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Effects upon *in vivo* nicotine metabolism reveal functional variation in *FMO3* associated with cigarette consumption

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Abstract

Background—Flavin-containing monooxygenases (FMO) catalyze the metabolism of nucleophilic heteroatom containing drugs and xenobiotics including nicotine. Rare mutations in *FMO3* are responsible for defective *N*-oxygenation of dietary trimethylamine leading to trimethylaminuria, and common genetic variation in *FMO3* has been linked to interindividual variability in metabolic function that may be substrate specific.

Methods—A genetic model of *CYP2A6* function is used as a covariate to reveal functional polymorphism in *FMO3* that indirectly influences the ratio of deuterated nicotine metabolized to cotinine following oral administration. The association is tested between *FMO3* haplotype and cigarette consumption in a set of nicotine dependent smokers.

Results—*FMO3* haplotype, based on all common coding variants in Europeans, significantly predicts nicotine metabolism and accounts for approximately 2% of variance in the apparent percent of nicotine metabolized to cotinine. The metabolic ratio is not associated with *FMO2* haplotype or an *FMO1* expression quantitative trait locus (eQTL). Cross validation demonstrates calculated *FMO3* haplotype parameters to be robust and significantly improve the predictive nicotine metabolism model over *CYP2A6* genotype alone. Functional classes of *FMO3* haplotypes, as determined by their influence on nicotine metabolism to cotinine, are also significantly associated with cigarettes per day (CPD) in nicotine dependent European Americans (n=1,025, p=0.04), and significantly interact (p=0.016) with *CYP2A6* genotype to predict CPD.

Conclusion—These findings suggest that common polymorphisms in *FMO3* influence nicotine clearance, and that these genetic variants in turn influence cigarette consumption.

Introduction

Human flavin-containing monooxygenase (FMO) enzymes are involved in the oxidation of a broad variety of heteroatom-containing substrates. The targets of FMO3, the most

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prominently expressed member in adult liver [1, 2], include dietary-derived tertiary amines, commonly prescribed drugs, agrichemicals, and nicotine. Rare mutation in *FMO3* is associated with trimethylaminuria, 'fish odor syndrome', and there is some evidence that common variation in the gene also reduces enzyme activity and contributes to mild or transient symptoms [3]. However, the locus is highly polymorphic with many common coding and non-coding variants having putative functional effects [4, 5], including non-synonymous variants that appear to interact *in cis* [6]. Alterations in *in vitro* FMO3 function associated with these variants appear to be substrate specific [7, 8]. There is also evidence for rare *FMO3* variants associated with increased catalytic activity in both African and European populations [4, 9–12].

Nicotine is metabolized via three pathways (reviewed in [13]): 1) C-oxidation by the cytochrome P450, CYP2A6, and to a lesser extent CYP2B6 [14] in liver, and by CYP2A13, which is expressed primarily in the lung [13, 15], 2) glucuronidation by the UDP-Glucuronosyltransferases [16, 17] and 3) N-oxidation by FMO enzymes. These pathways play greater or lesser roles in overall nicotine clearance depending on individual genotype [18–20], and differences in allele frequencies across ethnicities [21, 22]. For example, in the average smoker, approximately 3–5% of nicotine equivalents are excreted as nicotine-glucuronide [23, 24] but homozygotes for the *CYP2A6* deletion allele have been reported to excrete up to 46% of absorbed nicotine as nicotine-glucuronide [18]. Similarly 4–7% of nicotine equivalents are excreted as nicotine N′-oxide [23, 24], the product of FMO activity, in most smokers, but *CYP2A6* deletion homozygotes can excrete up to 31% of absorbed nicotine as nicotine N′-oxide [18].

Common variation in the gene encoding CYP2A6, the dominant enzyme responsible for nicotine metabolism in most smokers, is also robustly associated with smoking behavior (reviewed in [25]). But the great complexity of the *CYP2A6* locus and the resultant variety of allelic activity, demonstrate the difficulty in investigating associations with such a highly heterogeneous gene. Common *CYP2A6* alleles include complete and partial deletions, duplications, amino acid changes that reduce or completely eliminate catalytic activity, and variants that alter expression or splicing efficiency. This degree of common variation in the locus makes single SNP analyses, or investigations based on partial genotyping, difficult to interpret.

