td>
</tr>
<tr>
<td>n</td>
<td>65</td>
<td>65</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>16.1 ± 0.6</td>
<td>17.2 ± 0.9</td>
<td>15.1 ± 0.9</td>
<td>16.0 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clearance</td>
<td>10.0 ± 0.5</td>
<td>9.3 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.9 ± 0.9&lt;sup&gt;l&lt;/sup&gt;</td>
<td>9.1 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC</td>
<td>6103 ± 270</td>
<td>6414 ± 419</td>
<td>5378 ± 369</td>
<td>6588 ± 613</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>260 ± 15</td>
<td>261 ± 22</td>
<td>245 ± 22</td>
<td>275 ± 33</td>
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</table>

#### Urinary APAP Metabolite Excretion

<table>
<thead>
<tr>
<th></th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>65</td>
<td>65</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>% of oral dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%APAP</td>
<td>3.1 ± 0.12</td>
<td>3.2 ± 0.12</td>
<td>3.37 ± 0.16</td>
<td>3.00 ± 0.16&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>%APAPG</td>
<td>35.0 ± 0.9</td>
<td>35.8 ± 0.9</td>
<td>32.9 ± 1.4</td>
<td>36.3 ± 1.4</td>
</tr>
<tr>
<td>%APAPS</td>
<td>24.1 ± 0.9</td>
<td>22.1 ± 0.9</td>
<td>25.0 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.9 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

#### Metabolite ratios

<table>
<thead>
<tr>
<th></th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
<th>Basal F&amp;V</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>65</td>
<td>65</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>APAPG ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.2 ± 1.2</td>
<td>58.9 ± 1.2</td>
<td>53.5 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.7 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>APAPG/APAP&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11.8 ± 0.6</td>
<td>11.9 ± 0.6</td>
<td>10.0 ± 0.7</td>
<td>12.9 ± 0.9&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
<tr>
<td>APAPS/APAP&lt;sup&gt;*&lt;/sup&gt;</td>
<td>8.1 ± 0.4</td>
<td>7.1 ± 0.3</td>
<td>7.5 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Different superscript letters represent significant diet differences within a genotype (<i>P</i>&lt;0.05)

<sup>1,2,3</sup>Different superscript numbers represent significant genotype differences within diet (<i>P</i>&lt;0.05)
\[^{\text{APAPG/(APAP+APAPG+APAPS)}*100}\]

*Significant overall diet effect for \textit{UGT1A6} genotype \((P<0.05)\)

\[C_{\text{max}}, \text{mg/l}; \text{Clearance, } 1/h; \text{AUC}_{0-\infty}, \text{mg\cdot min/l}; \text{T}_{1/2}, \text{min}\]
Table 3. Salivary APAP pharmacokinetics and urinary APAP metabolite excretion by *UGT2B1* genotypes (LS means ± SE)

<table>
<thead>
<tr>
<th>UGT2B15 *1/*1</th>
<th>UGT2B15 *1/*2</th>
<th>UGT2B15 *2/*2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>F&amp;V</td>
</tr>
<tr>
<td><strong>Salivary Pharmacokinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>n=16</strong></td>
<td><strong>n=16</strong></td>
<td><strong>n=33</strong></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>15.9 ± 1.2</td>
<td>17.0 ± 1.3</td>
</tr>
<tr>
<td>Clearance</td>
<td>10.2 ± 1.0</td>
<td>10.3 ± 1.0</td>
</tr>
<tr>
<td>AUC</td>
<td>5913 ± 504</td>
<td>5787 ± 493</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>307 ± 31&lt;sup&gt;f&lt;/sup&gt;</td>
<td>282 ± 28</td>
</tr>
</tbody>
</table>

| **Urinary APAP Metabolite Excretion** |       |     |       |     |       |     |
| **n=16** | **n=16** | **n=33** | **n=33** | **n=17** | **n=17** |
| % of oral dose |       |     |       |     |       |     |
| %APAP       | 3.0 ± 0.2 | 2.9 ± 0.2 | 3.0 ± 0.1 | 3.2 ± 0.1 | 3.4 ± 0.2 | 3.5 ± 0.2 |
| %APAPG      | 40.2 ± 1.6<sup>f</sup> | 40.9 ± 1.6<sup>f</sup> | 35.1 ± 1.1<sup>2</sup> | 36.0 ± 1.1<sup>2</sup> | 29.3 ± 1.5<sup>3</sup> | 30.4 ± 1.5<sup>3</sup> |
| %APAPS<sup>*</sup> | 22.1 ± 1.6<sup>4</sup> | 19.0 ± 1.5<sup>b,f</sup> | 23.3 ± 1.1<sup>a,2</sup> | 21.6 ± 1.1<sup>b,2</sup> | 28.5 ± 1.5<sup>a,3</sup> | 26.9 ± 1.5<sup>b,3</sup> |

| **Metabolite ratios** |       |     |       |     |       |     |
| APAPG ratio<sup>9</sup> | 61.3 ± 1.8<sup>4</sup> | 65.9 ± 1.8<sup>b,f</sup> | 57.3 ± 1.2<sup>a,2</sup> | 59.4 ± 1.2<sup>b,2</sup> | 47.9 ± 1.7<sup>a,3</sup> | 50.1 ± 1.8<sup>b,3</sup> |
| APAPG/APAP | 13.9 ± 1.2<sup>f</sup> | 15.5 ± 1.3<sup>f</sup> | 12.2 ± 0.7<sup>2</sup> | 11.7 ± 0.7<sup>2</sup> | 8.9 ± 0.7<sup>3</sup> | 9.0 ± 0.7<sup>3</sup> |
| APAPS/APAP* | 7.6 ± 0.7<sup>a</sup> | 6.8 ± 0.6<sup>b</sup> | 8.0 ± 0.5<sup>a</sup> | 6.8 ± 0.4<sup>b</sup> | 8.7 ± 0.7 | 8.0 ± 0.7 |

<sup>a,b</sup>Different superscript letters represent significant diet differences within a genotype (P<0.05)

<sup>1,2,3</sup>Different superscript numbers represent significant genotype differences within diet (P<0.05)

<sup>c</sup>APAPG/(APAP+APAPG+APAPS)*100

<sup>*</sup>Significant overall diet effect for *UGT2B15* (P<0.05)

C<sub>max</sub>, mg/l; Clearance, 1/h; AUC<sub>0-∞</sub>, mg·min/l; T<sub>1/2</sub>, min