JPET Fast Forward. Published on June 29, 2017 as DOI: 10.1124/jpet.117.242107
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Race, gender, and genetic polymorphism contribute to variability in acetaminophen pharmacokinetics, metabolism, and protein-adduct concentrations in healthy African-American and European-American volunteers

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Running title: Race, gender, genes and APAP pharmacokinetics

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Counts:

Text pages: 15

Tables: 5

Figures: 6

References: 27

Abstract words: 250

Introduction words: 738

Discussion words: 1134

Nonstandard abbreviations: SNP – single nucleotide polymorphism; UGT – UDP-glucuronosyltransferase; CYP – cytochrome P450; SULT – sulfotransferase.

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ABSTRACT

Over 30 years ago, black Africans from Kenya and Ghana were shown to metabolize acetaminophen faster by glucuronidation and slower by oxidation compared with white Scottish Europeans. The objectives of this study were to determine whether similar differences exist between African-Americans and European-Americans, and to identify genetic polymorphisms that could explain these potential differences. Acetaminophen plasma pharmacokinetics and partial urinary metabolite clearances via glucuronidation, sulfation, and oxidation were determined in healthy African-Americans (18 men, 23 women) and European-Americans (34 men, 20 women) following a one-gram oral dose. There were no differences in acetaminophen total plasma, glucuronidation, or sulfation clearance values between African-Americans and European-Americans. However, median oxidation clearance was 37% lower in African-Americans versus European-Americans (0.57 versus 0.90 mL/min/kg; P=0.0001). Although acetaminophen total or metabolite clearance values were not different between genders, shorter plasma half-life values (by 11 to 14%; P<0.01) were observed for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate in women versus men. The UGT2B15*2 polymorphism was associated with variant allele number proportional reductions in acetaminophen total clearance (by 15 to 27%; P<0.001) and glucuronidation partial clearance (by 23 to 48%; P<0.001). UGT2B15 *2/*2 genotype subjects also showed higher acetaminophen protein-adduct concentrations than *1/*2 (by 42%; P=0.003) and *1/*1 (by 41%; P=0.003) individuals. Finally, CYP2E1 *1D/*1D genotype African-Americans had lower oxidation clearance than *1C/*1D (by 42%; P=0.041) and *1C/*1C (by 44%; P=0.048) African-Americans. Consequently, African-Americans oxidize acetaminophen more slowly than European-Americans, which may be partially explained by the CYP2E1*1D polymorphism. UGT2B15*2 influences acetaminophen pharmacokinetics in both African-Americans and European-Americans.

INTRODUCTION

Acetaminophen is one of the most commonly used non-prescription drugs for treating mild pain and fever in the United States of America (Ameer and Greenblatt, 1977; Kaufman et al., 2002). This drug is also the most common cause of acute liver failure in this country resulting from intentional or inadvertent drug overdose (Larson et al., 2005). Recent studies in our laboratory using patient DNA samples collected by the Acute Liver Failure Study Group have identified polymorphisms associated with acetaminophen-induced acute liver failure in genes encoding enzymes that metabolize acetaminophen, including UDP-glucuronosyltransferase (UGT) 1A and cytochrome P450 (CYP) 3A5 (Court et al., 2013; Court et al., 2014). Interestingly, acetaminophen-induced liver failure patients identified by the Acute Liver Failure Study Group were primarily female (74%) and white (88%), with only 5% of subjects identified as African-American (Larson et al., 2005). This differs from the reported race distribution (~60% white and ~12% African-American) and gender distribution (~51% women) for the United States population over the same study period (census.gov). Although there are likely alternate explanations for this observation, it is possible that these demographic differences reflect variable susceptibility to toxicity because of race and gender differences in acetaminophen metabolism. Unfortunately, relatively few studies have characterized such metabolic differences.

Over 30 years ago, it was shown that blacks from Ghana and Kenya excreted similar amounts (based on 24 h fractional urinary recovery) of APAP sulfate, higher amounts of APAP glucuronide, and lower amounts of APAP glutathione conjugates (derived from NAPQI) compared with whites from Scotland (Critchley et al., 1986). This finding was suggested to explain the lower incidence of APAP hepatotoxicity in Africans compared with whites (Critchley et al., 2005). Race-associated differences in acetaminophen metabolism may be a consequence of differences in the population frequencies of genetic polymorphisms. As far as we are aware, studies of APAP pharmacokinetics or metabolism have not been reported for African-Americans.

There is some evidence for gender-associated differences in APAP pharmacokinetics and metabolism. After controlling for body size differences, about 20% higher acetaminophen oral clearance

was also observed in young men versus young women, but not in elderly men versus women (Divoll et al., 1982). However, another study showed no differences in weight-adjusted acetaminophen clearance after intravenous acetaminophen administration, but did show higher weight-adjusted acetaminophen volume of distribution values in men versus women (Abernethy et al., 1982). Higher APAP glucuronide fractional excretion and lower APAP sulfate excretion was observed in men compared with women in both Scottish white and Ghanaian populations, but not in the Kenyan population (Critchley et al., 1986). Using human liver microsomes, we also showed approximately 50% higher APAP glucuronidation in livers from male donors compared with female donors (Court et al., 2001).

Several studies have evaluated associations of acetaminophen pharmacokinetics with drug metabolizing enzyme genotypes. Urinary acetaminophen glucuronide metabolite concentrations were not associated with Gilbert's syndrome (caused by the UGT1A1*28 promoter variant) in a study of 23 healthy white European volunteers (Rauchschwalbe et al., 2004). Slightly higher acetaminophen clearance was associated with a UGT1A6*2/UGT1A1*28 haplotype in a study of 15 Thai beta-thalassemia patients (Tankanitlert et al., 2007). The UGT2B15*2 (rs1902023) polymorphism was associated with decreased acetaminophen glucuronide concentrations in urine samples from 66 healthy white and Asian volunteers in the USA (Navarro et al., 2011). This finding was confirmed by a study of 109 Pakistani volunteers that showed lower blood acetaminophen glucuronide to acetaminophen concentration ratios with an increasing number of UGT2B15*2 alleles (Mehboob et al., 2016). However, studies evaluating genetic associations with acetaminophen sulfation or oxidation have not been reported.

Although at therapeutic doses acetaminophen-derived NAPQI largely undergoes conjugation with glutathione, it can also covalently bind with liver proteins. An HPLC with electrochemical detection assay has been developed that can quantitatively measure acetaminophen protein-adduct concentrations in patient blood samples following therapeutic and toxic acetaminophen concentrations (Davern et al., 2006; Heard et al., 2011; James et al., 2013). This assay has been used to identify cases of acetaminophen-induced acute liver failure in which the precipitating cause was initially indeterminate (James et al., 2006;

Khandelwal et al., 2011). Currently, it is unclear whether race, gender or genetics influences acetaminophen protein-adduct concentrations.

The main objective of this study was to determine whether acetaminophen pharmacokinetics, metabolism, and protein-adduct formation differ between African-American and European-American men and women. We also evaluated whether selected polymorphisms in candidate genes for enzymes involved in acetaminophen glucuronidation, sulfation, and oxidation were associated with differences in acetaminophen metabolic clearance and protein-adduct formation.

MATERIALS AND METHODS

Subjects

The study protocol and consent form were reviewed and approved by the Institutional Review Boards serving Tufts University School of Medicine (Boston, MA) and Promedica Clinical Research Center (Boston, MA). Healthy self-declared European-American (or white or Caucasian) and African-American (or black) men and women aged 18 to 64 years were recruited from the local population. The planned enrolment was 100 with an approximately equal distribution of subjects by gender and race. Exclusion criteria included a history of medical disease, any clinically significant abnormality detected by physical examination or routine laboratory analysis, HIV or hepatitis (B or C) infection, a history of tobacco use or consuming an average of 3 or more alcohol drinks per day, pregnancy or the use of drugs or herbal medications known to alter acetaminophen metabolism. Specific medications included isoniazid, disulfiram, phenobarbital, phenytoin, carbamazepine, rifampicin, valproic acid, probenecid, and St. John's wort. Additionally, all subjects were asked to refrain from consuming any type of caffeinated products or grapefruit juice on the study day.

