Title: Predictors of variation in CYP2A6 mRNA, protein, and enzyme activity in a human liver bank: influence of genetic and non-genetic factors

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Running Title: Impact of genetic and non-genetic factors on CYP2A6

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ABSTRACT

Cytochrome P450 2A6 (CYP2A6) metabolizes several clinically relevant substrates, including nicotine, the primary psychoactive component in cigarette smoke. Smokers vary widely in their rate of inactivation and clearance of nicotine, altering numerous smoking phenotypes. We aimed to characterize independent and shared impact of genetic and non-genetic sources of variation in CYP2A6 mRNA, protein, and enzyme activity in a human liver bank (n=360). For the assessment of genetic factors, we quantified levels of CYP2A6, cytochrome P450 oxidoreductase (POR), and aldo-keto reductase 1D1 (AKR1D1) mRNA, and CYP2A6 and POR protein. CYP2A6 enzyme activity was determined through measurement of cotinine formation from nicotine and 7-hydroxycoumarin formation from coumarin. Donor DNA was genotyped for *CYP2A6*, *POR*, and *AKR1D1* genetic variants. Non-genetic factors assessed included: gender, age, and liver disease. CYP2A6 phenotype measures were positively correlated to each other (r values ranging from 0.47-0.88, $P<0.001$). Female donors exhibited higher CYP2A6 mRNA expression relative to males ($P<0.05$). Donor age was weakly positively correlated with CYP2A6 protein (r=0.12, $P<0.05$) and activity (r=0.20, $P<0.001$). CYP2A6 reduce-of-function genotypes, but not POR or AKR1D1 genotype, were associated with lower CYP2A6 protein ($P<0.001$) and activity ($P<0.01$). AKR1D1 mRNA was correlated with CYP2A6 mRNA (r=0.57, $P<0.001$), protein (r=0.30, $P<0.001$), and activity (r=0.34, $P<0.001$). POR protein was correlated with CYP2A6 activity (r=0.45, $P<0.001$). Through regression analyses, we accounted for 17% ($P<0.001$), 37% ($P<0.001$), and 77% ($P<0.001$) of the variation in CYP2A6 mRNA, protein, and activity, respectively. Overall, several independent and shared sources of variation in CYP2A6 activity *in vitro* have been identified, which could translate to variable hepatic clearance of nicotine.

INTRODUCTION

Cytochrome P450 2A6 (CYP2A6) metabolizes several clinically relevant substrates, including nicotine, tegafur, letrozole, efavirenz, valproic acid, and pilocarpine (Messina et al., 1997; Ikeda et al., 2000; Kiang et al., 2006; Endo et al., 2007; di Iulio et al., 2009; Murai et al., 2009). Nicotine metabolism by CYP2A6 is of interest as nicotine is the primary psychoactive component in cigarette smoke and the main source of tobacco dependence (Stolerman and Jarvis, 1995). Smokers can vary widely in their rate of inactivation and clearance of nicotine (Malaiyandi et al., 2005), with variations in the rate of nicotine metabolism associating with differences in smoking behaviors, cessation, and response to cessation pharmacotherapies (Schoedel et al., 2004; Patterson et al., 2008; Schnoll et al., 2009). The major pathway of nicotine inactivation is its conversion to cotinine, primarily catalyzed by CYP2A6 (Messina et al., 1997).

Genetic and non-genetic factors contribute to variation in CYP2A6 enzyme activity and rate of nicotine metabolism. The *CYP2A6* gene, encoding the CYP2A6 enzyme, is highly polymorphic, and *CYP2A6* genetic variation is associated with variable rates of nicotine metabolism *in vitro* and *in vivo* (Al Koudsi et al., 2010; Binnington et al., 2012), and accordingly with differences in smoking behavior (Wassenaar et al., 2011; Chen et al., 2014). A significant proportion of variation in CYP2A6 activity can be attributed to *CYP2A6* genetic variation (heritability estimates of 60-80%) (Swan et al., 2009; Loukola et al., 2015), however unaccounted for variation remains even after controlling for additional factors (age, sex, race/ethnicity, body mass index, cigarettes per day, and total nicotine equivalents) (Chenoweth et al., 2014a; Park et al., 2016). Unidentified *CYP2A6* genetic variation, or variation in other genes regulating CYP expression or function, may account for the 35% missing variation.

AKR1D1, an enzyme involved in bile acid synthesis (Schuetz et al., 2001; Lee et al., 2009), has been identified as a potential regulator of CYP activity (Yang et al., 2010). *AKR1D1* SNP rs1872930, associated with higher *AKR1D1* mRNA expression, is associated with increased

expression and activity of several CYPs *in vitro* (Chaudhry et al., 2013). To our knowledge, the relationship between rs1872930 and CYP2A6 has not yet been investigated. Cytochrome P450 oxidoreductase (POR), an enzyme that donates electrons to CYPs during their catalytic cycle (Hu et al., 2012), also contributes to variation in multiple CYP activities, including CYP2A6 (Gomes et al., 2009; Chenoweth et al., 2014b; Lv et al., 2016).

Combined effects of *CYP2A6*, *AKR1D1*, and *POR* genetic variation should also be considered. *POR* SNP rs1057868 interacts with *CYP2A6* genotype, with rs1057868 only associating with faster CYP2A6 activity among individuals not possessing known *CYP2A6* reduce-of-function genetic variants (Chenoweth et al., 2014b). Due to potential shared effects of each genetic factor on CYP2A6 activity, we aimed to elucidate both the independent and combined influences of *CYP2A6*, *POR*, and *AKR1D1* genetic variation on CYP2A6 mRNA expression, protein levels, and enzyme activity. We also aimed to investigate relationships between POR and AKR1D1 expression, independent of genotype, with variation in CYP2A6 expression and activity.

Several non-genetic factors, including gender, age, and liver disease, have been associated with altered CYP2A6 expression, function, and nicotine pharmacokinetics. Here we will examine independent and combined impacts of these variables in a large human liver bank (n=360) with extensive CYP2A6 phenotyping. Relative to men, females had higher microsomal CYP2A6 protein levels in a smaller human liver bank (n=67) (Al Koudsi et al., 2010), and women smokers exhibited greater CYP2A6-mediated nicotine metabolism (Benowitz et al., 2006), consistent with estrogen-mediated induction of *CYP2A6* transcription (Benowitz et al., 2006; Higashi et al., 2007). Individuals 65 and older have been shown to have lower nonrenal nicotine clearance than those ages 22-43 (Molander et al., 2001), however there was no association between age and CYP2A6 protein or activity when investigated in a smaller human liver bank (n=67) (Al Koudsi et al., 2010). Nonalcoholic fatty liver disease was associated with higher CYP2A6 mRNA expression and enzyme activity *in vitro* (Fisher et al., 2009).

Despite several known sources of variation in CYP2A6 activity, substantial variation remains to be characterized (Swan et al., 2009). Considering the clinical relevance of CYP2A6, it is important to further characterize causes of variability in CYP2A6 activity and thus the rate of nicotine metabolism, which can be used to tailor smoking cessation treatment (Lerman et al., 2015). We aimed to assess independent and shared impacts of *CYP2A6*, *POR*, and *AKR1D1* genotypes and levels, as well as gender, age, and liver disease on CYP2A6 mRNA expression, protein expression, and enzyme activity in a large human liver bank.

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MATERIALS AND METHODS

Chemicals and reagents

Lodoacetamide, dithiothreitol, and sequencing grade trypsin were purchased from Pierce Biotechnology (Rockford, IL). Ammonium bicarbonate was purchased from Acros Organics (Geel, Belgium). Sodium deoxycholate (98% purity) was obtained from MP Biomedicals (Santa Ana, CA). Synthetic light peptides for CYP2A6 and POR quantification were procured from New England Peptides (Boston, MA), with purity established by amino acid analysis. Heavy stable isotope labeled amino acids, [$^{13}C_6{}^{15}N_2$]-lysine and [$^{13}C_6{}^{15}N_4$]-arginine, were purchased from Pierce Biotechnology, Inc. (Rockford, IL). LC-MS grade acetonitrile (99.9% purity) and formic acid (≥99.5% purity) were purchased from Fischer Scientific (Fair Lawn, NJ). (–)-Nicotine hydrogen tartrate, (–)-cotinine, coumarin, 7-hydroxycoumarin, and 4-hydroxycoumarin were purchased from Sigma-Aldrich (St. Louis, MO); chemical structures illustrated in Emami and Dadashpour, 2015. Nicotine-D_4 and cotinine-D_3 were purchased from Toronto Research Chemicals (Toronto, ON); chemical structures illustrated in Hukkanen et al., 2005.