With the above difficulties in mind, we here approach a similarly complex locus including the *FMO3* gene, which is believed to be primarily responsible for hepatic nicotine N-oxidation. Utilizing a genetic predictive model that accounts for ~70% of the variance in metabolism of oral nicotine to cotinine based on *CYP2A6* genotype [26], we are able to categorize *FMO3* haplotypes based on all common coding polymorphism, according to their significant indirect influences on this parallel metabolic pathway. Furthermore, we find that *FMO3* haplotypes categorized by this method are significantly associated with cigarette consumption among nicotine dependent smokers, indicating a larger potential role for this gene in tobacco-related disease and treatment than previously believed.

Materials and methods

This study complies with the Code of Ethics of the World Medical Association and obtained informed consent from participants and approval from the appropriate Washington University institutional review boards. Participant recruitment from the Collaborative Genetic Study of Nicotine Dependence (COGEND)[27] and *CYP2A6* genotyping were previously described [26, 28]. COGEND is a multi-site project in the United States designed to recruit dependent smokers (Fagerstrom test for nicotine dependence (FTND) > 4) and non-dependent smokers (FTND=0). All subjects analyzed in this study were self-identified as being of European American ancestry, and race was verified using EIGENSTRAT [29]. Participant characteristics for the metabolism experiment are summarized in Supplementary Table 1. Cigarettes per day (CPD) was assessed by direct interview with the question "On the days you smoked in the past 12 months, about how many cigarettes did you usually have per day".

Deuterated nicotine metabolism experiment

As previously described [26], 189 European American subjects between 27 and 44 years of age were given 2mg of [30,30]-D2-nicotine in 4 oz of juice, and blood was drawn approximately 30, 150, and 240 min later. Plasma was collected and frozen at −20°C until analysis. D2-nicotine and D2-cotinine were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a modification of a previously described method [30]. Changes to the method included the addition of D9-trans-30-hydroxycotinine (a gift from Dr Peyton Jacob, University of California, San Francisco, USA) to the plasma samples as an internal standard and elution of the solid phase extraction column with methanol containing 2% ammonium hydroxide/base. An aliquot was removed for nicotine analysis and the remainder was evaporated to dryness, and resuspended in one-tenth the volume of ammonium hydroxide/base for cotinine analysis. LC/MS/MS for nicotine was as previously described [30]. Cotinine was eluted at 2.53 min. The limits of quantitation for D2-nicotine and D2-cotinine were 0.25 ng/ml plasma. D2-nicotine and D2-cotinine plasma concentrations were determined for all participants at 30, 150, and 240min after oral D2-nicotine administration, summarized in Supplemental Table 2.

Statistical Analysis

Statistical analyses were performed using the software package 'R' (R Foundation for Statistical Computing, Vienna, Austria). All t-tests performed were two-sided. Application of the predictive model of CYP2A6 activity was previously described [26, 28]. Briefly, all analyses of measured metabolism are performed on a metabolism metric, the ratio of deuterated (D_2)cotinine/(D_2cotinine+ D_2nicotine) determined 30 minutes following oral administration of D_2nicotine. The original model parameters were derived from the regression, log (1 − metric) = log(α) + log(βH1) + log(βH2) where α is the intercept, βH1 represents the first *CYP2A6* haplotype and βH2 represents the second *CYP2A6* haplotype for each subject. For a new model, new parameters for all *CYP2A6* haplotypes and for the *FMO3* haplotype classes were derived from the regression log (1 − metric) = log(α) + log(βH1) + log(βH2) + log(βH3) + log(βH4) where βH3 represents the first *FMO3* haplotype class and βH4 represents the second *FMO3* haplotype class for each subject. The p-value for

the improvement of the new model over the original model ($p=6.4\times10^{-5}$) was determined from the Chi-squared distribution of the test statistic D, defined as two times the difference between the log likelihood computed for the two competing models.