Study procedures

On the day of the study, subjects reported to the study unit at 7 AM, received a light breakfast, and had a catheter placed into a forearm vein for serial collection of blood samples. Baseline plasma, serum,

and urine samples were collected and subsequently stored at -80° C. Two 500 mg acetaminophen tablets (Extra Strength Tylenol, McNeil, Fort Washington, PA) were then administered at 8 AM under direct observation with a glass of water. No food or drink (except water) was permitted until 2 h after acetaminophen administration. Plasma samples were collected at 1, 2, 3, 4, 5, 6, 8, 10, and 12 h after acetaminophen dosing and stored at -80° C with the baseline sample. All voided urine was collected throughout the study into a refrigerated container up until 12 hours after acetaminophen administration. The final urine volume collected was then measured, an aliquot was stored at -80° C, and the subject was discharged. Subjects returned 4 days after acetaminophen administration (on Day 5) for collection of a second serum sample. Serum samples were assayed for alanine aminotransferase levels within 24 h of collection by a commercial laboratory (Quest Diagnostics, Madison, NJ). Plasma and urine samples stored at -80° C were assayed for acetaminophen and metabolite concentrations within 12 months of collection.

Acetaminophen and metabolite concentration assays

Plasma samples were assayed for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate concentrations by HPLC with UV absorbance detection using the same method we have recently reported in detail (Zhao et al., 2015). Calibration curves were consistently linear ($R^2 > 0.99$) over the assayed range with the limit of quantitation was 0.1 µg/mL for each analyte. Quality control samples spiked with low and high analyte concentrations showed excellent precision (<15% coefficient of variation) and accuracy (89% to 104% of nominal). Concentrations in unknown plasma samples were determined in duplicate on two separate occasions and the results were averaged.

Acetaminophen, acetaminophen glucuronide, acetaminophen sulfate, acetaminophen mercapturate, and acetaminophen cysteinate concentrations were determined in urine samples by HPLC with mass spectrometry detection. Pure standards and the respective stable isotope labelled internal standards were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Briefly, 5 µL of urine was diluted to 500 µL volume with 0.5% (v/v) formic acid in water. After adding internal standards, including acetaminophen-D4 (20 ng), acetaminophen-D3 glucuronide (500 ng), acetaminophen-D3 sulfate (200 ng),

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and acetaminophen mercapturate-D5 (200 ng), the sample was mixed, and 20 µL was analysed by HPLC (Agilent 1100, Agilent Technologies, Santa Clara, CA) with a triple quadrupole mass spectrometry detector (AB-Sciex API4000, Applied Biosystems Life Technologies, Framingham, MA). The mobile phase consisted of 0.5% v/v formic acid in water (A) mixed with acetonitrile (B) that was pumped at 400 µL per minute through a 2.0 mm x 150 mm 4 µ C18 column (Synergi Fusion RP, Phenomenex, Torrance, CA). Separation of analytes was achieved through use of a mobile phase gradient starting at an A:B ratio of 75:25 until 0.5 min, linearly changing to 5:95 at 1.5 min, and returning to 75:25 at 1.6 min with a total run time of 5 min. Positive ion mass transitions monitored included m/z 152→110 (acetaminophen), m/z 156→114 (acetaminophen-D4), m/z 328→152 (acetaminophen glucuronide), m/z 331→155 (acetaminophen-D3 glucuronide), m/z 232→152 (acetaminophen sulfate), m/z 235→155 (acetaminophen-D3 sulfate), m/z 335→152 (acetaminophen mercapturate), m/z 340→152 (acetaminophen mercapturate-D5), and m/z 271→140 (acetaminophen cysteinate). Retention times for acetaminophen cysteinate, acetaminophen glucuronide, acetaminophen mercapturate, acetaminophen, and acetaminophen sulfate (and the applicable respective labelled isotopes) were 1.0, 1.1, 1.5, 1.6, and 1.7 min, respectively. Acetaminophen mercapturate-D5 was used as the internal standard for acetaminophen cysteinate since an isotope labelled derivative was not commercially available.

Calibration curves were generated using drug-free urine spiked with known concentrations of acetaminophen (0.5 – 50 µg/mL), acetaminophen glucuronide (25 – 2500 µg/mL), acetaminophen sulfate (5 – 500 µg/mL), acetaminophen mercapturate (2.5 – 250 µg/mL), and acetaminophen cysteinate (2.5 – 250 µg/mL). Curves were linear ($R^2 > 0.99$) over the assayed range. The assay showed excellent precision (<15% coefficient of variation) and accuracy (<10% deviation from nominal) over the concentrations assayed. Analyte concentrations in unknown urine samples were determined in duplicate on two separate occasions and the results were averaged.

Acetaminophen protein-adduct assay

Baseline and 8-hour post-administration samples were analyzed within six months of collection by HPLC with electrochemical detection as described previously (Davern et al., 2006; Khandelwal et al., 2011). Briefly, samples were gel filtered and hydrolyzed to release acetaminophen-cysteine from acetaminophen protein-adducts. Following protein precipitation and extraction, samples were injected on the HPLC system, resolved on a 150 mm C_{18} column (Symmetry, Waters, Milford, MA) using a mobile phase containing 8% v/v methanol and 50 mM sodium acetate in water (pH 4.8), and detected using a coulometric electrochemical detector (Esa Corp, Chelmsford, MA). Final acetaminophen protein-adduct concentrations were reported as nmol APAP-cys / mL serum.

Pharmacokinetic calculations

A plot of log-transformed plasma concentration versus time was constructed for each analyte for each subject. The terminal log-linear phase of the plasma concentration curve was identified visually. The beginning and ending time points were designated as the regression interval. The slope (beta) of the terminal phase over the designated regression interval was calculated by log-linear regression and used to calculate the elimination half-life ($t_{½}$ = (ln 2)/beta). The total area under the plasma concentration curve (AUC) was calculated using the linear trapezoidal method from time zero to 12 h, and extrapolated to infinity by addition of the terminal segment calculated by dividing the final measured concentration by beta. The weight-normalized apparent oral clearance of acetaminophen was calculated by dividing the acetaminophen dose by the acetaminophen total AUC and by the subject's body weight. The molar fraction of unchanged acetaminophen, acetaminophen glucuronide, acetaminophen sulfate, and oxidative acetaminophen metabolites (summed mercapturate and unbound cysteinate) in the 0 to 12 h urine samples from each subject was used to calculate the partial urinary clearance of acetaminophen and each metabolite from the apparent oral plasma clearance of acetaminophen. These latter calculations assume that renal excretion is the predominant mechanism for elimination of acetaminophen metabolites and that the majority of acetaminophen metabolites are excreted in the urine within 12 hours after administration. This latter

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assumption is supported by prior work that showed 84% of the radioactivity of a dose of radiolabelled acetaminophen was recovered in the urine within 12 hours and 90% within 24 hours (Mitchell et al., 1974).

Genotyping

DNA was extracted using a spin column kit (QIAamp DNA Blood Mini Kit, Qiagen, Germantown, MD) from the buffy coat fraction of blood samples collected each subject. DNA samples were then genotyped using a real-time PCR instrument (CFX96 Touch, Bio-Rad, Hercules, CA) by allele discrimination assays (Applied Biosystems TaqMan SNP Genotyping Assay, Thermo Fisher Scientific, Waltham, MA). Variants assayed included the UGT1A6*2 haplotype SNPs consisting of rs6759892 (S7A; C__1432204), rs2070959 (T181A; C__15868110), and rs1105879 (R184S; C__1173642), as well as UGT1A9 rs6714486 (-275T>A; C__27843087), UGT1A-3'UTR rs8330 (c.2042C>G; C__7607429), UGT2B15*2 rs1902023 (D85Y; C__27028164), CYP3A5*3 rs776746 (C__26201809) and CYP2E1*4 rs6413419 (V179I; C__30443971). The UGT1A1 -53(TA) x 5, 6, 7, or 8 variable length dinucleotide insertion/deletion polymorphism was genotyped by Genescan fragment length analysis as previously reported (Girard et al., 2005). The CYP2E1*1D tandem repeat polymorphism (6 repeats in the *1C allele and 8 repeats in the *1D allele) was assayed by PCR with agarose gel sizing (Court et al., 2014), while the SULT1A1*2 polymorphism (rs9282861, R213H) was assayed by a PCR-RFLP method (Court et al., 2014). Assay accuracy was confirmed through direct sequencing of PCR product from representative samples. All observed genotype frequencies were determined to be consistent with expected genotype frequencies according to the Hardy-Weinberg distribution ($P > 0.05$; Chi-square test).