Human liver bank

Human liver tissue samples are from two liver banks: (1) the St. Jude Liver Resource at the St. Jude Children's Research Hospital (Memphis, TN, USA) (n=295), and (2) the University of Washington Human Liver Bank (Seattle, WA, USA) (n=65). The St Jude Liver Resource human liver tissues were obtained through the Liver Tissue Cell Distribution System, Minneapolis, Minnesota and Pittsburgh, Pennsylvania, which was funded by NIH Contract # HHSN276201200017C. Details on the selection of the livers and investigator blinding for sample analyses have been described previously (Shirasaka et al., 2015). Age, gender, and ethnicity were known for most ($\geq$90%) of the liver donors. The donors ranged in age from 0-87 years (mean 40 years, SD $\pm$ 22 years). Of the donors with known gender, 58% were male. The liver bank consists of 92% Caucasian, 3% African American, <1% Asian, <1% Hispanic, and 5% unknown ethnicity donors. Cause of death, medications used, and liver pathology was known

for less than 50% of donors. Smoking status was unknown for >88% of donors and therefore was not assessed as a predictor of CYP2A6 phenotypes in the present study

CYP2A6, POR, and AKR1D1 mRNA quantification

RNA Isolation

Liver RNA was isolated and purified using a NucleoSpin® miRNA kit (Macherey-Nagel, Duren, Germany; Clonetech Labs, Mountain View, CA).), according to manufacturer's protocol. Briefly, ~30 mg liver tissue was combined with 4°C Lysis buffer, homogenized using a TissueLyser LT (Qiagen, Valencia, CA), and allowed to sit at room temperature for 5 minutes. The solution was then added to a column. After centrifugation to bind the large RNA to the column, the column was treated with an rDNAse solution at room temperature for at least 15 minutes. Meanwhile, the flow-through containing the small RNA was treated, in order to precipitate out the protein. The small RNA was then bound to a new column. Following three wash steps of each column, the resulting large and small RNA were each eluted, quantitated and bioanalyzed for quality control using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Only RNA with an RNA integrity score greater or equal to 7.0 was submitted for sequencing.

TruSeq Stranded mRNA Preparation

Next-generation sequencing libraries were prepared from 1.25 µg of total RNA in an automated, high-throughput format using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA). All the steps required for sequence library construction have been automated and performed on a Sciclone NGSx Workstation (Perkin Elmer, Waltham, MA). During library construction, ribosomal RNA was depleted by means of a poly-A enrichment and first and second strand cDNA syntheses were performed. Each library was then uniquely barcoded using the Illumina adapters and amplified using a total of 13 cycles of PCR. After amplification and cleanup, library concentrations were quantified using the Quant-it dsDNA Assay (Life Technologies, Carlsbad, CA). Libraries were subsequently normalized and pooled based on Agilent 2100 Bioanalyzer results (Agilent Technologies, Santa Clara, CA). Pooled libraries were

size selected using a Pippin Prep (Sage Science, Beverly, MA) and then balanced by mass and pooled in batches of 96 with a final pool concentration of 2-3 nM for sequencing on the HiSeq 2500.

Read Processing and Analysis Pipeline

The Northwest genome sequencing lab processing pipeline included the following elements: (1) base calls generated in real-time on the HiSeq or NextSeq instrument; (2) Illumina RTA-generated BCL files converted to FASTQ files; (3) custom scripts developed in-house and used to process the FASTQ files and to output de-multiplexed FASTQ files by lane and index sequence; (4) sequence read and base quality checked using the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); (5) sequences aligned to hg19 with reference transcriptome Ensembl v67 using Tophat (Kim et al., 2013) followed by mate-fixing; and (6) custom scripts for quality assessment generate metrics. All aligned read data were subject to the following steps: (1) lane level bam data files were merged using the Picard MergeSamFiles tool and suspected PCR duplicates were marked, not removed, in the alignment files using the Picard MarkDuplicates tool (http://broadinstitute.github.io/picard/); (2) local realignment performed around indels, and base quality score recalibration was run using GATK tools (McKenna et al., 2010); (3) variant detection performed with the GATK Unified Genotyper version 2.6.5 (DePristo et al., 2011); (4) aligned data were used for isoform assembly and quantitation with Cufflinks (Kim et al., 2013; Trapnell et al., 2013); genomic features were quantitated with featureCounts (Liao et al., 2014); and (5) gene-specific quantitation data were used for further analysis.

CYP2A6 and POR protein quantification

Simultaneous quantification of CYP2A6 and POR was carried out using a LC-MS/MS proteomics method (Prasad and Unadkat, 2014). The surrogate peptides were selected and light and heavy peptides (Table 1) containing labeled [$^{13}C_6{}^{15}N_2$]-lysine or [$^{13}C_6{}^{15}N_4$]-arginine

residues were procured. Liver microsomal samples were diluted to 2 mg/ml, and 40 µg microsomal protein was digested as described before (Shuster et al., 2014). Briefly, microsomal protein was denatured and reduced with 4 µl of 100 mM dithiothreitol, 10 µl of sodium deoxycholate (2.6 % w/v) and 10 µl of ammonium bicarbonate buffer (100 mM) at 95°C for 5 min. The denatured protein was then alkylated by 4 µl of 200 mM iodoacetamide at room temperature. The digestion was performed by addition 10 µl of trypsin (protein:trypsin ratio, 25:1) at 37°C for 22 hours. The reaction was quenched by the addition of 20 µl of peptide internal standard cocktail (prepared in 50% acetonitrile in water containing 0.1% formic acid) and 10 µl of the neat solvent, i.e., 50% acetonitrile in water containing 0.1% formic acid. The samples were vortexed and centrifuged at 3500 × g for 5 min. The calibration curves were generated using serial dilutions of light peptide standard in phosphate buffer (50 mM Kpi, 0.25 M sucrose, 10 mM EDTA, pH 7.4) to replace microsomal sample.

Triple-quadrupole LC-MS instrument (Agilent 6460A) coupled to an Agilent 1290 Infinity LC system (Agilent Technologies), in ESI positive ionization mode was used for quantification. 2 µg of the trypsin digest was injected onto the column (Kinetex 1.7 µ, C18 100A; 100 × 2.1 mm, Phenomenex, Torrance, CA). Mobile phase and gradient program were exactly the same as described before (Shuster et al., 2014). Surrogate light and heavy (internal standards) peptides were monitored using instrument parameters provided in Supplementary Table 1. The LC-MS/MS data were processed using MassHunter (Agilent Technologies) and Skyline (University Of Washington) software.

CYP2A6 enzyme activity assays

Human liver microsomes were prepared, and total protein concentrations were quantified, as described previously (Shirasaka et al., 2015). CYP2A6 enzyme activity was determined by quantifying the rate of metabolism of two known substrates of this enzyme, nicotine and coumarin. Linear conditions for the rate of nicotine metabolism (i.e. the rate of

cotinine formation from nicotine) were established for the following assay conditions: 0.5 mg/ml microsomal protein, 50 µM Tris-HCl buffer (pH 7.4), 30 µM nicotine, 1 mM NADPH, 10 µl cytosol (source of aldehyde dehydrogenase), and water to a final volume of 100 µl, for an incubation time of 20 minutes at 37°C. The reaction was terminated with 20 µl of 20% Na_2CO_3, and 20 ng of nicotine-D_4 and cotinine-D_3 internal standards were added. Samples were extracted and analyzed using LC-MS/MS, as described previously (Jacob et al., 2011; Craig et al., 2014).

