

## **The ability of plasma cotinine to predict nicotine and carcinogen exposure is altered by differences in CYP2A6: the influence of genetics, race and sex**

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### **Conflict of Interest**

NLB has been a paid consultant to pharmaceutical companies that market medications for smoking cessation treatment, and has served as a paid expert witness in litigation against tobacco companies. RFT has participated in one day advisory meetings for Novartis and McNeil. DKH has received grant funding from Nabi Biopharmaceuticals to conduct a clinical trial. GES participated in a one-day advisory meeting for Pfizer in 2008.

## ABSTRACT

**Background:** Cotinine, a nicotine metabolite, is a biomarker of tobacco, nicotine and carcinogen exposure. However a given cotinine level may not represent the same tobacco exposure; for example, African Americans have higher cotinine levels than Caucasians after controlling for exposure.

**Methods:** Cotinine levels are determined by the amount of cotinine formation and the rate of cotinine removal which are both mediated by the enzyme CYP2A6. Since CYP2A6 activity differs by sex (estrogen induces CYP2A6) and genotype, their effect on cotinine formation and removal were measured in non-smoking Caucasians (Study 1, n=181) infused with labeled nicotine and cotinine. The findings were then extended to *ad libitum* smokers (Study 2, n=163).

**Results:** Study 1: Reduced CYP2A6 activity altered cotinine formation less than cotinine removal resulting in ratios of formation to removal of 1.31 and 1.12 in *CYP2A6* reduced and normal metabolizers (P=0.01), or 1.39 and 1.12 in males and females (P=0.001), suggesting an overestimation of tobacco exposure in slower metabolizers. Study 2: Cotinine again overestimated tobacco and carcinogen exposure by  $\geq 25\%$  in *CYP2A6* reduced metabolizers ( $\approx 2$  fold between some genotypes) and in males.

**Conclusions:** In people with slower, relative to faster, CYP2A6 activity cotinine accumulates resulting in substantial differences in cotinine levels for a given tobacco exposure.

**Impact:** Cotinine levels may be misleading when comparing those with differing *CYP2A6* genotypes within a race, between races with differing frequencies of *CYP2A6* gene variants (i.e. African Americans have higher frequencies of reduced function variants contributing to their higher cotinine levels) or between the sexes.

## ***INTRODUCTION***

In humans, cotinine is metabolically formed from nicotine in a reaction catalyzed by CYP2A6 and is further metabolized to *trans*'-3-hydroxycotinine (3HC) by CYP2A6 (1, 2). Cotinine is routinely used as an objective index of tobacco and tobacco-derived carcinogen exposure. The use of cotinine is particularly useful because of wide individual variability in the relationship between self-reported cigarettes smoked per day and systemic exposure to tobacco and tobacco-derived carcinogen (3). Higher plasma cotinine levels have been associated with increased lung cancer risk (4), and variation in cotinine levels have recently been used as evidence that the mechanism mediating the association between genetic variation in *CHRNA5-A3-B4* and lung cancer risk is almost entirely through the modulation of tobacco consumption (5).

The systemic intake of nicotine is correlated with exposure to tobacco-derived carcinogen (3). When used as a biomarker of tobacco-derived carcinogen exposure, it is generally assumed that plasma cotinine levels reflect intake of nicotine, and that variability in the relationship between cotinine levels and nicotine intake is random among smokers. However, some research suggests that a particular level of cotinine does not predict the same level of tobacco exposure among different groups of smokers. For example, directional inconsistencies have been observed between plasma cotinine levels and other indicators of tobacco exposure in genetic association studies involving *CYP2A6*. Caucasian smokers with one or more reduced function *CYP2A6* alleles (i.e. *CYP2A6* reduced metabolizers, or RM) smoke fewer cigarettes per day compared to the *CYP2A6* normal metabolizers (NM), consistent with titration of smoke intake to obtain desired levels of nicotine in the body (6, 7). Yet despite smoking fewer cigarettes per day, smokers with reduced CYP2A6 activity have similar plasma cotinine levels compared to the smokers with faster CYP2A6 activity (8). Another example is found in studies of African American light smokers,

where similar levels of cigarettes per day are reported between *CYP2A6* genotypes (i.e. *CYP2A6* RM have similar cigarettes per day compared to *CYP2A6* NM), but the plasma cotinine levels are significantly higher in *CYP2A6* RM compared to *CYP2A6* NM (9). Furthermore, gene variants at the *CYP2A6* loci (i.e. tag SNPs) that are associated with decreased lung cancer risk are paradoxically associated with increased, rather than decreased, plasma cotinine levels (10). These observations suggest that *CYP2A6* reduced activity genotype may increase plasma cotinine levels in addition to its role in reducing nicotine inactivation, tobacco consumption and nitrosamine metabolic activation (11).