Statistical analyses

Statistical analyses were performed using Sigmaplot 12 software (Systat, San Jose, CA). All data were summarized as median and range (demographic data) or as median and interquartile range (pharmacokinetic data) values. Comparisons between groups were performed using parametric tests unless the Shapiro-Wilk test indicated non-normality or the Brown-Forsythe test indicated unequal variance, in which case an equivalent non-parametric test was used (indicated below). A *P* value of less than 0.05 was

considered significant. Demographic characteristics were compared between racial and gender groups by either Mann-Whitney rank sum test (continuous data) or by Chi-square test (frequency data). Spearman rank order correlation was used to evaluate associations between acetaminophen total plasma clearance, partial clearance, and protein-adduct concentrations. Pharmacokinetic parameters were log-transformed prior to statistical comparisons between demographic and enzyme genotype groups. These groups were compared by either t-test (two groups) or ANOVA (three groups). In instances where ANOVA indicated a significant difference between groups, multiple pairwise comparisons (Holm-Sidak method) were performed to identify the groups that were different from each other. After log transformation of plasma acetaminophen protein-adduct concentrations distributions remained non-normal (Shapiro-Wilk test $P<0.05$), consequently these data were analysed by non-parametric methods, including either Mann-Whitney rank sum test (2 groups) or Kruskal-Wallis ANOVA on ranks (3 groups) with post-hoc testing (when appropriate) by Dunn's multiple comparisons procedure. Multiple linear regression was used to evaluate the possible contributions of race, gender, BMI, and enzyme genotypes to variability in acetaminophen and metabolite clearance, as well as acetaminophen protein-adduct formation.

RESULTS

The demographic characteristics of the study participants are summarized in Table 1. Of 104 subjects that met enrolment criteria and entered the study, 98 subjects (55 European-Americans and 43 African-Americans) completed the study. The primary reason for failure to complete the study was an inability to obtain satisfactory venous access for serial blood samples. Data from three subjects (one European-American man and two African-American women) were excluded since pre-dose plasma samples contained significant concentrations of acetaminophen. Urine samples from two African-Americans (one man and one woman) and the DNA sample from one European-American man were lost to analysis, and so acetaminophen partial clearances and genotypes (respectively) could not be determined for those subjects. As shown in Table 1, there was a similar distribution of age, weight, drinking history, and female hormonal contraceptive use between European-Americans and African-Americans. However, there was a

higher proportion of women (56% versus 37%) and a higher median body mass index (26 versus 24 kg/m^2) in the African-American group compared with the European-American group (respectively). Table 1 also gives baseline serum ALT values and the change in serum ALT values at 4 days after acetaminophen administration. Baseline ALT values did not differ between racial groups and there was no change in ALT values for either racial group after acetaminophen administration ($P > 0.05$).

A plot showing mean (± standard error) plasma concentrations of acetaminophen, and its glucuronide and sulfate metabolites for all subjects measured up to 12 h after dosing is shown in Figure 1. Median (interquartile range) acetaminophen plasma clearance, glucuronidation clearance, sulfation clearance, oxidation clearance, and unchanged acetaminophen renal clearance values for all subjects were 5.5 (4.8 – 6.6), 3.0 (2.3 - 4.0), 1.5 (1.1 – 1.9), 0.76 (0.57 - 1.0), and 0.17 (0.14 – 0.23) mL / min / kg. Spearman correlation analysis (Figure 2) showed the strongest correlation of acetaminophen plasma clearance with acetaminophen glucuronidation clearance (Rs = 0.90, $P < 0.001$) and sulfation clearance (Rs = 0.50, $P < 0.001$), with much weaker correlations with acetaminophen oxidation (Rs = 0.23, $P = 0.03$), and unchanged renal clearance (Rs = 0.21, $P = 0.04$). Median (interquartile range) plasma acetaminophen protein-adduct concentrations were 0.021 (0.014 – 0.027) nmoles / mL for all subjects. Acetaminophen protein-adduct concentrations were most strongly and negatively correlated with acetaminophen glucuronidation clearance (Rs = -0.60, $P < 0.001$) followed by acetaminophen plasma clearance (Rs = -0.46 $P < 0.001$) (Figure 3), but were not correlated with acetaminophen sulfation (Rs = -0.07, $P = 0.5$), oxidation (Rs = 0.18, $P = 0.10$) or unchanged renal clearance (Rs = -0.04, $P = 0.7$) (data not shown).

Pharmacokinetic parameters for European-American and African-American subjects are compared in Table 2. No differences were observed between these groups except for acetaminophen oxidation partial clearance, which showed a 37% lower median value ($P = 0.0001$) in African-Americans compared with European-Americans. Pharmacokinetic parameters were also compared between genders (Table 3). Shorter median half-life values were observed for acetaminophen (by 11%; $P = 0.0009$), acetaminophen glucuronide (by 14%; $P = 0.0002$), and acetaminophen sulfate (by 11%; $P = 0.002$) in women compared with men. Lower median plasma metabolite/acetaminophen AUC ratios were also observed for

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acetaminophen glucuronide (by 17%; $P = 0.049$) and acetaminophen sulfate (by 14%; $P = 0.025$) in women compared with men.

The minor allele frequencies for the enzyme variants assayed in the study subjects (given in Table 4) were in general agreement with reported dbSNP database values (https://www.ncbi.nlm.nih.gov/projects/SNP/) for the corresponding racial group. Higher minor allele frequencies ($P<0.05$, Chi-square test) were observed for UGT1A rs8330, UGT1A9 rs6714486, CYP2E1*1D and CYP2E1*4 in African-American compared with European-American subjects, while lower frequencies were found for UGT1A6*2 and CYP3A5*3. On the other hand, UGT1A1 (TA)n, UGT2B15*2, and SULT1A1*2 allele frequencies did not differ between racial groups.

Associations were then evaluated between acetaminophen pharmacokinetic parameters and enzyme genotypes. These genetic variants were chosen based on prior evidence for functional impact on the enzymes known to be involved in acetaminophen metabolism (Court et al., 2014). In instances where the number of individuals with the homozygous variant genotype were small (<5 individuals), data were also analyzed by combining the heterozygous and homozygous variant groups (variant carrier) to increase statistical power. As shown in Figure 4A, acetaminophen plasma clearance varied by UGT2B15*2 genotype in direct proportion to the number of variant T alleles (ANOVA, $P < 0.001$; Supplemental Table 1). UGT2B15 *2/*2 individuals showed 27% lower median acetaminophen clearance than *1/*1 individuals ($P < 0.001$) and 15% lower than *1/*2 individuals ($P = 0.021$), while *1/*2 individuals were 14% lower than *1/*1 individuals ($P = 0.03$). However none of the other enzyme genotypes evaluated were associated with acetaminophen plasma clearance (Supplemental Table 1). A similar but stronger association (ANOVA, $P < 0.001$; Supplemental Table 2) was observed between UGT2B15*2 genotype and glucuronidation partial clearance (Figure 4B). UGT2B15 *2/*2 genotype individuals showed 48% lower median glucuronidation clearance than *1/*1 individuals ($P < 0.001$) and 32% lower than *1/*2 individuals ($P < 0.001$), while *1/*2 individuals were 23% lower than *1/*1 individuals ($P = 0.03$). None of the other glucuronidation enzyme polymorphisms evaluated were associated variation in glucuronidation partial clearance (Supplemental Table 2). A similar trend was also observed for plasma

glucuronide/acetaminophen AUC ratios with lower median ratios associating with an increasing number of variant UGT2B15*2 alleles (ANOVA, $P < 0.001$; Supplemental Table 2). Again, none of the other glucuronidation enzyme polymorphisms correlated with glucuronide/acetaminophen AUC ratios (Supplemental Table 2).

As shown in Supplemental Table 3, although no association (ANOVA, $P = 0.41$) was observed between the SULT1A1*2 variant and sulfation partial clearance, there was a weak association (ANOVA, $P = 0.04$) between this variant and plasma sulfate/acetaminophen ratios. SULT1A1 *2/*2 genotype individuals showed 30% lower (P = 0.04) and 22% lower (P = 0.043) median AUC ratios than A/G and *1/*1 individuals (respectively), while A/G and *1/*1 individuals were not different ($P = 0.75$).