In order to allow for adequate detection of 7-hydroxycoumarin and avoid substrate depletion, linear conditions for the rate of coumarin metabolism (i.e. the rate of 7-hydroxycoumarin formation from coumarin, a very rapidly metabolized CYP2A6 substrate) were established at multiple incubation times and protein concentrations using a number of substrate concentrations. For donors who exhibited a relatively slow rate of *in vitro* nicotine metabolism (cotinine formation velocity of <0.1 nmol/min/mg), 0.05 mg/ml microsomal protein and 15 min incubation was used. For donors exhibiting intermediate rates of nicotine metabolism (cotinine formation velocity of 0.1-0.3 nmol/min/mg), the assay was adjusted to 0.02 mg/ml microsomal protein for a 10 minute incubation, and for donors with fast rates of nicotine metabolism (cotinine formation velocity of >0.3 nmol/min/mg), 0.01 mg/ml microsomal protein for a 7 min incubation was used. All other assay conditions were identical among slow, intermediate, and fast metabolizers, including 50 mM Tris-HCl buffer (pH 7.4), 2 µM coumarin, 1 mM NADPH, and water to a final volume of 200 µl with an incubation at 37°C. The reactions were terminated with 40 µl of trichloroacetic acid (20% w/v), and 25 ng of 4-hydroxycoumarin internal standard was added. Samples were extracted and analyzed using high performance liquid chromatography, as described previously, (Li et al., 1997) with minor modifications. Limits of quantification for nicotine, cotinine, coumarin, and 7-hydroxycoumarin were 1 ng/ml, 1 ng/ml, 50 ng/ml, and 10 ng/ml, respectively.

CYP2A6, POR, and AKR1D1 genotyping

DNA was extracted using the DNeasy tissue kit. DNA from all donors was genotyped for the *CYP2A6* alleles **2*, **4*, **9*, and **12*, whereas DNA from African American and unknown ethnicity donors were also genotyped for *CYP2A6 *17*, **20*, **23*, **25*, **28*, and **35*, and Asian and unknown ethnicity donors were additionally genotyped for *CYP2A6 *7*, **8*, and **10*, as these alleles have zero to extremely low frequencies among Caucasians (Mwenifumbo and Tyndale, 2007). Genotyping for the *CYP2A6* alleles was conducted using a two-step allele-specific polymerase chain reaction approach, except for *CYP2A6*2*, which was genotyped using a TaqMan SNP genotyping assay (Applied Biosystems) and real-time polymerase chain reaction; *CYP2A6* genotyping approaches have been described in detail previously (Wassenaar et al., 2016). All donors were genotyped for *POR* SNPs rs17148944, rs2868177, rs1057868 and *AKR1D1* SNP rs1872930 using allele-specific TaqMan SNP genotyping assays (Applied Biosystems).

Statistical Analyses

The following data were non-normally distributed, and therefore non-parametric statistical tests were used: CYP2A6 mRNA, AKR1D1 mRNA, POR mRNA, CYP2A6 protein, POR protein, nicotine metabolism, and coumarin metabolism. Correlations were determined via Spearman rank correlations. We used the Mann Whitney test to analyze the association of gender, liver disease, and *AKR1D1* genotype with CYP2A6 mRNA, CYP2A6 protein, and CYP2A6 activity. The Kruskal-Wallis test was used to determine associations between *CYP2A6* genotype and CYP2A6 mRNA, CYP2A6 protein, and CYP2A6 activity, as well as between POR SNP genotypes and POR mRNA, POR protein, and CYP2A6 activity. We ran separate linear regression models for CYP2A6 mRNA, CYP2A6 protein, and CYP2A6 activity to calculate the proportion of variation in each that is accounted for by each variable in the model. The liver bank is comprised of samples collected and processed at two different research sites; the phenotype means were different between sites. For the purposes of illustrating the combined data we have corrected for overall site differences using the following conversion factor: mean

phenotype measure (CYP2A6 mRNA, AKR1D1 mRNA, POR mRNA, CYP2A6 protein, POR protein, rate of nicotine metabolism, and rate of coumarin metabolism) from University of Washington site divided by the mean phenotype measure from St. Jude site, which was multiplied by each sample from the University of Washington site (the smaller liver bank). Findings are illustrated using the site-corrected measures. Additional regression models were run in which the original, non-corrected phenotype data was included, along with the liver bank site (University of Washington or St. Jude) as a covariate. Both versions of each model (corrected vs. uncorrected data with site as covariate) produced very similar results, indicating that the conversion factor had a minimal effect but allowed for combining the data sets for illustration purposes. Analyses were conducted with GraphPad Prism (v6.0) and SPSS (v22), and statistical tests were considered significant for $P<0.05$.

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RESULTS

Moderate to strong correlation between CYP2A6 mRNA, protein, and activity

The two *in vitro* measures of CYP2A6 activity, velocity of cotinine formation from nicotine, and velocity of 7-hydroxycoumarin formation from coumarin, were strongly positively correlated (Spearman r=0.86, *P*<0.001; Fig. 1A). The rate of nicotine metabolism serves as the primary measure of CYP2A6 activity in all analyses, and all coumarin metabolism data has been included in the supplementary materials. CYP2A6 mRNA and protein expression were moderately positively correlated (Spearman r=0.47, *P*<0.001; Fig. 1B). CYP2A6 protein was strongly positively correlated with both measures of CYP2A6 activity (nicotine metabolism: Spearman r=0.88, *P*<0.001; Fig. 1C; coumarin metabolism: Spearman r=0.81, *P*<0.001; Supplementary Fig. 1). All correlations were similar among males and females, and among wild-type (*CYP2A6 *1/*1*) donors and the whole sample (donors with wild-type and variant genotypes included); the rate of nicotine and coumarin matabolism were strongly correlated in females (r=0.86, *P*<0.001) and males (r=0.86, *P*<0.001); CYP2A6 mRNA and CYP2A6 protein were moderately correlated in females (r=0.53, *P*<0.001) and males (r=0.42, *P*<0.001); CYP2A6 protein and CYP2A6 enzyme activity (cotinine formation) were strongly correlated in females (r=0.88, *P*<0.001) and males (r=0.88, *P*<0.001); CYP2A6 mRNA and protein for the wild-type donors only were moderately correlated (r=0.50, *P*<0.001), CYP2A6 mRNA and enzyme activity (cotinine formation from nicotine) were moderately correlated (r=0.49, *P*<0.001), and CYP2A6 protein and enzyme activity were strongly correlated (r=0.87, *P*<0.001). The mean, standard deviation, and range for each CYP2A6 phenotype measure were as follows: CYP2A6 mRNA 373 $\pm$ 481 (0.2-4940) FPKM values, CYP2A6 protein 22.5 $\pm$ 19.4 (0.0-121.1) pmol/mg, CYP2A6 activity (nicotine metabolism) 0.10 $\pm$ 0.10 (0.0007-0.55) nmol/min/mg, and CYP2A6 activity (coumarin metabolism) 0.52 $\pm$ 0.68 (0.01-4.51) nmol/min/mg.

Impact of non-genetic factors on CYP2A6 mRNA, protein, and activity

Gender was associated with differences in CYP2A6 mRNA expression, protein levels, and enzyme activity. Female liver donors exhibited higher CYP2A6 mRNA levels compared to males (P<0.05; Fig. 2A). Although not statistically significant, there was a trend for higher CYP2A6 protein (P<0.1; Fig. 2B) and enzyme activity (P<0.1 for both nicotine and coumarin metabolism; Fig. 2C and Supplementary Fig. 2) among females relative to males

There was no association between donor age and CYP2A6 mRNA expression (Spearman r=0.04, P>0.1; Fig. 3A), while age was weakly positively correlated with both CYP2A6 protein expression (Spearman r=0.12, P<0.05; Fig. 3B) and enzyme activity (nicotine metabolism: Spearman r=0.20, P<0.001; Fig. 3C; coumarin metabolism: Spearman r=0.13, P<0.05; Supplementary Fig. 3).

Liver disease was defined as being positive for at least one of the following conditions: hepatitis, liver injury, biliary atresia, cirrhosis, fat accumulation, fibrosis, or hepatoma. There was no association between liver disease state and CYP2A6 mRNA (normal vs. disease, means=383 and 299 FPKM values, respectively, P>0.1), protein (normal vs. disease, means=21.6 and 20.0 pmol/mg, respectively, P>0.1), or enzyme activity (normal vs. disease, nicotine means=0.10 and 0.09 nmol/min/mg, respectively, coumarin means=0.55 and 0.47 nmol/min/mg, respectively, P>0.1 for both nicotine and coumarin metabolism).