Variation in plasma cotinine levels is also observed between the sexes. The male participants of the National Health and Nutrition Examination Surveys have significantly higher plasma cotinine levels compared to the female participants even after adjusting for cigarettes per day, machine-determined nicotine delivery of cigarettes, race, age, body mass index, poverty status, and the use of either menthol or regular cigarettes (12). Since *CYP2A6* activity differs between the sexes, this systematic variation in cotinine levels between the sexes could be the result of the difference in *CYP2A6* activity (13).

Notable variation in cotinine levels is also observed between races. African American smokers generally have higher plasma cotinine levels compared to Caucasian smokers after adjusting for the number of cigarettes smoked per day and the machine-determined nicotine delivery in cigarettes (14), which could be partially due to the slower cotinine clearance in African Americans compared to Caucasians (15). Since the prevalence of *CYP2A6* RM is high in African Americans compared to Caucasians, it is possible that the racial variation in cotinine levels originates from the difference in *CYP2A6* activities between racial groups.

Steady state plasma cotinine levels are determined by three factors: the daily intake of nicotine, the fraction of nicotine converted to cotinine ( $f_{\text{NIC} \rightarrow \text{COT}}$ ), and the systemic clearance of cotinine. The steady state plasma cotinine level is described by the following equation:

$$[\text{COT}_{\text{plasma}}] = \text{Dose}_{\text{NIC}} \times \frac{f_{\text{NIC} \rightarrow \text{COT}}}{\text{Cl}_{\text{COT}}} \quad (1)$$

where  $\text{COT}_{\text{plasma}}$  represents the steady state plasma cotinine levels,  $\text{Dose}_{\text{nic}}$  is the daily nicotine intake,  $f_{\text{NIC} \rightarrow \text{COT}}$  is the fraction of nicotine converted to cotinine (i.e. cotinine formation), and  $\text{Cl}_{\text{COT}}$  represents systemic plasma cotinine clearance (i.e. cotinine removal) (16). Hence, the steady state plasma cotinine levels are proportional to the ratio of  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$  and the  $\text{Dose}_{\text{nic}}$ .

Cotinine is both formed and removed by CYP2A6. Thus, both  $f_{\text{NIC} \rightarrow \text{COT}}$  and cotinine clearance are highly dependent on CYP2A6 activity. We hypothesize that reduced CYP2A6 activity will increase the ratio of  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ , and alter the quantitative relationship between plasma cotinine and nicotine and tobacco-derived carcinogen exposure in cigarette smokers.

In this study, we use *CYP2A6* genotype as the primary indicator of CYP2A6 activity. *CYP2A6* genetic variants are known to alter *in vivo* CYP2A6 activity as well as nicotine and cotinine clearance (17-23). In addition to *CYP2A6* genotype, we also use the nicotine metabolite ratio (NMR) as a secondary indicator of CYP2A6 activity (24). For the evaluation of nicotine intake in *ad libitum* smokers, we use urinary total nicotine equivalents (TNE) (25). For the evaluation of tobacco-derived carcinogen in *ad libitum* smokers, we used total urinary (methylnitrosamino)-1-(3) pyridyl-1-butanol (NNAL), 1-hydroxyfluorene and 1-hydroxypyrene levels (26, 27).

## MATERIALS AND METHODS:

### *Study design*

Data for the present analysis were taken from two studies that have been previously published (21, 28).

**Study 1:** The pharmacokinetics of nicotine and cotinine were characterized in 181 non-smoking Caucasian male and female sets of twins (both monozygotic and dizygotic). Demographic variable comparisons are shown in supplementary table 1. A comprehensive description of the study procedures has been published previously (29, 30). Briefly, participants received a simultaneous 30-minute infusion of deuterium-labeled nicotine and cotinine in the fasting condition. Blood samples were collected for the measurement of labeled nicotine and cotinine for determination of pharmacokinetic parameters and DNA was extracted for *CYP2A6* genotyping. The cotinine and 3HC levels were measured in the 480-minute plasma sample for determination of the NMR (21). The pharmacokinetic calculations can be found in the supplementary methods.

**Study 2:** The relationship between plasma cotinine, nicotine intake and carcinogen exposure was studied in daily *ad libitum* smokers (Supplementary table 1). In a cross sectional study of 163 Alaska Native smokers, the plasma cotinine levels urinary TNE, total NNAL and PAH metabolites were measured. A comprehensive description of the study procedures has been published elsewhere (28).

### **Tobacco and tobacco derived carcinogen exposure biomarkers**

To evaluate the quantitative relationship between plasma cotinine and tobacco exposure in cigarette smokers, we used urinary total nicotine equivalents (TNE) from Study 2 participants as

the reference biomarker of nicotine and tobacco exposure as it is the summation of multiple nicotine metabolic pathways. TNE is the total urinary level of nicotine and 8 of its metabolites (i.e. nicotine, nicotine glucuronide, cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, nicotine-*N*-oxide, cotinine-*N*-oxide, and nornicotine). Together, the 9 analytes account for about 90% of nicotine (determined from a transdermal administered nicotine dose (31)), and creatinine adjusted spot urinary TNE correlates with daily tobacco consumption (32). TNE is not influenced by the different rates of nicotine and cotinine metabolism since it measures the nicotine metabolites generated via different metabolic pathways.