Genotyping and Haplotype Determination

CYP2A6 nomenclature follows official recommendations (http://www.cypalleles.ki.se) except that *CYP2A6*1A* is defined by the A allele of rs1137115 throughout. FMO genotyping was previously performed using a custom designed array [27] with the exception of rs1963273 which was genotyped using the KBioscience Competitive Allele Specific PCR genotyping system (KASPar, KBioscience, Hoddesdon, Herts, UK) following standard procedures with custom designed primers: common primer CTACCCTAATCCAAGCTCCTCTCAT, allelic GAAGGTGACCAAGTTCATGCTCAAGATTAGAAGTGGGAAGACCTG and GAAGGTCGGAGTCAACGGATTGCAAGATTAGAAGTGGGAAGACCTA. 8μl KASPar assay reactions were measured with the 7900HT Fast Real Time PCR System (Applied Biosytems, Foster City, CA, USA). *FMO3* haplotypes were determined using PHASE version 2.1.1 [31, 32]. Linkage disequilibrium was determined using Haploview [33].

Results

FMO polymorphisms and haplotypes associated with the ratio of nicotine metabolized to cotinine

We began by constructing haplotypes of *FMO1*, *FMO2* and *FMO3* based on all common (≥2% minor allele frequency in Europeans) coding SNPs in these genes [34]. Haplotypes of *FMO3*, the gene associated with the majority of hepatic *FMO* activity [35], are associated with the residual variance in the metabolism metric, the ratio of deuterated (D_2)cotinine/ (D_2cotinine+ D_2nicotine) in plasma 30 minutes after oral D_2nicotine administration, after accounting for *CYP2A6* diplotype. Five common haplotypes defined by five common coding SNPs occur among Europeans. Multivariate regression analyses indicate they fall into two statistically significantly different categories (Table 1). Dividing haplotypes into these two categories, essentially the reference haplotype and the compound minor-allele haplotype rs2266782A/rs2266780G (amino acid changes E158K & E308G) haplotype together, versus all other haplotypes (i.e. 1 and 3 versus 2,4,5,6 and 7, Table 1), creates a variable more strongly associated with the metabolism metric (p=0.003) than any single SNP tested in the *FMO* locus. Shifting the two singleton haplotypes, for which we cannot estimate parameters, into the other category (i.e. 1,3,6 and 7 versus 2,4 and 5,6) is similarly significantly associated with the metric (p=0.002).

Recalculating parameters for the predictive model of the metabolism metric including the *FMO3* haplotype variable significantly ($p=6.4\times10^{-5}$) improves the fit of the model. Adding the *FMO3* variable (haplotypes 1&3 versus 2,4,5,6&7) to the model also improves ($p<0.05$) fit in a simple cross-validation where all subjects are divided by sex (n=89 vs. 100) or current smoking status (n=102 vs. 86), parameters are recalculated in each subset, and applied to the other. This demonstrates that the model is not merely over-fitting the data, and

that the effect of *FMO3* haplotype is consistent among different subsets. We wanted to generate an improved model to predict the metabolism metric that includes both *CYP2A6* and *FMO3* haplotype variables for several reasons: 1) to more precisely define a covariate to aid in identifying further factors contributing to variance in nicotine metabolism, 2) to more precisely define relative parameter estimates for *CYP2A6* haplotypes to better predict *CYP2A6* function by accounting for the indirect effects of *FMO3* haplotype, and 3) to estimate the remaining variance in the metric unexplained by polymorphism in these two genes.

FMO enzymes are not responsible for the metabolism of nicotine to cotinine, but variation in FMO activity could influence the ratio of nicotine metabolized to cotinine via substrate metabolism rerouting [18]; i.e. just as a greater percent of nicotine equivalents are excreted as nicotine-N-oxide in smokers that are deficient in CYP2A6 nicotine C-oxidation activity, subjects deficient in FMO nicotine N-oxidation activity may metabolize a higher percentage of absorbed nicotine to cotinine. The influence of *FMO3* haplotype upon the metric is indirect, most likely as faster metabolizing *FMO3* alleles that more quickly remove nicotine from the denominator of the cotinine: nicotine ratio. Overall, the *FMO3* haplotype variable accounts for 4% of the residual variance in the metabolism metric after accounting for *CYP2A6* genotype.

Haplotypes of *FMO2*, the primary FMO expressed in lung, were not associated with the nicotine metabolism metric (data not shown); greater than 99.8% of European Americans are homozygous for a premature stop codon (rs6661174) that truncates the final 63 amino acids of the wildtype FMO2 protein [34, 36], Nor were there associations with rs742350/ rs1126692 the common *FMO1* synonymous coding SNPs, or with rs1963273, a reported *FMO1* eQTL [37, 38].