CYP2A6 genotype is associated with CYP2A6 protein and enzyme activity, but not CYP2A6 mRNA

The *CYP2A6* genetic variants **2*, **4*, **9*, **10*, **12*, and **17* were identified among one or more liver donors, while **7*, **8*, **20*, **23*, **25*, **28, or **35* were not. Liver donors were grouped according to their *CYP2A6* genotype (e.g. **1/*9*), where wild-type (**1/*1*) donors were those who did not possess any tested *CYP2A6* genetic variant, and the “all variants” group included all donors that possessed one or more *CYP2A6* genetic variant. CYP2A6 mRNA expression did not differ across all genotypes (P>0.1; Fig. 4A), and there was no difference between the wild-type and the all variants groups (P>0.1; Fig. 4A). There was an apparent gene-dose effect on

CYP2A6 mRNA expression with increasing copies of the *CYP2A6*9* allele (mean FPKM values: **1/*1* 386, **1/*9* 326, **9/*9* 213); this TATA box variant has previously been associated with decreased CYP2A6 transcription (Pitarque et al., 2001). Although no individual genotypes were significantly different with respect to CYP2A6 mRNA, protein, or activity, *CYP2A6* genotype was associated with differences in CYP2A6 protein and activity such that liver donors possessing one or more *CYP2A6* genetic variant exhibited lower CYP2A6 protein expression (P<0.001; Fig. 4B) and enzyme activity (P<0.01 for both nicotine and coumarin metabolism; Fig. 4C and Supplementary Fig. 4) relative to the wild-type *CYP2A6*1/*1* donors. Additionally, there was a gene-dose effect on the CYP2A6 phenotypes for *CYP2A6*9* (CYP2A6 protein: **1/*1* 24.3, **1/*9* 20.8, **9/*9* 8.9 pmol/mg; CYP2A6 activity: **1/*1* 0.11, **1/*9* 0.09, **9/*9* 0.03 nmol/min/mg; Fig. 4A-C) and *CYP2A6*2* (CYP2A6 protein: **1/*1* 24.3, **1/*2* 13.0, **2/*2* 3.0 pmol/mg; CYP2A6 activity: **1/*1* 0.11, **1/*2* 0.06, **2/*2* 0.01 nmol/min/mg; Fig. 4A-C). A wide degree of variation in CYP2A6 mRNA (0.9-4940 FPKM values), protein (0.0-121.1 pmol/mg), and enzyme activity (nicotine metabolism: 0.002-0.55 nmol/min/mg; coumarin metabolism: 0.02-4.51 nmol/min/mg) was observed in the wild-type genotype group.

AKR1D1 mRNA expression, but not genotype, is associated with CYP2A6 mRNA, protein, and activity

Based on work by Chaudhry and colleagues (Yang et al., 2010; Chaudhry et al., 2013), we investigated the correlation between AKR1D1 mRNA expression and CYP2A6 mRNA, protein, and enzyme activity, and further the association between the *AKR1D1* SNP rs1872930 with AKR1D1 mRNA expression, CYP2A6 mRNA, protein, and enzyme activity. AKR1D1 mRNA expression was moderately correlated with CYP2A6 mRNA expression (Spearman r=0.57, P<0.001; Fig. 5A), protein levels (Spearman r=0.30, P<0.001; Fig. 5B), and enzyme activity (nicotine metabolism: Spearman r=0.34, P<0.001; Fig. 5C; coumarin metabolism: Spearman r=0.30, P<0.001; Supplementary Fig. 5). There was no difference in the association between AKR1D1 mRNA and CYP2A6 mRNA, protein, or enzyme activity in females versus males;

AKR1D1 mRNA and CYP2A6 mRNA were moderately correlated in females (r=0.49, *P*<0.001) and males (r=0.60, *P*<0.001); AKR1D1 mRNA and CYP2A6 protein were weakly to moderately correlated in females (r=0.26, *P*<0.001) and males (r=0.34, *P*<0.001); AKR1D1 mRNA and CYP2A6 enzyme activity were moderately correlated in females (r=0.30, *P*<0.001) and males (r=0.34, *P*<0.001). There was no association between rs1872930 and AKR1D1 mRNA (TT vs TC genotype, means=33.8 and 32.5 FPKM values, respectively, *P*>0.1), CYP2A6 mRNA (TT vs TC genotype, means=363 and 389 FPKM values, respectively, *P*>0.1), CYP2A6 protein (TT vs TC genotype, means=22.1 and 23.2 pmol/mg, respectively, *P*>0.1), or CYP2A6 activity (TT vs TC genotype, nicotine means=0.10 and 0.10 nmol/mg/min, respectively, coumarin means= 0.51 and 0.52 nmol/mg/min, respectively, *P*>0.1 for both nicotine and coumarin metabolism). The mean, standard deviation, and range of AKR1D1 mRNA expression were follows: 33.7 $\pm$ 32.3 (0.1-152) FPKM values.

POR protein levels, but not genotype, are positively correlated with CYP2A6 activity

POR protein and CYP2A6 activity were moderately correlated (r=0.45, *P*<0.001 for both nicotine and coumarin metabolism; Fig. 6A and Supplementary Fig. 6). Based on the literature (Gomes et al., 2009; Chenoweth et al., 2014b; Lv et al., 2016), we investigated the association of three POR SNPs (rs17148944, rs2868177, rs1057868) with POR mRNA expression, POR protein levels, and, due to the observed association between POR protein and CYP2A6 activity, also with CYP2A6 enzyme activity. *POR* SNP rs2868177 was associated with higher POR mRNA expression (AA, AG, GG genotype means=202, 221, and 240 FPKM values, respectively, *P*<0.05), however there were no other associations between POR genotypes and POR and CYP2A6 phenotypes (*P* values provided in Supplementary Table 2). Associations between *POR* SNP rs17148944 and POR mRNA, POR protein, and CYP2A6 activity are shown in Figure 6 B-D as representative examples. There was a weak correlation between AKR1D1 mRNA and POR mRNA expression (Spearman r=0.17, *P*<0.01) and POR protein levels (Spearman r=0.21, *P*<0.001), suggesting minimal overlap in the influence of POR and AKR1D1

on CYP2A6 activity. The mean, standard deviation, and range for each POR phenotype measure were as follows: POR mRNA expression 215 ± 89 (95-795) FPKM values, and POR protein 23.7 ± 12.3 (1.2-67.3) pmol/mg.

A significant proportion of variation in CYP2A6 mRNA, protein, and activity is accounted for by genetic and non-genetic predictors

Using linear regression analyses, we assessed the individual and combined contribution of genetic and non-genetic variables to variation in CYP2A6 mRNA, protein, and enzyme activity. In each regression model, we included only those variables that were significantly (P<0.05), or trending (P<0.1) toward being, associated with each CYP2A6 phenotype in univariate analyses, or variables that have been previously associated with CYP2A6 mRNA, protein, or enzyme activity in the literature. All models presented here are derived from phenotype data corrected for liver bank site differences, as illustrated above and described in the Methods. We have compared this to models in which uncorrected phenotype data was used with the addition of site as a covariate and found only minor insignificant differences between the two modeling approaches.

When modeling influences on CYP2A6 mRNA expression in the human livers, AKR1D1 mRNA expression was the only significant independent contributor to variation in CYP2A6 mRNA, accounting for 16.0% of this variation (P<0.001; Table 1); neither gender or genotype were significant predictors despite both estrogen and CYP2A6*9 working at a transcriptional level (Pitarque et al., 2001; Higashi et al., 2007). Overall, this model accounted for 17% of the variation in CYP2A6 mRNA expression (R^2=0.17, P<0.001; Table 1).

In order to investigate factors that influence CYP2A6 protein levels, we ran two versions of the regression model, one without and one with CYP2A6 mRNA included as a predictor variable, in order to identify factors that influence CYP2A6 protein independently or as a byproduct of their influence on CYP2A6 mRNA. In model 1, genotype, AKR1D1 mRNA levels, and age were all significant predictors (Table 2). When CYP2A6 mRNA was added (model 2),

the overall proportion of variation in CYP2A6 protein that we were able to account for increased from 17% (R^2=0.17, *P*<0.001) to 37% (R^2=0.37, *P*<0.001; Table 2). Additionally, as expected the *CYP2A6*9* allele was no longer a significant independent predictor of CYP2A6 protein, with the proportion of variation accounted for by this genetic variant decreasing from 1.3% (*P*<0.05) to 0.5% (*P*>0.1; Table 2). There was a substantial decrease in the contribution of AKR1D1 mRNA to variation in CYP2A6 protein when CYP2A6 mRNA was included as a predictor in the model. The proportion of variation accounted for by AKR1D1 mRNA decreased from 9.1% (*P*<0.001) to 1.0% (*P*<0.05; Table 2). CYP2A6 mRNA independently accounted for 20.0% of the variation in CYP2A6 protein levels (*P*<0.001; Table 2).