Initial analysis of the association of acetaminophen oxidation partial clearance with oxidation enzyme genotypes in all subjects showed significant correlations with all genotypes evaluated (Supplemental Table 4). However, since both oxidation partial clearance (Table 2) and the frequencies of these polymorphisms (Table 4) varied by race, this result could simply be a consequence of stratification of subjects by race-associated genetic variants. Subsequent analysis within each racial group showed a weak association (ANOVA, $P = 0.048$) of the CYP2E1*1D variant with oxidation clearance in African-American subjects (Figure 5 and Supplemental Table 5). Individuals with the CYP2E1 *1D/*1D genotype showed 42% lower ($P = 0.041$) and 44% lower ($P = 0.048$) median oxidation clearance than *1C/*1D and *1C/*1C individuals (respectively), while *1C/*1D and *1D/*1D individuals were not different ($P = 0.74$). No significant associations were found for other genotypes in African-American subjects, or for any genotype in European-American subjects (Supplemental Table 5). However, it should be noted that the frequencies of these polymorphisms were much lower in European-American subjects and none of them were homozygous for the variant allele of any of the oxidative enzyme polymorphisms tested.

Finally, we evaluated enzyme genotype associations with plasma acetaminophen-adduct concentrations measured at 8 h after drug administration in all subjects (Supplemental Table 6). This corresponded to the time at which the acetaminophen-adduct plasma concentration was predicted to be highest (i.e. the T_{max}) based on the results of a previous study (James et al., 2013). As shown in Figure 6,

only UGT2B15*2 genotype was significantly correlated with acetaminophen protein-adduct concentration (ANOVA, P = 0.003), with *2/*2 genotype individuals showing 41% higher concentrations than *1/*1 individuals (P = 0.003) and 42% higher than *1/*2 individuals (P = 0.014), while *1/*2 individuals were not different from *1/*1 individuals (P = 1.0).

Given that multiple factors could contribute to pharmacokinetic variability, we conducted a multivariate analysis by linear regression incorporating independent variables shown to be associated with pharmacokinetic parameters in the preceding univariate analyses, including race, gender, as well as UGT2B15*2, SULT1A2*2, and CYP2E1*1D genotypes. As shown in Table 5, UGT2B15*2 genotype was the only variable that was predictive of acetaminophen plasma clearance, glucuronidation clearance and acetaminophen protein-adduct concentrations, accounting for 17%, 29%, and 19% (respectively) of the observed variability. Both gender and UGT2B15*2 genotype were predictive of glucuronide to acetaminophen AUC ratios (30% of the observed variability), while both race and CYP2E1*1D genotype were predictive of acetaminophen oxidative clearance (23% of the observed variability). None of the independent variables evaluated were associated with acetaminophen sulfation clearance or sulfate to acetaminophen AUC ratios.

DISCUSSION

The major findings of this study are that race, gender and some genetic polymorphisms contribute to individual variability in acetaminophen pharmacokinetics, metabolism, and plasma acetaminophen protein-adduct concentrations. The effect of race was relatively modest resulting in 37% lower acetaminophen oxidation in African-Americans, without discernible differences in acetaminophen glucuronidation, sulfation, or total plasma clearance. These results largely agree with a prior study that showed lower (by 44 to 53%) 24 h fractional urinary recovery of glutathione-derived acetaminophen conjugates in black Ghanaians (5.2% of total excreted) and Kenyans (4.4% of total excreted) versus Scottish whites (9.3% of total excreted), and unchanged sulfate recovery (Critchley et al., 1986). However, they also reported slightly higher (by 7.4%) acetaminophen glucuronide recovery for black Africans compare

with white Europeans. Although we also observed a trend for higher median acetaminophen glucuronidation clearance values in African-Americans (3.3 mL/min/kg) versus European-Americans (2.9 mL/min/kg), this difference (14.4%) did not reach statistical significance ($P = 0.42$). One possible reason for this discrepancy is that the African-American population in the USA is more genetically diverse than Ghanaian and Kenyan populations, which could increase individual variability in acetaminophen metabolism and somewhat limit the ability to detect a population difference. Other reasons could be methodological in that we used a somewhat lower acetaminophen dose (1 gram versus 1.5 gram), collected urine for 12 h instead of 24 h, and studied a somewhat smaller number of African-Americans (41) versus Ghanaians (67) and Kenyans (20).

We also observed shorter plasma half-life values for acetaminophen, acetaminophen glucuronide, and acetaminophen sulfate, and lower metabolite/acetaminophen AUC ratios in women versus men, without any difference in acetaminophen or metabolite clearance. These findings are consistent with gender-related differences in drug and metabolite distribution rather than any effect on drug clearance and/or metabolism. Lower weight-adjusted acetaminophen volume of distribution has been previously reported in women compared with men (Divoll et al., 1982) and is likely a reflection of the relatively poor distribution of acetaminophen (and metabolites) into fatty tissue, which tends to provide a higher proportion of body weight in women than in men (Abernethy et al., 1982).

The UGT2B15*2 polymorphism was identified as a determinant of acetaminophen clearance and glucuronidation partial clearance. This finding is consistent with prior work that showed an association of the UGT2B15*2 allele with lower acetaminophen glucuronide to acetaminophen concentration ratios in urine (Navarro et al., 2011) and blood (Mehboob et al., 2016). Our work extends upon those findings to indicate an effect on total acetaminophen oral clearance, which reflects the important contribution of glucuronidation to acetaminophen clearance (55% in this study). UGT2B15*2 was also associated with increased plasma concentrations of acetaminophen protein-adduct. Presumably this is because UGT2B15*2 causes decreased acetaminophen glucuronidation leading to increased availability of acetaminophen for oxidative metabolism to NAPQI, which then binds to proteins that can subsequently be

detected in the plasma. In support of this, plasma acetaminophen protein-adduct concentrations were most strongly and negatively correlated with acetaminophen glucuronidation.

SULT1A1*2 was associated with decreased plasma acetaminophen sulfate to acetaminophen AUC ratios. This association, although relatively weak, remained significant even after accounting for possible race and gender effects in the multivariate analysis. Although we did see a trend toward lower sulfation clearance in SULT1A1 *2/*2 individuals this did not reach the statistical significance threshold in the univariate or multivariate analyses. The SULT1A1*2 variant has rarely been studied in the context of drug pharmacokinetics (Daniels and Kadlubar, 2014). Several studies have examined the association of SULT1A1*2 with the pharmacokinetics of tamoxifen and major oxidative metabolites (Jin et al., 2005; Gjerde et al., 2008). Although all of these failed to show a significant influence of this gene variant, none of the studies measured the sulfation metabolites directly. A more recent study evaluated the association of four different SULT1A1 gene variants with plasma pharmacokinetics of ABT-751, an experimental cancer treatment (Innocenti et al., 2013). Although they found no association of SULT1A1*2 with ABT-751 pharmacokinetics, they did find a significant association of a SULT1A1 gene copy number variant (CNV) with increased ABT-751 clearance and ABT-751 sulfate to ABT-751 AUC ratios. Consequently, future studies are needed to evaluate associations of acetaminophen sulfation with other SULT1A1 variants, particularly the SULT1A1 CNV.

Analysis of CYP genotype associations with acetaminophen oxidation partial clearance was initially confounded by race effects since all of the CYP genotypes examined differed markedly in frequencies between in African-Americans and European-Americans. However, after controlling for race effects, either by stratification by race or by multivariate analysis, the only variant that showed a significant association with reduced oxidation clearance was CYP2E1*1D. Given the much higher frequency of CYP2E1*1D in black African-derived populations, it is likely that this allele accounts for a proportion of the race-related variability in acetaminophen oxidation. However, since race remained a significant covariate in the multivariate analysis it is likely that other factors influencing acetaminophen oxidation in African-derived populations, including additional genetic polymorphisms, have yet to be discovered. We

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did not observe a significant association between CYP2E1*1D genotype and acetaminophen protein-adduct concentrations. This may be because we assayed acetaminophen protein-adduct concentrations at a single time point corresponding to the expected T_{max} (8 h), rather than over the entire sampling time.