Reduced C-oxidation activity caused by CYP2A6 loss of function has been shown to be associated with greater FMO mediated N-oxidation [18] due to substrate metabolism rerouting. It is therefore reasonable to posit that, among populations of *CYP2A6* slow metabolizers, genetic variation in *FMO3* will exert a larger effect upon overall nicotine clearance, compared to populations of *CYP2A6* fast metabolizers. Without direct measurements of nicotine-N-oxide available, we attempted to investigate this question by comparing the effect size of the *FMO3* haplotype variable upon the ratio D_2cotinine/ (D_2cotinine+ D_2nicotine) among different subsets of subjects divided into faster and slower *CYP2A6* genotype groups (Table 2). Although this study does not include a sample size sufficient to adequately address this question (for example, it only includes three *CYP2A6* null homozygotes), it is interesting to note that the effect size of the *FMO3* haplotype upon the ratio among carriers of *CYP2A6* loss-of-function alleles is twice as large (0.022±0.009) as among carriers of only fully functional *CYP2A6* alleles (0.011±0.006, Table 2).

FMO3 haplotype is significantly associated with CPD in nicotine dependent European Americans

To determine whether the *FMO3* haplotype categories predicted to differ based on the nicotine metabolism experiment would also improve prediction of CPD in conjunction with predicted *CYP2A6* activity, we determined *FMO3* haplotype in 2,002 European Americans

from the COGEND dataset with measurements of CPD and Fagerström Test of Nicotine Dependence (FTND) scores. Surprisingly, the *FMO3* haplotype variable significantly predicts CPD among nicotine dependent subjects (n=1,025, p=0.04), corresponding to a relatively large difference between homozygotes for the two haplotype classes (haplotypes 1&3 vs. all others, 2.0 CPD, p=0.017, Figure 1). Inclusion of rare *FMO3* rs2266780G haplotypes (Table 1) with haplotypes 1 and 3 did not affect the results of any analysis.

FMO3 haplotype also significantly interacts with predicted *CYP2A6* activity, a continuous trait, to predict CPD (p=0.016), indicating a significant effect of *FMO3* haplotype among faster metabolizing *CYP2A6* genotypes but no effect among slower metabolizing *CYP2A6* genotypes; this can be demonstrated by dividing subjects arbitrarily at different points along the scale of CYP2A6 activity (Figure 2). For example, the difference in CPD between homozygotes for the two *FMO3* haplotype classes is 3.5 CPD (p=0.006) among normal metabolizers (n=77 vs. 221, subjects excluding carriers of *CYP2A6*1A,*2,*4,*9,*12* and **38* alleles), as opposed to −0.9 CPD (p=0.6) among slow metabolizers (n=38 vs. 76, carriers of *CYP2A6*2,*4,*9,*12* and **38* alleles). Another way to describe the data is that normal *CYP2A6* metabolizers homozygous for the class of *FMO3* haplotypes including common haplotypes 2, 4 and 5 smoke as few cigarettes on average (20.1), as all slow *CYP2A6* metabolizers (20.6) (Figure 2).

Consistent with our previous observations regarding *CYP2A6* [28], *FMO3* haplotype does not predict nicotine dependence (FTND 0 vs. FTND≥4) in this case/control set, nor does it predict CPD after including FTND 0 subjects (n=977).

Discussion

The *FMO*s were formerly considered important in hepatic nicotine metabolism, but have since been relegated to minor status due to the low amounts of nicotine-*N*-oxide relative to cotinine and cotinine metabolites excreted by most smokers [17, 18]. Here we provide evidence that genetic polymorphism in *FMO3* affects enzyme activity strongly enough to be detected via an indirect effect upon another nicotine metabolism pathway, the conversion of nicotine to cotinine, despite the fact that FMOs do not catalyze this reaction. Interestingly, as with *CYP2A6*, our data indicate a diversity of common *FMO3* haplotypes confounding unbiased single-SNP analyses. Variants necessary to define the key haplotypes include both synonymous and non-synonymous changes and are therefore likely to influence *in vivo* enzyme activity by a variety of mechanisms. Previous investigation of the effects of polymorphism upon *FMO3* function, both *in vivo* and *in vitro*, have focused on only a few non-synonymous SNPs, and different substrates, i.e. trimethylamine [3], ranitidine [6], benzydamine, methyl p-tolyl sulfide, and sulindac sulfide [8]. These studies demonstrated substrate-specific effects, as well as a large share of variation in *FMO3* activity still unaccounted for. While our results regarding nicotine metabolism confirm the important role of amino acid changes E158K, V257M and E308G, the relative influences of different haplotypes do not relate straight-forwardly to prior results for other substrates. The differences in the effects of these variants upon nicotine metabolism, compared to other substrates, may reflect the dearth of genotyping in earlier studies as well as differences in substrate specificity. Studies of other metabolism genes have even demonstrated opposite