Lastly, we quantified the contribution of predictor variables to CYP2A6 enzyme activity. We ran three versions of this model: (1) model 1, (2) model 2 with the addition of CYP2A6 mRNA as a predictor, and (3) model 3 with the further addition of CYP2A6 mRNA and CYP2A6 protein as predictors. Overall, each model accounted for 35%, 46%, and 77% of the variation in CYP2A6 enzyme activity, respectively (nicotine metabolism, R^2=0.35, 0.46, 0.77, *P*<0.001; Table 3; coumarin metabolism, R^2=0.26, 0.32, 0.57, *P*<0.001; Supplementary Table 3). In model 1 genotype, AKR1D1 mRNA, age, and POR protein were all significant predictors (Table 3). We again observed decreased contributions of both the *CYP2A6*9* allele and AKR1D1 mRNA toward CYP2A6 enzyme activity when CYP2A6 mRNA was added (model 2). In the third model, with CYP2A6 protein included as a predictor variable, the independent contribution of all other variables decreased below 1%, except for CYP2A6 protein, which independently contributed to 31.9% of the variation in nicotine (nicotine metabolism *P*<0.001; Table 3, coumarin metabolism 25.0% *P*<0.001; Supplementary Table 3). Aside from CYP2A6 protein, POR protein was the only remaining significant independent predictor of CYP2A6 enzyme activity, accounting for 0.6% (nicotine metabolism, *P*<0.05; Table 3; courmain metabolism, 0.8% *P*<0.05; Supplementary Table 3).

DISCUSSION

In a large human liver bank, we have identified several genetic and non-genetic factors that contribute to variation in CYP2A6 mRNA, protein, and ultimately CYP2A6 enzyme activity, which is an important determinant of the rate of nicotine metabolism. We confirmed that our CYP2A6 phenotype measures were correlated with, and predictive of one another. Using regression models we were able to account for over 75% of the variation in CYP2A6 activity, as well as assessing the novel roles of AKR1D1 and POR in these CYP2A6 phenotypes.

Consistent with the literature, female gender was associated with higher CYP2A6 mRNA expression, relative to males donors in the liver bank, in univariate analyses with similar trends for protein levels, and enzyme activity. However, gender was not a significant independent predictor of any CYP2A6 phenotype based on regression modeling. It is possible that other factors are blunting the relationship between gender and CYP2A6 in our models, or that there is a relatively large impact of pre- and post-menopausal women in this analysis, where previous data suggests similar levels of activity (Benowitz et al., 2006). 20.1% and 42.5% of females in this liver bank are below 16 years and above 50 years old, respectively.

The age of liver donors was positively, but weakly, associated with CYP2A6 protein levels and enzyme activity (Figure 3). It is unclear if this relationship denotes a true age effect on CYP2A6, or if this increase results from unknown covariates, for example greater inducer exposure among older donors. For example, there is an overall increase in polypharmacy with increasing age (Hajjar et al., 2007), and several drugs are known CYP2A6 inducers, such as phenobarbital, dexamethasone, or rifampin (Maurice et al., 1991; Rae et al., 2001).

Additionally, there may be dietary differences between age groups, with elderly individuals consuming more CYP2A6-inducing foods, or a role for estrogen in increasing levels over puberty (Hakooz and Hamdan, 2007; Higashi et al., 2007). However, when modeling predictors of CYP2A6 phenotypes, age was a significant independent predictor of both CYP2A6 protein and activity, even when accounting for gender, suggesting that a female puberty effect is not

responsible for the observed positive association between age and CYP2A6 protein levels and enzyme activity.

CYP2A6 genotype was associated with reduced CYP2A6 protein and enzyme activity in both univariate and regression analyses. We observed a step-wise decrease in CYP2A6 mRNA expression, protein levels, and enzyme activity with increasing copies of the *CYP2A6*9* allele (i.e. from **1/*1* to **1/*9* to **9/*9*). *CYP2A6*9* is a SNP present in the TATA box of the *CYP2A6* promoter and is associated with decreased CYP2A6 transcription (Pitarque et al., 2001), consistent with our results. There was also a step-wise decrease in CYP2A6 protein and enzyme activity associated with *CYP2A6*2*, which is a nonsynonymous SNP in exon 3, resulting in the failure of the enzyme to incorporate heme necessary for catalytic function, resulting in a less stable enzyme, vulnerable to degradation (Yamano et al., 1990). Both the *CYP2A6*2* and *CYP2A6*9* alleles were significant independent predictors of CYP2A6 protein levels and enzyme activity in our regression models. However, when CYP2A6 mRNA was included as a covariate in both the protein and activity models, the impact of the *9 allele on each phenotype decreased, and was no longer significant, suggesting that *9 exhibits a secondary effect on CYP2A6 protein and activity, via its direct influence on CYP2A6 mRNA. Conversely, the **2* allele remains a significant predictor of both CYP2A6 protein and activity when CYP2A6 mRNA was added to the model, suggesting a mechanism independent of CYP2A6 mRNA expression. Within the wild-type (**1/*1*) group, which possesses no tested *CYP2A6* genetic variants, we still observed a large range in CYP2A6 mRNA, protein, and enzyme activity. This implies that there are additional uncharacterized sources of variation, which may be due to environmental exposures, or as a result of undetected genetic variation present at the *CYP2A6* gene locus or potentially other regulatory loci.

AKR1D1 has been implicated as being a potential regulator of the expression of CYPs (Chaudhry et al., 2013), and our findings support a relationship between AKR1D1 and CYP2A6 mRNA expression. AKR1D1 mRNA expression was associated with increasing CYP2A6 mRNA,

protein, and enzyme activity, while the specific genetic variation (*AKR1D1* SNP rs1872930) was not. AKR1D1 mRNA levels accounted for a significant proportion (16%) of the variation in CYP2A6 mRNA expression (Table 1). AKR1D1 was also a significant predictor of CYP2A6 protein levels and enzyme activity, however the proportion of variation in CYP2A6 protein and activity accounted for by AKR1D1 mRNA decreased more than 8-fold when CYP2A6 mRNA was added to each model. This suggests that the relationship between AKR1D1 mRNA and CYP2A6 phenotypes is largely due to the influence of AKR1D1 mRNA on CYP2A6 mRNA expression. The association between AKR1D1 on CYP2A6 mRNA expression may result from the role of AKR1D1 in bile acid synthesis and/or the reduction of steroid hormones, which can act as ligands of nuclear hormone receptors. For example AKR1D1 is responsible for 5β-reduction of progesterone, resulting in the activation of nuclear hormone receptor PXR (Bertilsson et al., 1998). Bile acids can activate nuclear hormone receptors, such as PXR and CAR, which can regulate the transcription of several genes, including CYPs (Schuetz et al., 2001). These data confirm a significant role for AKR1D1 on the regulation of multiple CYPs, suggesting a broader role for it in altering drug metabolism. Whether the variation contributes to altered nicotine pharmacokinetics and subsequent smoking behaviours remains to be tested.

POR protein was positively correlated with CYP2A6 activity, remaining a significant independent predictor of CYP2A6 activity when accounting for other sources of variation (Figure 6A, Table 3). There was no association between the POR genotypes tested and CYP2A6 enzyme activity. The role of POR as an electron donor to CYPs during the process of substrate metabolism is consistent with the observed positive correlation of POR and CYP2A6 enzyme activity. However, the contribution of POR to CYP2A6 activity decreased from 21.5% to 14.0% to less than 1% when CYP2A6 mRNA and CYP2A6 protein were added to the models, respectively (Table 3). This suggests a potential role of POR in regulating mRNA or protein levels, or that POR and CYP2A6 may have a common regulatory feature, as opposed to a direct influence on CYP2A6 enzyme activity.