The genetic variants associated with acetaminophen metabolism in the current study (UGT2B15*2, SULT1A1*2, and CYP2E1*1D; but not UGT1A rs8330 or CYP3A5*3) differ from those associated with acetaminophen-induced liver failure in our prior studies (UGT1A rs8330 and CYP3A5*3; but not UGT2B15*2, SULT1A1*2, or CYP2E1*1D) (Court et al., 2013; Court et al., 2014). The most likely reason for this difference is the higher acetaminophen dose (usually over 10 grams) consumed in acetaminophen-induced liver failure patients leading to much higher plasma concentrations compared with the therapeutic dose (1 gram) used here. Consequently, alternate glucuronidation, sulfation, and oxidation enzymes may become quantitatively more important for acetaminophen metabolism at these higher concentrations. Interestingly, although UGT2B15 appears to be the predominant acetaminophen glucuronidation enzyme at therapeutic concentrations (0.1 mM or less), recombinant enzyme studies indicate that there is substantial substrate inhibition of UGT2B15 at higher concentrations (10 mM or higher) (Mutlib et al., 2006). Consequently, other hepatic acetaminophen glucuronidation enzymes, including UGT1A1, UGT1A6, and UGT1A9, which do not demonstrate substrate inhibition, appear to predominate at these higher substrate concentrations (Mutlib et al., 2006). These enzymes are all affected by the rs8330 variant located in the 3'-UTR region of the UGT1A gene.

In summary, our results indicate that acetaminophen total clearance, and partial metabolic clearance by glucuronidation and sulfation is similar in healthy adult African-Americans compared with European-Americans. However, African-Americans, like black Africans from Kenya and Ghana, metabolize acetaminophen by oxidation more slowly than European-derived populations. This difference may be partially explained by the CYP2E1*1D polymorphism, which is more prevalent in black African derived populations. UGT2B15*2 was also identified as a determinant of lower acetaminophen clearance by glucuronidation and higher circulating acetaminophen protein-adduct concentrations in both European-American and African-American populations.

ACKNOWLEDGEMENTS

None.

AUTHOR CONTRIBUTIONS

Participated in research design: Court, Greenblatt.

Conducted experiments: Court, Zhu, Masse, Duan, James.

Contributed new reagents or analytic tools: None.

Performed data analysis: Court, Masse, Harmatz.

Wrote or contributed to the writing of the manuscript: Court, James, Greenblatt.

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FOOTNOTES

This work was supported by the National Institutes of Health, National Institute of General Medical Sciences [Grants R01-GM061834 and R01-GM102130] to M.H.C.

FIGURE LEGENDS

Figure 1. Mean (± standard error) plasma concentrations of acetaminophen (APAP) and its glucuronide and sulfate metabolites (APAP-glucuronide, APAP-sulfate) at corresponding times across all subjects administered one gram of acetaminophen by mouth.

Figure 2. Correlation of acetaminophen (APAP) total plasma clearance with partial urinary clearance by glucuronidation (A), sulfation (B), oxidation (C), and unchanged renal excretion (D) measured in 95 subjects (54 European-Americans and 41 African-Americans; 52 men and 43 women) administered one gram of acetaminophen by mouth. Acetaminophen oxidation clearances are calculated from the sum of the glutathione derived cysteinate and mercapturate metabolites. Also shown are the Spearman correlation coefficients (Rs) and associated *P* values.

Figure 3. Correlation of total acetaminophen plasma clearance (A) and partial glucuronidation clearance (B) with plasma acetaminophen protein-adduct concentrations measured in 95 subjects (54 European-Americans and 41 African-Americans; 52 men and 43 women) at 8 h after administering one gram of acetaminophen by mouth. Also shown are the Spearman correlation coefficients (Rs) and associated *P* values.

Figure 4. Association of UGT2B15 genotype with acetaminophen (APAP) total plasma clearance (A) and partial urinary clearance by glucuronidation (B). Shown are box and whiskers plots of data grouped by UGT2B15 genotype, including genotypes *1/*1 (27 subjects), *1/*2 (45 subjects with total clearance data; 43 subjects with glucuronidation clearance data), and *2/*2 (22 subjects). Also shown are the *P* values that were significant (<0.05) for comparisons between genotype groups by ANOVA with Holm-Sidak multiple comparisons testing on log-transformed data.

Figure 5. Association of race (European-American or European-American) and CYP2E1*1D genotype with partial urinary clearance of acetaminophen (APAP) by oxidation. Shown are box and whiskers plots of data grouped by CYP2E1*1D genotype and race, including genotypes *1C/*1C (49 European-Americans and 22 African-Americans), *1C/*1D (4 European-Americans and 14 African-Americans), and *1D/*1D (3 African-Americans). No European-Americans had the *1D/*1D genotype.

Also shown are the *P* values that were significant (<0.05) for the comparison between European-American and African-American subjects by *t*-test on log-transformed data, and for comparisons between genotype groups within African-American subjects by ANOVA with Holm-Sidak multiple comparisons testing on log-transformed data.

Figure 6. Association of UGT2B15 genotype with acetaminophen (APAP) protein-adduct concentrations. Shown are box and whiskers plots of data grouped by UGT2B15 genotype, including genotypes *1/*1 (27 subjects), *1/*2 (44 subjects), and *2/*2 (21 subjects). Also shown are the *P* values that were significant (>0.05) for comparisons between genotype groups by Kruskal-Wallis ANOVA with Dunn's multiple comparisons testing on rank-transformed data.

JPET Fast Forward. Published on June 29, 2017 as DOI: 10.1124/jpet.117.242107
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TABLES

Table 1. Demographic characteristics of study subjects.

Parameter	European-Americans (N=54)	African-Americans (N=41)	*P* value*
Age, years [median (range)]	30 (18 - 60)	43 (19 - 61)	0.07
Men/Women [N (%)]	34/20 (63/37%)	18/23 (44/56%)	0.01
Weight, kg [median (range)]	72 (52 - 100)	73 (55 - 112)	0.19
Body mass index, kg/m^2 [median (range)]	24 (17 - 30)	26 (20 - 36)	0.01
Nondrinkers [N (%)]	14 (26%)	8 (20%)	0.34
Hormonal contraceptive use [N (% of women)]	5 (25%)	3 (13%)	0.19
Serum ALT at baseline, U/L [median (range)]	18 (9 - 63)	15 (7 - 43)	0.17
Change in ALT at 4 days, U/L [median (range)]	0 (-41 - +9)	0 (-10 - +10)	0.90

*Mann-Whitney rank sum test or Chi-square test for gender, drinking history, and hormonal contraceptive use.

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Table 2. Comparisons of acetaminophen (APAP) pharmacokinetic parameters in study subjects by race.

Parameter	European-Americans (N = 54)	African-Americans (N = 41)	*P* value*
APAP $t_{1/2}$ (h)	2.5 (2.4 - 2.8)	2.5 (2.4 - 3.1)	0.24
APAP plasma clearance (mL/min/kg)	5.3 (4.8 - 6.4)	5.9 (5.1 - 6.9)	0.52
APAP renal clearance (mL/min/kg)	0.16 (0.14 - 0.20)	0.20 (0.15 - 0.24)	0.31
APAP glucuronide $t_{1/2}$ (h)	3.4 (3.1 - 4.2)	3.4 (3.0 - 4.0)	0.38
Glucuronidation clearance (mL/min/kg)	2.9 (2.3 - 3.8)	3.3 (2.4 - 4.1)	0.42
Glucuronide/APAP AUC (ratio)	2.8 (2.1 - 3.7)	2.7 (2.1 - 3.9)	0.66
APAP sulfate $t_{1/2}$ (h)	3.1 (2.9 - 3.6)	3.2 (2.8 - 3.9)	0.34
Sulfation clearance (mL/min/kg)	1.4 (1.1 - 1.7)	1.7 (1.3 - 2.2)	0.06
Sulfate/APAP AUC (ratio)	0.88 (0.71 - 1.04)	0.94 (0.68 - 1.23)	0.35
Oxidation clearance (mL/min/kg)	0.90 (0.65 - 1.15)	0.57 (0.49 - 0.77)	0.0001
APAP protein-adduct (nmol/mL)	0.021 (0.016 - 0.027)	0.018 (0.010 - 0.026)	0.11

Data presented as median (interquartile range)
*t-test on log-transformed data or Mann-Whitney rank sum test for plasma APAP protein-adduct concentrations

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Table 3. Comparisons of acetaminophen (APAP) pharmacokinetic parameters in study subjects by gender.