effects of common polymorphisms upon enzyme activity toward different substrates [39]. Clearly more remains to be discovered about the association between genotype and *in vivo FMO3* activity regarding all target substrates; for example *in vitro* luciferase assays indicate the potential influence of common non-coding variants upon *FMO3* expression [4], and frequent alternative *FMO3* splicing has been demonstrated [40], with common variants predicted to influence splicing-efficiency [4], but this has not yet been investigated vis-à-vis genotype.

Importantly, the relative activities of different *FMO3* haplotypes determined from their association with nicotine metabolism also significantly predict cigarette consumption. Although the small effect of *FMO3* haplotype upon the metric measured here is likely to underestimate *FMO3*'s total influence on variance in nicotine clearance, given past evidence it is not obvious how differences in *FMO3* activity could result in such large differences in CPD. Furthermore, counter-intuitively, *FMO3*'s effect upon variance in CPD is greatest among *CYP2A6* fast metabolizers, whereas its effects upon variance in overall nicotine clearance is likely to be strongest in slower metabolizers [18]. One possible explanation for this is that because the relationship between nicotine blood level and receptor occupancy is not linear [41], small effects upon nicotine clearance are more significant in subjects who metabolize nicotine most rapidly. Another possibility is that our results properly reflect the genetic heterogeneity resulting in differing enzyme activity demonstrated in liver, but that the key FMO3 activity influencing CPD occurs in another tissue such as the brain, where CYP2A6 is not detected. Although relatively low compared to liver, *FMO3* expression [42], as well as FMO activity [43], has been demonstrated in human brain tissue. Such a partition between the activities of CYP2A6 and FMO3 might explain the interaction we see between them in predicting CPD; i.e. perdurance of nicotine in the brain due to lower FMO activity might result in lower CPD regardless of overall lower circulating nicotine levels in the blood of subjects with high hepatic CYP2A6 activity; likewise faster local clearance of nicotine by FMO in the brain might be trumped by consistent replenishment of nicotine in subjects with slower hepatic nicotine metabolism. Common polymorphism in another nicotine metabolism gene, *CYP2B6*, has been associated with enzyme levels in the brain [44], suggesting that further investigation of the potential role of nicotine metabolism in the central nervous system is warranted.