Although this liver bank was extensively characterized, this study did not assess several genetic and environmental variables that have been associated with CYP2A6 and nicotine metabolism and may account for a portion of the 23% missing variation in CYP2A6 activity. For example, we have focused on the assessment of common (MAF>1%) established *CYP2A6* genetic variants, however the majority of genetic variants found in pharmacogenes, including CYP2A6, are thought to be rare (MAF<1%) (Kozyra et al., 2016), suggesting that a substantial portion of the 23% unidentified variation in CYP2A6 activity may be due to unknown *CYP2A6* rare variants. Variation in other genes regulating CYP expression or function, including AKRs or nuclear hormone receptors, may also, in part, account for the 23% missing variation. Several enzymes in the AKR1 family, including AKR1C1-1C4, may be associated with variation in CYP2A6 expression due to their contribution to metabolism and biosynthesis of steroids (estrogen, testosterone, progesterone, unavailable in this study), steroid hormones, and bile acids, ultimately controlling concentrations of active ligands at nuclear receptors, ligand occupancy, and trans-activation of receptors (as reviewed by Rizner and Penning, 2014). Cigarette smoking, an environmental factor, is associated with a reduction in nicotine clearance *in vivo*, such that nicotine clearance increases 14% after 4 days of abstinence in regular smokers (Benowitz and Jacob, 1993; Benowitz and Jacob, 2000). Similarly, grapefruit juice reduces nicotine's metabolism to cotinine, mediated by CYP2A6, by 15% (Hukkanen et al., 2006).

In conclusion, we have identified several sources of variation in CYP2A6 activity *in vitro* that could translate to variable hepatic clearance of several clinically important drugs, namely nicotine. We were able to account for 77% of the variation in CYP2A6 activity in our models. Unaccounted for variation may result from unknown genetic variation at the highly polymorphic *CYP2A6* gene locus and/or in additional regulatory genes, as well as environmental factors. Characterizing sources of variation in CYP2A6 activity is important as variation in the rate of

nicotine metabolism and clearance can ultimately influence smoking behavior, and affect a smoker's ability to quit smoking.

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AUTHORSHIP CONTRIBUTIONS

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Contributed new reagents or analytic tools: B.P., K.C., E.S.

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Wrote or contributed to the writing of the manuscript: J-A.T., R.F.T., B.P., P.S., K.C., A.C., E.S., K.E.T.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. (A) Correlation of two measures of CYP2A6 enzyme activity (the velocity of cotinine formation from nicotine vs. the velocity of 7-OH-coumarin formation from coumarin, nmol/min/mg). (B) Correlation between CYP2A6 mRNA levels (FPKM values, fragments per kilobase per million reads) and CYP2A6 protein levels (pmol/mg). (C) Correlation between CYP2A6 protein levels and CYP2A6 enzyme activity (cotinine formation from nicotine). r and *P* values are based on Spearman correlations.

Figure 2. Association of gender with (A) CYP2A6 mRNA levels (FPKM values), (B) CYP2A6 protein levels (pmol/mg), and (C) CYP2A6 enzyme activity (cotinine formation from nicotine, nmol/min/mg). * represents *P* values of <0.05 based on Mann Whitney tests.

Figure 3. Correlation of age with (A) CYP2A6 mRNA levels (FPKM values), (B) CYP2A6 protein levels (pmol/mg), and (C) CYP2A6 enzyme activity (cotinine formation from nicotine, nmol/min/mg). r and *P* values are based on Spearman correlations.

Figure 4. Association of *CYP2A6* genotype with (A) CYP2A6 mRNA levels (FPKM values), (B) CYP2A6 protein levels (pmol/mg), and (C) CYP2A6 enzyme activity (cotinine formation from nicotine, nmol/min/mg). Horizontal lines represent the mean for each genotype. Three data points exceed the y-axis limit for *CYP2A6*1/*1* group in part A, 2 data points exceed the y-axis limit for *CYP2A6*1/*1* group in part B, and 2 data points exceed the y-axis limit for *CYP2A6*1/*1* group in part C; all points were included in the mean and statistical tests. ** represents *P*<0.01, *** represents *P*<0.001, based on Mann Whitney tests.

Figure 5. (A) Correlation between AKR1D1 mRNA expression (FPKM values) and (A) CYP2A6 mRNA expression (FPKM values), (B) CYP2A6 protein levels (pmol/mg), and (C) CYP2A6 activity (cotinine formation from nicotine, nmol/min/mg). r and *P* values based on Spearman correlations.

Figure 6. (A) Correlation between POR protein levels (pmol/mg) and CYP2A6 activity (cotinine formation from nicotine, nmol/min/mg). r and *P* values based on Spearman correlations. (B-D)

Association between *POR* SNP rs17148944 and (B) POR mRNA expression (FPKM values), (C) POR protein levels (pmol/mg) and (D) CYP2A6 activity (cotinine formation from nicotine, nmol/min/mg). *P* values for B-D all >0.1, based Kruskal-Wallis tests.

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Table 1. Linear regression analysis of CYP2A6 mRNA expression (FPKM values) [a, b].

Predictor variable	B	Beta	95% CI	% variation accounted for [c]	*P* value
*CYP2A6*9* Genotype [d]	-117.9	-0.09	-264.8 to 25.2	0.8%	>0.1
Gender (M=1, F=0)	-12.0	-0.01	-118.5 to 98.5	0.01%	>0.1
AKR1D1 mRNA (FPKM)	6.0	0.4	4.3 to 7.6	16.0%	**<0.001**

a. Cases excluded pairwise

b. R^2=0.17, *P*<0.001

c. % variation accounted for by each variable is determined by: (Part Correlation)^2 x 100

d. *CYP2A6*9* genotype coded as 0 for *CYP2A6*1/*1* genotype, 1 for *CYP2A6*1/*9* genotype, and 2 for *CYP2A6*9/*9* genotype.

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Table 2. Linear regression analysis of CYP2A6 protein levels (pmol/mg) [a].

Predictor variable	B	Beta	95% CI	% variation accounted for [d]	*P* value
Model 1[b]					
*CYP2A6*2* Genotype[e]	-11.8	-0.2	-20.0 to -3.7	2.7%	**<0.01**
*CYP2A6*9* Genotype[e]	-6.1	-0.1	-12.2 to -0.08	1.3%	**<0.05**
*CYP2A6*12* Genotype[e]	-11.4	-0.1	-23.2 to 0.5	1.2%	<0.1
Gender (M=1, F=0)	-2.2	-0.06	-6.8 to 2.3	0.3%	>0.1
AKR1D1 mRNA (FPKM)	0.2	0.3	0.1 to 0.3	9.1%	**<0.001**
Age	0.1	0.1	0.01 to 0.2	1.6%	**<0.05**
Model 1 + CYP2A6 mRNA[c]					
*CYP2A6*2* Genotype[e]	-13.6	-0.2	-20.8 to -6.4	3.5%	**<0.001**
*CYP2A6*9* Genotype[e]	-3.8	-0.07	-9.1 to -1.5	0.5%	>0.1
*CYP2A6*12* Genotype[e]	-8.0	-0.08	-18.4 to 2.3	0.6%	>0.1
Gender (M=1, F=0)	-2.1	-0.05	-6.1 to 1.9	0.3%	>0.1
AKR1D1 mRNA (FPKM)	0.07	0.1	0.001 to 0.1	1.0%	**<0.05**
Age	0.1	0.1	0.01 to 0.2	1.2%	**<0.05**
CYP2A6 mRNA (FPKM)	0.02	0.5	0.02 to 0.02	20.0%	**<0.001**

a. Cases excluded pairwise

b. R^2=0.17, *P*<0.001

c. R^2=0.37, *P*<0.001

d. % variation accounted for by each variable is determined by: (Part Correlation)^2 x 100

e. *CYP2A6*2, CYP2A6*9,* and *CYP2A6*12* genotypes coded as 0 for *CYP2A6*1/*1* genotype, 1 for *CYP2A6*1/*2, CYP2A6*1/*9,* or *CYP2A6*1/*12* genotypes, and 2 for *CYP2A6*2/*2* or *CYP2A6*9/*9* genotypes.

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Table 3. Linear regression analysis of CYP2A6 enzyme activity (cotinine formation from nicotine, nmol/min/mg) [a].