Parameter	Men (N = 52)	Women (N = 43)	*P* value*
APAP $t_{1/2}$ (h)	2.7 (2.4 - 3.1)	2.4 (2.2 - 2.6)	0.0009
APAP plasma clearance (mL/min/kg)	5.6 (4.8 - 6.6)	5.4 (4.8 - 6.6)	0.74
APAP renal clearance (mL/min/kg)	0.16 (0.13 - 0.21)	0.19 (0.15 - 0.24)	0.067
APAP glucuronide $t_{1/2}$ (h)	3.7 (3.3 - 4.4)	3.2 (2.9 - 3.6)	0.0002
Glucuronidation clearance (mL/min/kg)	3.0 (2.4 - 4.1)	3.0 (2.3 - 3.8)	0.97
Glucuronide/APAP AUC (ratio)	3.0 (2.1 - 4.0)	2.5 (1.9 - 3.1)	0.049
APAP sulfate $t_{1/2}$ (h)	3.5 (3.0 - 3.9)	3.1 (2.8 - 3.2)	0.002
Sulfation clearance (mL/min/kg)	1.4 (1.2 - 1.8)	1.6 (1.1 - 1.9)	0.65
Sulfate/APAP AUC (ratio)	0.94 (0.79 - 1.14)	0.81 (0.61 - 1.03)	0.025
Oxidation clearance (mL/min/kg)	0.76 (0.56 - 0.97)	0.75 (0.58 - 1.13)	0.52
APAP protein-adduct (nmol/mL)	0.021 (0.015 - 0.027)	0.020 (0.013 - 0.028)	0.52

Data presented as median (interquartile range)

*t-test on log-transformed data or Mann-Whitney rank sum test for plasma APAP protein-adduct concentrations.

JPET Fast Forward. Published on June 29, 2017 as DOI: 10.1124/jpet.117.242107
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Table 4. Comparison of minor allele frequencies in study subjects by race.

Enzyme gene variant	European-Americans (N=53)	African-Americans (N=41)	*P* value*
UGT1A rs8330 C/G	0.21	0.51	<0.001
UGT1A1 (TA)N=5or6/7or8	0.37	0.40	0.47
UGT1A6*2 rs6759892; rs2070959; rs1105879	0.42	0.30	0.02
UGT1A9 rs6714486 T/A	0.05	0.14	0.007
UGT2B15*2 rs1902023 T/G	0.50	0.44	0.21
SULT1A1*2 rs9282861 G/A	0.36	0.28	0.07
CYP2E1*1D 6->8 repeats	0.04	0.27	<0.001
CYP2E1*4 rs6413419 G/A	0.01	0.16	<0.001
CYP3A5*3 rs776746 A/G	0.94	0.38	<0.001

*Chi-square test

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Table 5. Results of multivariate analysis by multiple linear regression evaluating the contributions of subject race, gender, and enzyme genotypes to variability in acetaminophen (APAP) pharmacokinetic parameters. Shown are the standardized coefficients (β), associated *P* values, and the regression coefficient of determination (R^2) and *P* value. All pharmacokinetic parameters (dependent variables) were log transformed prior to analysis, except for APAP protein-adduct, which was rank transformed. Race was coded as 0 = European-American, 1 = African-American; Gender was coded as 0 = man, 1 = woman; UGT2B15*2 genotype was coded as 0 = *1/*1, 1 = *1/*2, 2 = *2/*2; SULT1A1*2 genotype was coded as 0 = *1/*1 or *1/*2, 1 = *2/*2; CYP2E1*1D genotype was coded as 0 = *1C/*1C or *1C/*1D, 1 = *1D/*1D.

	Race		Gender		UGT2B15*2		SULT1A1*2		CYP2E1*1D			
Dependent variable	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	R^2	*P* value
APAP clearance	0.03	0.74	-0.01	0.89	-0.40	<0.001	-0.03	0.81	0.03	0.78	0.17	0.006
Glucuronidation clearance	0.04	0.72	-0.08	0.45	-0.53	<0.001	0.02	0.86	0.09	0.33	0.29	<0.001
Glucuronide/APAP AUC	0.04	0.67	-0.26	0.008	-0.51	<0.001	-0.01	0.89	0.08	0.40	0.31	<0.001
Sulfation clearance	0.23	0.04	0.03	0.80	-0.06	0.59	-0.12	0.30	-0.13	0.23	0.08	0.20
Sulfate/APAP AUC	0.17	0.11	-0.20	0.07	-0.01	0.89	-0.21	0.06	-0.05	0.65	0.12	0.05
Oxidation clearance	-0.36	<0.001	0.12	0.25	0.09	0.34	0.06	0.55	-0.23	0.02	0.23	<0.001
APAP protein-adduct	-0.08	0.44	0.04	0.72	0.37	<0.001	-0.18	0.09	-0.17	0.10	0.19	0.003

Figure 1

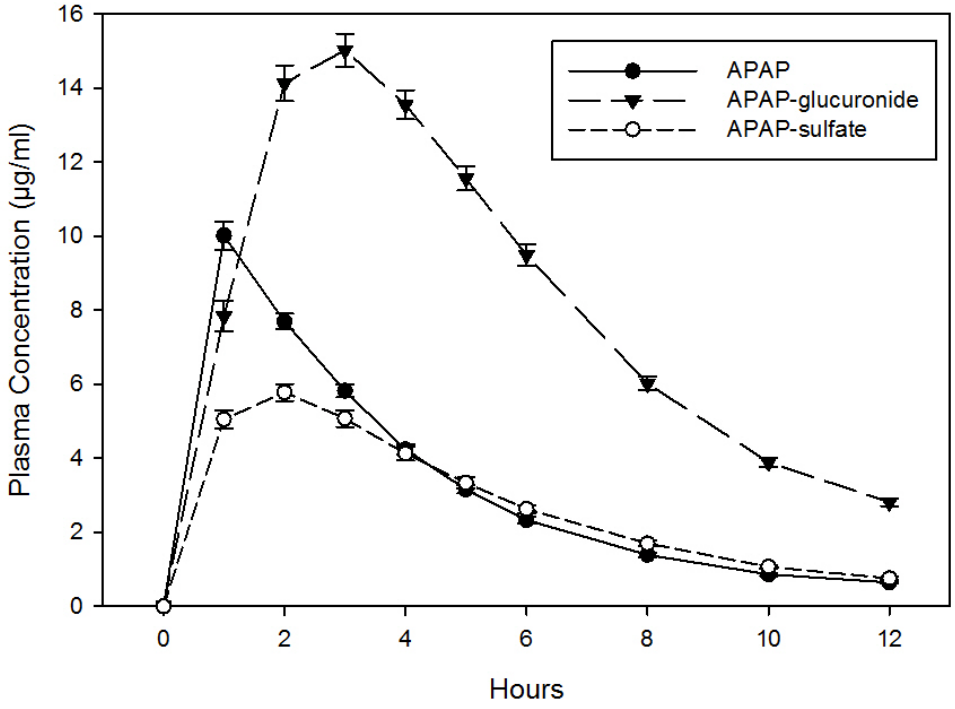
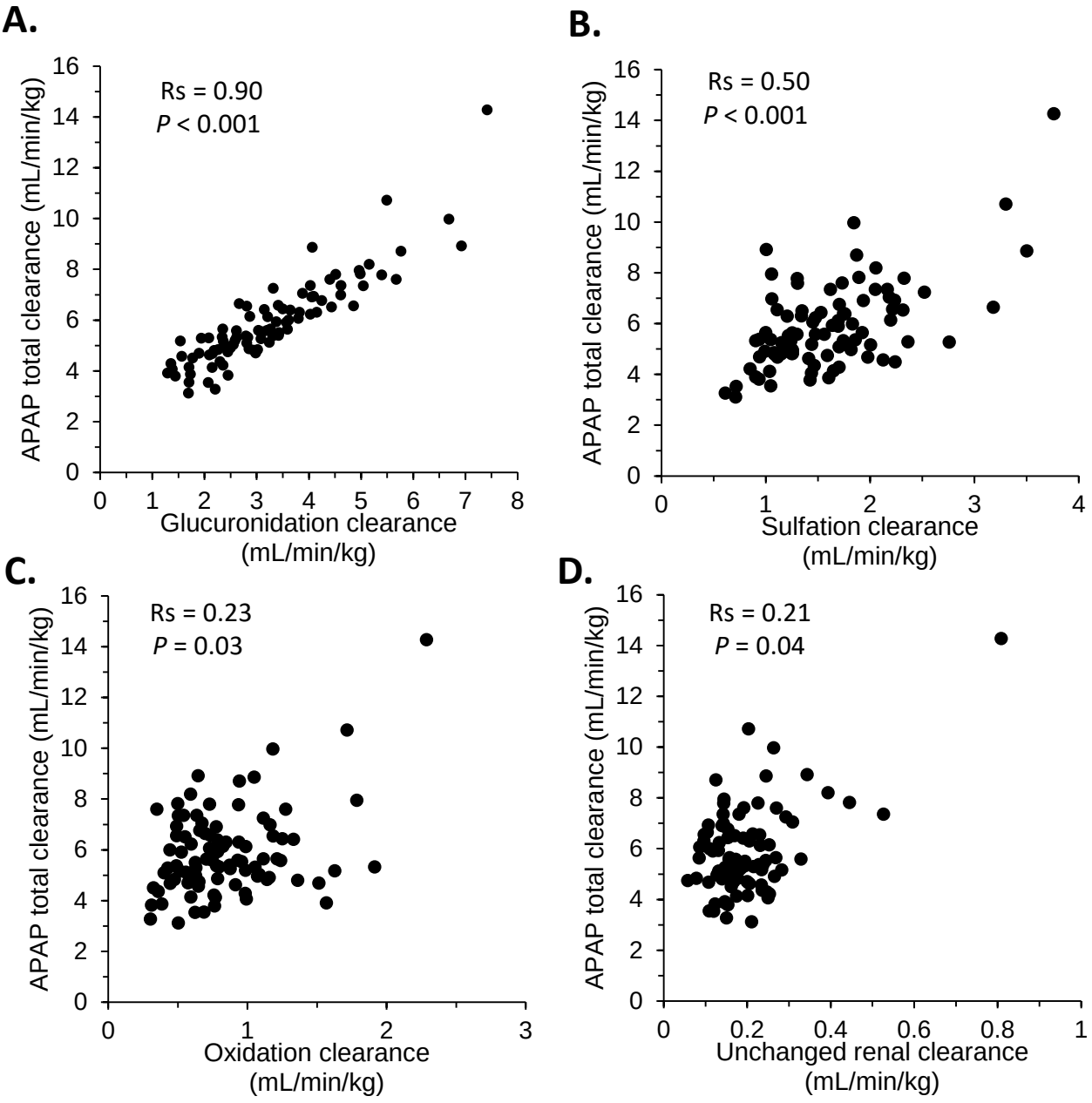