Overall, our results also underscore the pitfalls of single SNP analyses in discovering phenotype associations with highly polymorphic loci. As with *CYP2A6*, it appears that polymorphisms influencing protein function, mRNA expression, splicing, and stability, combine to produce the variance in *FMO3* function found in European Americans. Our results also demonstrate the usefulness of thoroughly determining the contribution of genetic variance in one gene (*CYP2A6*) to act as a covariate to detect the effects of variation in further genes (*FMO*s) upon a complex trait.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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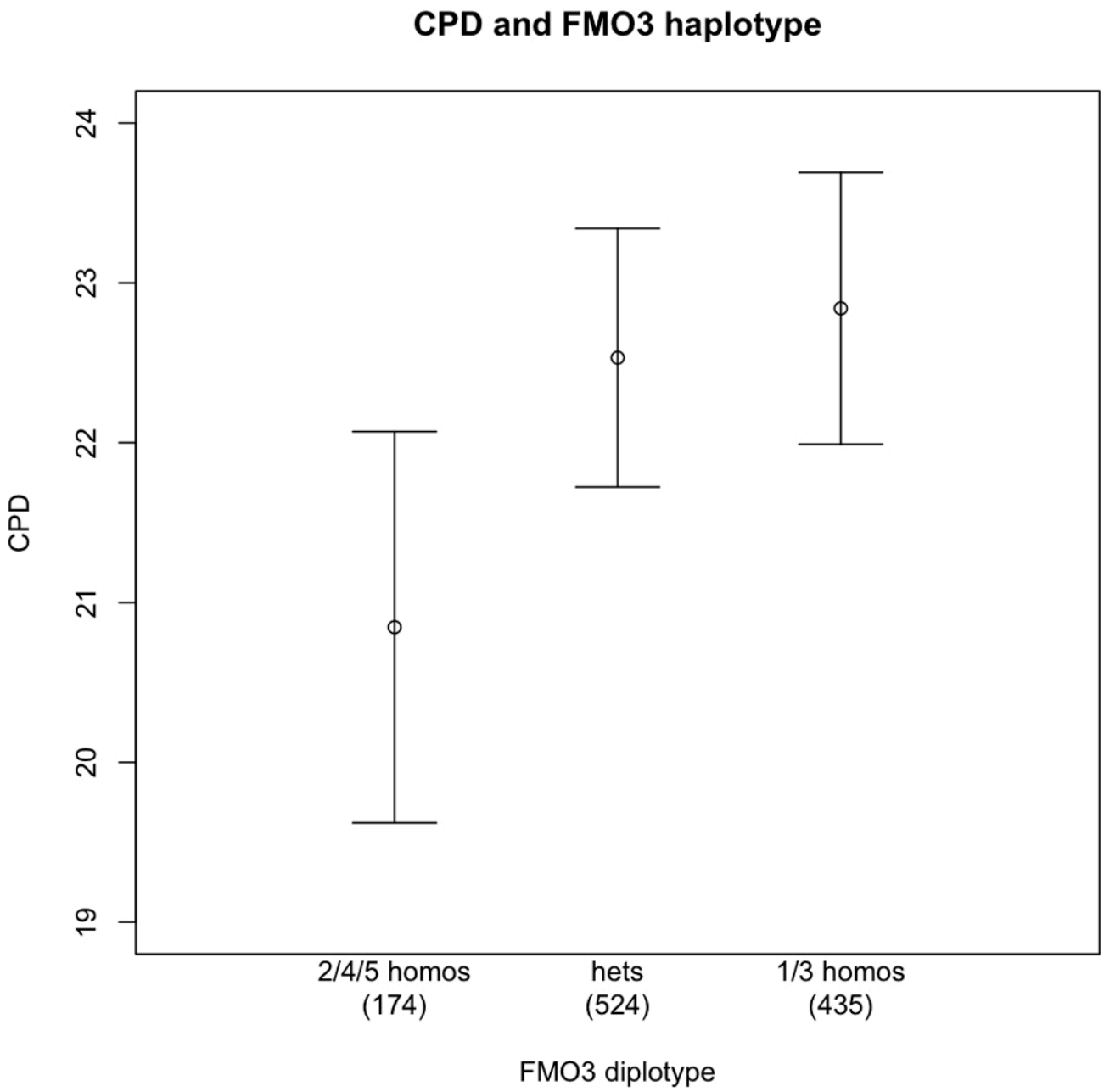

Figure 1.

Cigarettes per day among COGEND European American nicotine dependent smokers, divided by *FMO3* diplotype, ± 95% confidence intervals. 1/3 homozygotes carry only haplotypes 1 and 3 (Table 3), heterozygotes carry one haplotype 1 or 3 and one of the other haplotypes (2,4–10), 2/4/5 homozygotes carry only all other *FMO3* haplotypes (2,4&5, as well as the rare haplotypes 6–10). 1/3 homozygotes vs. 2/4/5 homozygotes, p=0.017; 2/4/5 homozygotes vs. heterozygotes, p=0.038