Predictor variable	B	Beta	95% CI	% variation accounted for [e]	*P* value
Model 1[b]					
*CYP2A6*2* Genotype[f]	-0.04	-0.1	-0.077 to -0.001	1.0%	**<0.05**
*CYP2A6*9* Genotype[f]	-0.03	-0.1	-0.059 to -0.003	1.2%	**<0.05**
*CYP2A6*12* Genotype[f]	-0.05	-0.09	-0.105 to 0.004	0.8%	<0.1
Gender (M=1, F=0)	0.004	0.02	-0.02 to 0.03	0.03%	>0.1
AKR1D1 mRNA (FPKM)	0.001	0.2	<0.001 to 0.001	3.3%	**<0.001**
Age	0.001	0.2	<0.001 to 0.001	3.5%	**<0.001**
POR Protein	0.004	0.5	0.003 to 0.005	21.5%	**<0.001**
Model 1 + CYP2A6 mRNA[c]					
*CYP2A6*2* Genotype[f]	-0.05	-0.1	-0.08 to -0.01	1.5%	**<0.01**
*CYP2A6*9* Genotype[f]	-0.02	-0.08	-0.047 to 0.005	0.5%	>0.1
*CYP2A6*12* Genotype[f]	-0.04	-0.07	-0.09 to 0.01	0.4%	>0.1
Gender (M=1, F=0)	0.003	0.02	-0.02 to 0.02	0.03%	>0.1
AKR1D1 mRNA (FPKM)	<0.001	0.07	<0.001 to 0.001	0.4%	>0.1
Age	0.001	0.2	<0.001 to 0.001	2.6%	**<0.01**
POR Protein	0.003	0.4	0.003 to 0.004	14.0%	**<0.001**
CYP2A6 mRNA (FPKM)	<0.001	0.4	<0.001 to <0.001	10.0%	**<0.001**
Model 1 + CYP2A6 mRNA + CYP2A6 protein[d]					
*CYP2A6*2* Genotype[f]	0.03	0.009	-0.02 to -0.03	0.008%	>0.1
*CYP2A6*9* Genotype[f]	<0.001	<0.001	-0.02 to 0.02	<0.001%	>0.1
*CYP2A6*12* Genotype[f]	0.007	0.01	-0.03 to 0.04	0.01%	>0.1

Gender (M=1, F=0)	0.009	0.05	-0.003 to 0.022	0.2%	>0.1
AKR1D1 mRNA (FPKM)	<0.001	0.02	<0.001 to <0.001	0.04%	>0.1
Age	<0.001	0.03	<0.001 to <0.001	0.06%	>0.1
POR Protein	0.001	0.09	<0.001 to 0.001	0.6%	**<0.05**
CYP2A6 mRNA (FPKM)	<0.001	0.4	<0.001 to <0.001	0.1%	>0.1
CYP2A6 Protein	0.004	0.8	0.004 to 0.005	31.9%	**<0.001**

a. Cases excluded pairwise

b. R^2=0.35, *P*<0.001

c. R^2=0.46, *P*<0.001

d. R^2=0.77, *P*<0.001

e. % variation accounted for by each variable is determined by: (Part Correlation)^2 x 100

f. *CYP2A6*2, CYP2A6*9,* and *CYP2A6*12* genotypes coded as 0 for *CYP2A6*1/*1* genotype, 1 for *CYP2A6*1/*2, CYP2A6*1/*9,* or *CYP2A6*1/*12* genotypes, and 2 for *CYP2A6*2/*2* or *CYP2A6*9/*9* genotypes.

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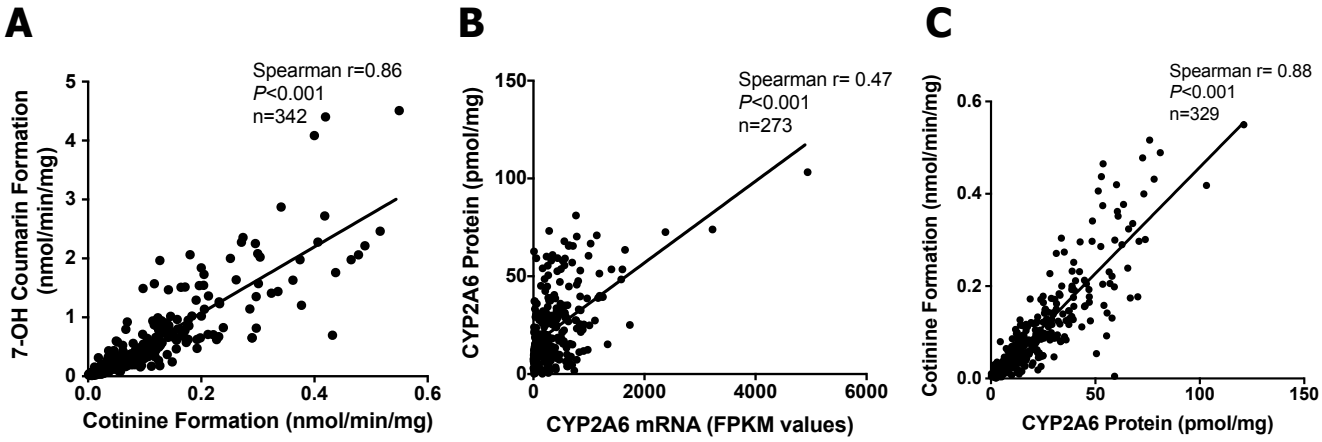

Figure 1

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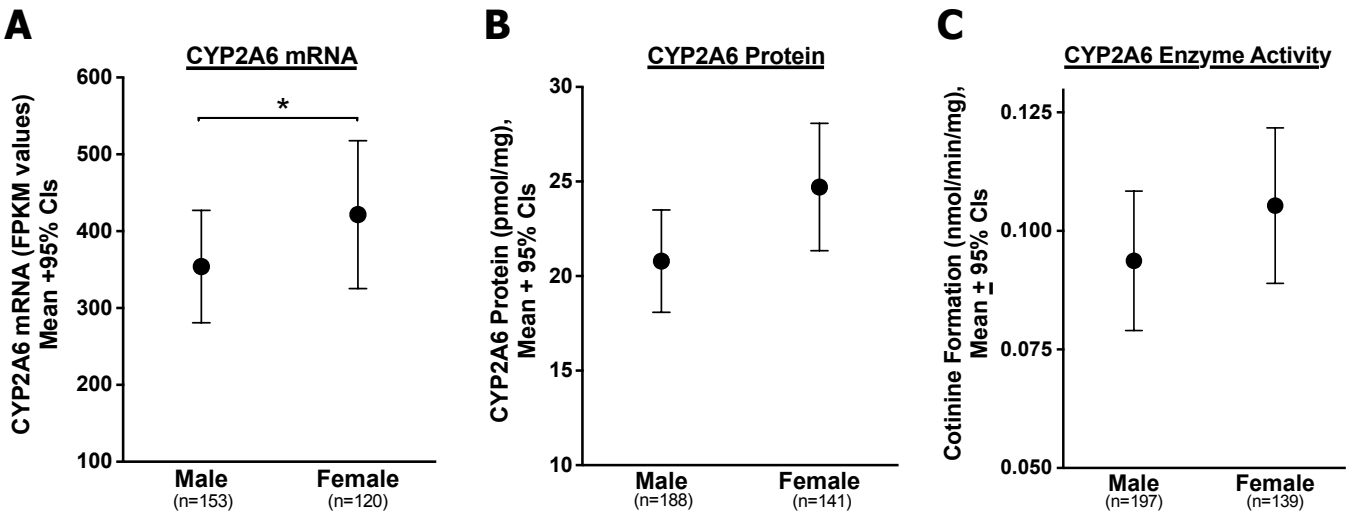

Figure 2

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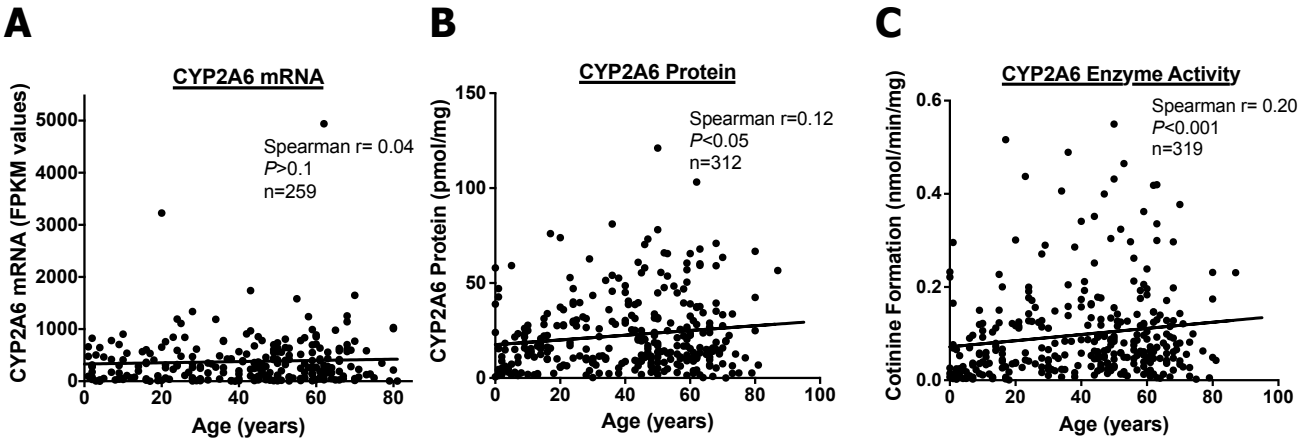