Plasma Concentration (µg/ml)
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
Hours
APAP
APAP-glucuronide
APAP-sulfate

Figure 2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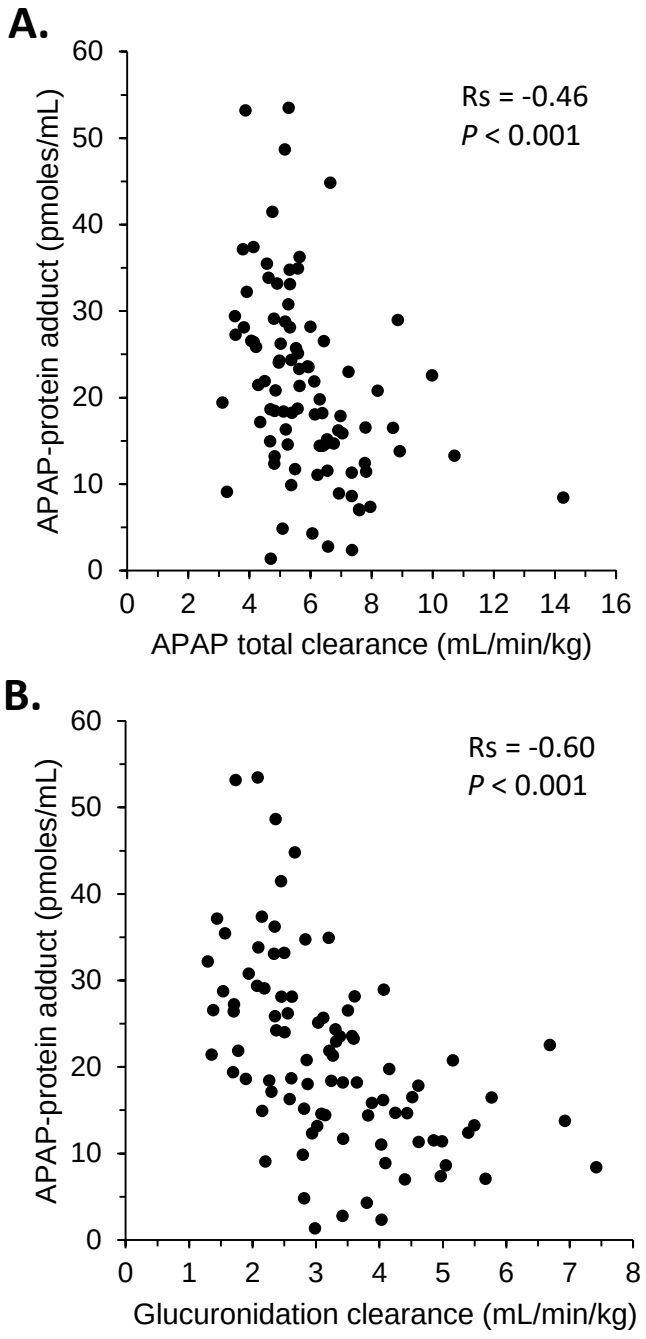
P < 0.001
APAP total clearance (mL/min/kg)
Glucuronidation clearance (mL/min/kg)
B.
Rs = 0.50
P < 0.001
APAP total clearance (mL/min/kg)
Sulfation clearance (mL/min/kg)
C.
Rs = 0.23
P = 0.03
APAP total clearance (mL/min/kg)
Oxidation clearance (mL/min/kg)
D.
Rs = 0.21
P = 0.04
APAP total clearance (mL/min/kg)
Unchanged renal clearance (mL/min/kg)

Figure 3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0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
B.
Rs = -0.60
P < 0.001
APAP-protein adduct (pmoles/mL)
Glucuronidation clearance (mL/min/kg)
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20
30
40
50
60
0
1
2
3
4
5
6
7
8