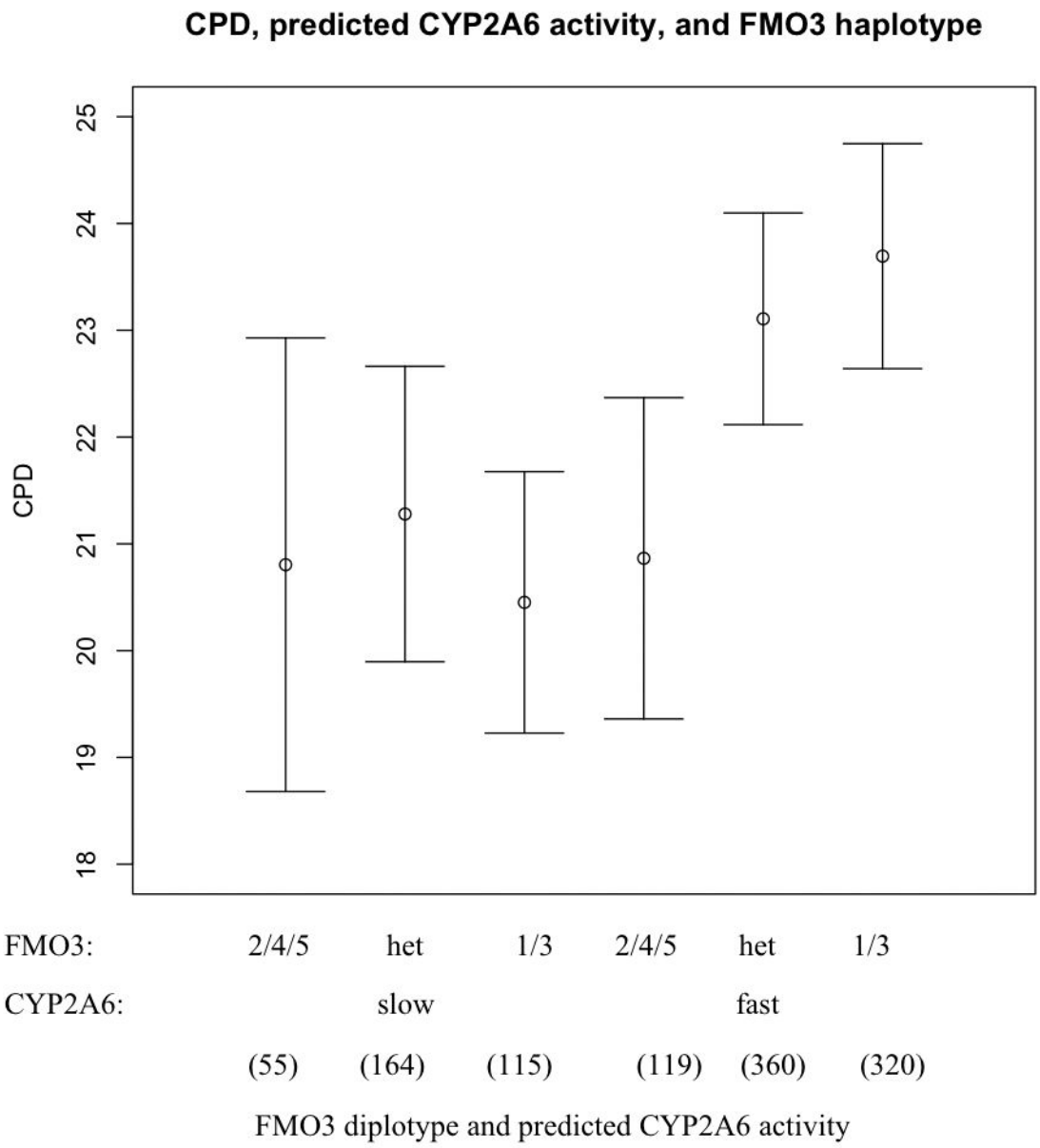

Figure 2.

Cigarettes per day among COGEND European American nicotine dependent smokers, divided by predicted *CYP2A6* function based on *CYP2A6* genotype, and *FMO3* diplotype, ± 95% confidence intervals. 1/3 homozygotes carry only *FMO3* haplotypes 1 and 3 (Table 3), heterozygotes carry one *FMO3* haplotype 1 or 3 and one of the other haplotypes (2,4–10), 2/4/5 homozygotess carry only all other *FMO3* haplotypes (2,4&5, as well as the rare haplotypes 6–10). 'CYP2A6 slow' are subjects with a predicted metabolism metric <0.85 [28], corresponding to all carriers of *CYP2A6*2,*4,*9,*12*, or **38* alleles, and **1A* homozygotes; 'CYP2A6 fast' are all other subjects with a predicted metabolism metric >0.85. CYP2A6 fast/FMO3 2/4/5 homozygotess vs. CYP2A6 fast/FMO3 1/3 homozygotes

p=0.006; CYP2A6 fast/FMO3 2/4/5 homozygotes vs. CYP2A6 fast/FMO3 heterozygotes p=0.027.