Figure 3

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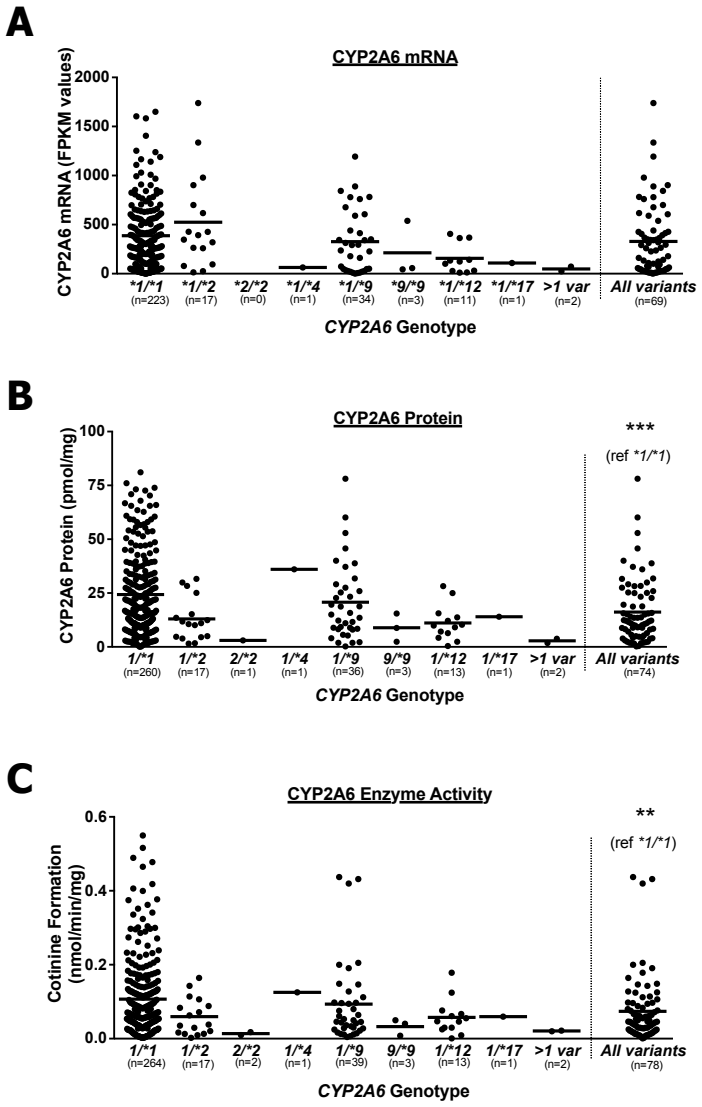

Figure 4

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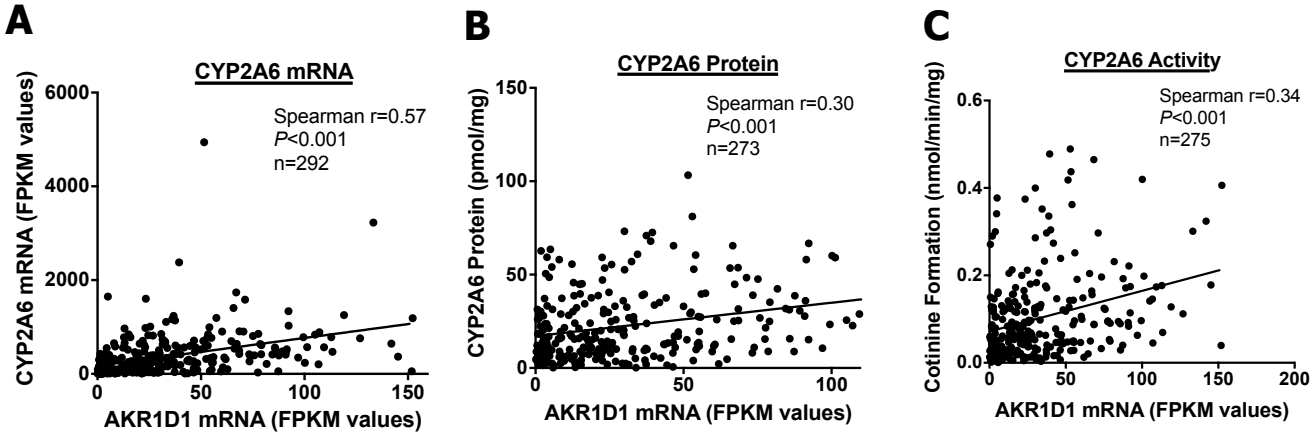

Figure 5

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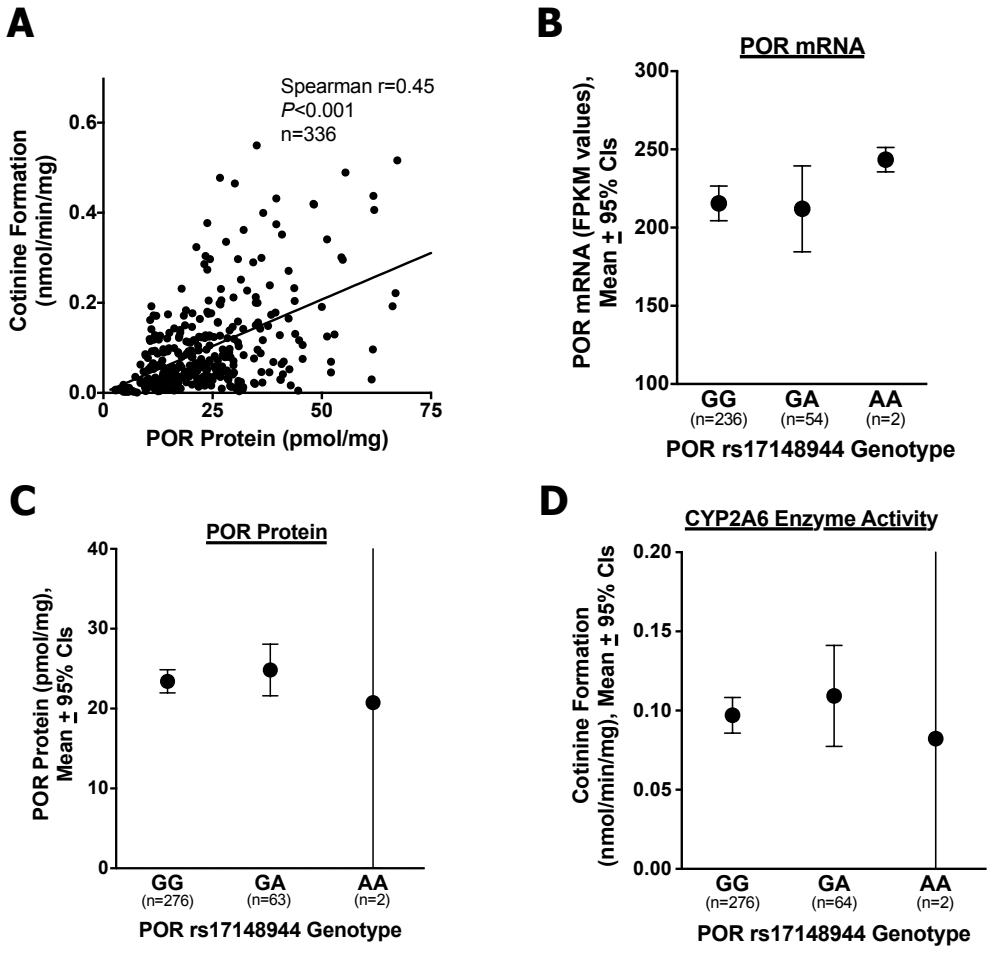

Figure 6