Figure 4

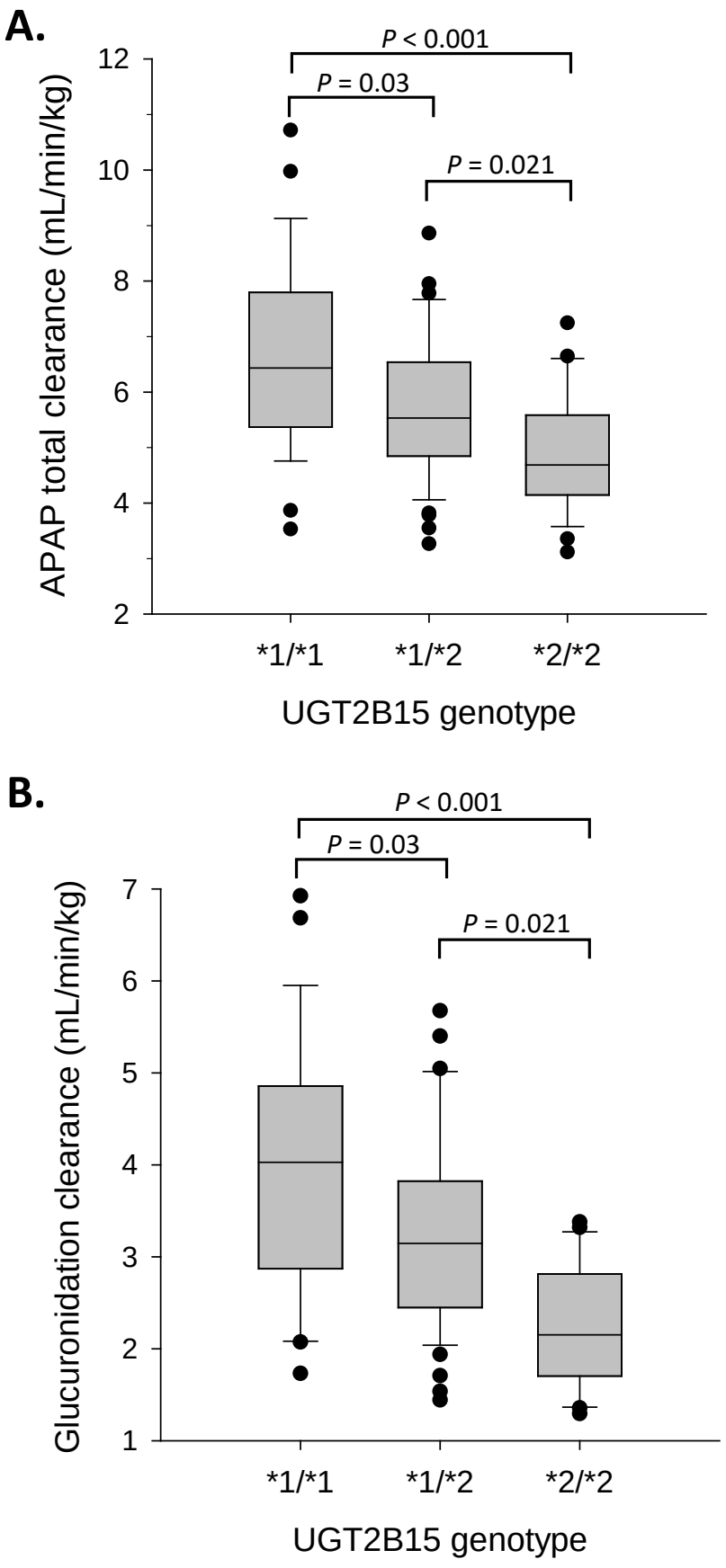

A.
P < 0.001
P = 0.03
P = 0.021
APAP total clearance (mL/min/kg)
12
10
8
6
4
2
*1/*1
*1/*2
*2/*2
UGT2B15 genotype
B.
P < 0.001
P = 0.03
P = 0.021
Glucuronidation clearance (mL/min/kg)
7
6
5
4
3
2
1
*1/*1
*1/*2
*2/*2
UGT2B15 genotype

Figure 5

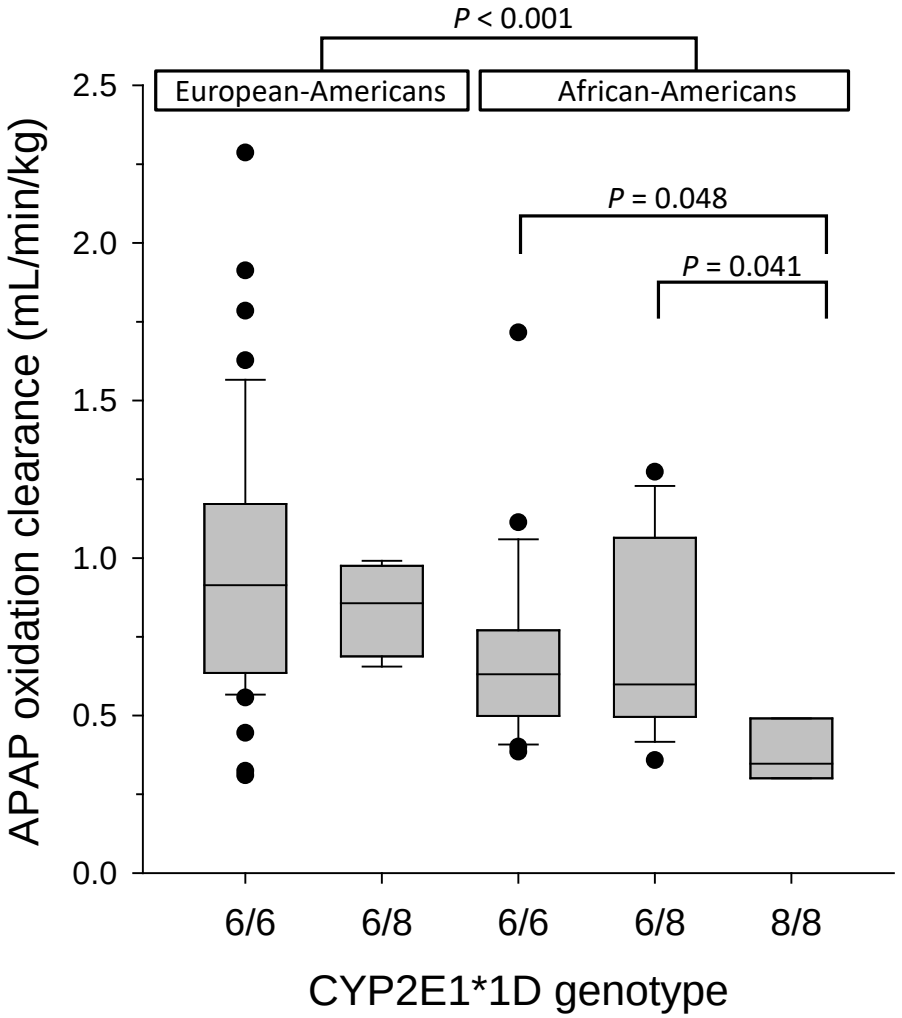
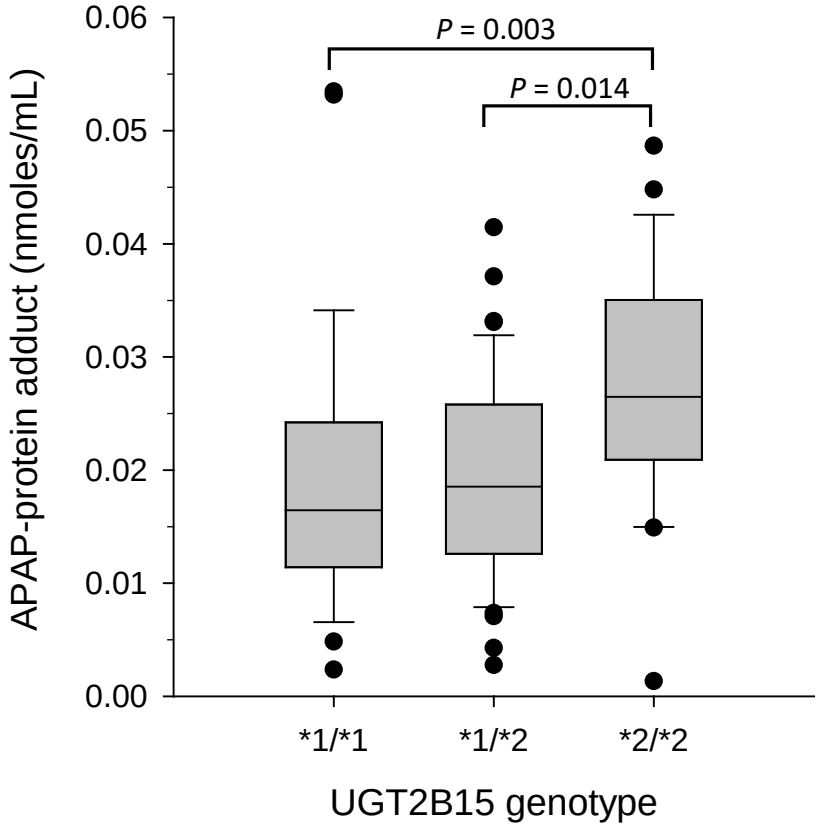

P < 0.001
European-Americans
African-Americans
P = 0.048
P = 0.041
APAP oxidation clearance (mL/min/kg)
2.5
2.0
1.5
1.0
0.5
0.0
6/6
6/8
6/6
6/8
8/8
CYP2E1*1D genotype

Figure 6


P = 0.003
P = 0.014
0.06
0.05
0.04
0.03
0.02
0.01
0.00
APAP-protein adduct (nmoles/mL)
*1/*1
*1/*2
*2/*2
UGT2B15 genotype