Table 1

FMO3 haplotypes associated with D_2cotinine/(D_2cotinine+ D_2nicotine)

FMO3 haplotype	rs1800822	rs2266782 (E158K)	rs1736557 (V257M)	rs909530	rs2266780 (E308G)	[a] metabolism alleles	parameter estimate relative to hap1	[b] p ≠ haps1&3	[c] p ≠ hap2,4&5	[d] COGEND alleles	COGEND frequency (%)
1	C	G	G	C	A	168			0.008	1855	45.2
2	C	**A**	G	C	A	83	−0.014	0.02		930	22.6
3	C	**A**	G	T	**G**	66	0.006		0.004	760	18.5
4	C	G	**A**	C	A	35	−0.017	0.03		296	7.2
5	T	G	G	T	A	20	−0.024	0.03		238	5.8
6	T	G	G	C	A	1				17	0.4
7	C	G	G	T	A	1				8	0.2
8	C	G	G	T	**G**	0				2	0.0
9	C	**A**	G	T	A	0				1	0.0
10	C	**A**	**A**	T	**G**	0				1	0.0

Polymorphic sites analyzed are given at the top of each column by rs number and amino acid changes when relevant. Haplotypes are ordered by frequency among COGEND European Americans.

[a]The number of each *FMO3* haplotype analyzed in the metabolism experiment.

[b]The probability that the parameter estimate for the haplotype is different by chance from *FMO3* haplotypes 1,3,6 and 7 combined. Rare haplotypes 6 and 7 are included in the reference in both analyses conservatively to avoid making assumptions about their effects. Haplotypes 2, 4 and 5 are shown to be independently significantly different from the reference.

[c]The probability that the parameter estimate for the haplotype is different by chance from *FMO3* haplotypes 2,4,5,6 and 7 combined. Rare haplotypes 6 and 7 are included in the reference in both analyses conservatively to avoid making assumptions about their effects. Haplotypes 1 and 3 are shown to be independently significantly different from the reference.

[d]The number of each *FMO3* haplotype found among all fully genotyped COGEND European American subjects (n=4,108 chromosomes).

Table 2

Association between *FMO3* haplotype class and D_2cotinine/(D_2cotinine+ D_2nicotine) divided by CYP2A6 activity estimated from *CYP2A6* genotype

Division by *CYP2A6* estimated activity [a]	n		Parameter estimate (± standard deviation) [b]		p [c]	
>0.90[d] & <0.90[e]	98	89	0.011±0.006	0.022±0.009	0.06	0.02
>0.85[f] & <0.85[g]	131	56	0.015±0.005	0.015±0.013	0.004	0.3
>0.80[h] & <0.80[i]	159	28	0.016±0.005	0.009±0.024	0.0007	0.7
all	188		0.016±0.005		0.003	

[a] Relative CYP2A6 activity predicted by diplotype [26].

[b] The estimated effect of one *FMO3* allele divided into two classes (1 & 3 vs. 2,4,5,6 & 7, Table 1), upon D_2cotinine/(D_2cotinine+ D_2nicotine) in a multivariate analysis with *CYP2A6* genotype.

[c] The probability that the number of alleles (0,1,2) of *FMO3,* divided into two classes (1 & 3 vs. 2,4,5,6 & 7, Table 1), is associated with D_2cotinine/(D_2cotinine+ D_2nicotine) in a multivariate analysis with *CYP2A6* genotype.

[d] Carriers of only full function *CYP2A6* alleles (**1B,*1D,*1H,*14*).

[e] Carriers of any partial-loss-of-function or null *CYP2A6* alleles (**1A,*9,*2,*4,*12,*38*).

[f] Carriers of only *CYP2A6* alleles (**1B,*1D,*1H,*14*), and **1A/*(**1B,*1D,*1H,*14*) heterozygotes.

[g] Carriers of *CYP2A6 *9,*2,*4,*12,*38* alleles and *CYP2A6*1A* homozygotes.

[h] Carriers of only *CYP2A6* alleles (**1B,*1D,*1H,*14*), and **1A/*(**1B,*1D,*1H,*14*) and **9/*(**1B,*1D,*1H,*14*) heterozygotes, and *CYP2A6*1A* homozygotes.

[i] Carriers of *CYP2A6* null alleles (**2,*4,*12,*38*), *CYP2A6*9* homozygotes, and **9/*1A* trans-heterozygotes.