To investigate the relationship between plasma cotinine levels and tobacco-derived carcinogen exposure, we used total urinary (methylnitrosamino)-1-(3) pyridyl-1-butanol (NNAL) levels as the biomarker for tobacco specific nitrosamines exposure in study 2 participants (33). NNAL is a reductive metabolite of the highly carcinogenic 4-(methylnitrosamino)-1-(3) pyridyl-1-butanone (NNK); prediagnostic levels are associated with subsequent lung cancer risk (34).

We also investigated the relationship between plasma cotinine levels and exposure to another class of tobacco-related carcinogens which are not nicotine-derived, the polycyclic aromatic hydrocarbons (33). The urinary levels of two hydroxylated polycyclic aromatic hydrocarbons, 1-hydroxypyrene and 1-hydroxyfluorene, were used as biomarkers of polycyclic aromatic hydrocarbons exposure. 1-Hydroxypyrene levels have been directly associated with cancer risk (35), and recent evidence suggests that 1-hydroxyfluorene is more specific to tobacco exposure than 1-hydroxypyrene (27).

### ***CYP2A6 genotyping***

Prevalent *CYP2A6* alleles with altered function were genotyped by two step-allele specific PCR reactions in the Pharmacogenetics laboratory at CAMH and University of Toronto as previously reported (7, 20, 36, 37). Those individuals with one or two copies of reduced function allele (\*2, \*4, \*7, \*9, \*10, \*12, \*17 and \*35) were classified as *CYP2A6* RM (20, 36, 37).

### ***The measurement of CYP2A6 activity***

The in vivo CYP2A6 activity was measured using NMR, which is the ratio between plasma 3HC and cotinine. NMR is widely used as an *in vivo* indicator of CYP2A6 activity (24, 36, 38, 39). In the context of our data analysis, the use of NMR may be confounded with plasma cotinine since it uses plasma cotinine as the denominator. Thus, we use NMR to extend our findings with *CYP2A6* genotype as it is a continuous variable and for comparisons to the literature.

### ***Tobacco and tobacco-derived carcinogen exposure measurements***

Plasma nicotine, cotinine and 3HC levels, as well as urinary nicotine and metabolites, total NNAL, and PAH metabolites were quantified by gas/liquid chromatography-tandem mass spectrometry in the Clinical Pharmacology Laboratory at the University of California San Francisco as previously described (24, 40-43). NMR stratification was done by a median split of plasma NMR within each study population; participants who were in the higher NMR stratum were considered the faster CYP2A6 activity group, while those in the lower NMR stratum were considered the slower CYP2A6 activity group.

### ***Statistical analysis***

Statistical analyses were performed using R statistical package (version 2.13, R foundation for statistical computing). In study 1 statistical comparisons between *CYP2A6* genotypes and sex were performed using mixed-effect linear regressions. All analyses controlled for non-



independence of data in twin pairs by modeling twinning as a random effect (21). Spearman's correlation was used to evaluate the relationship between 1) NMR and  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ ; 2) NMR and cotinine to TNE ratio; 3) Plasma cotinine and urinary TNE, NNAL, 1-hydroxypyrene and 1-hydroxyfluorene. Fisher's r to z transformation was used to compare the correlation coefficients. To minimize the influence of potential confounders such as race and smoking status on cotinine pharmacokinetics, the effects of *CYP2A6* genotype and sex were examined within non-smoking Caucasians in Study 1.

## RESULTS

### ***CYP2A6 Reduced function genetic variants decreased cotinine removal more than cotinine formation (Study 1)***

Fig. 1A compares cotinine formation ( $f_{\text{NIC} \rightarrow \text{COT}}$ ) between *CYP2A6* genotypes in non-smoking Caucasians. *CYP2A6* RM (n=33) had significantly lower  $f_{\text{NIC} \rightarrow \text{COT}}$  compared to *CYP2A6* NM (n=148). In *CYP2A6* RM, 67% of the nicotine dose was metabolized to cotinine compared to the 77% in *CYP2A6* NM, a 15% relative reduction,  $P=0.001$  (Fig. 1A). *CYP2A6* RM also had lower cotinine clearance compared to the *CYP2A6* NM (0.58 ml/min/kg in *CYP2A6* RM versus 0.77 ml/min/kg in *CYP2A6* NM, a 33% reduction,  $P=0.01$ , Fig. 1B). The ratio of the fractional conversion of nicotine (reflecting cotinine formation) to cotinine clearance (reflecting cotinine removal),  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ , was significantly higher in *CYP2A6* RM compared to *CYP2A6* NM (1.31 in *CYP2A6* RM versus 1.12 in *CYP2A6* NM,  $P=0.01$ , Fig. 1C). Of note, the resulting  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$  ratio is the conversion factor, or average correction needed in plasma cotinine levels to accurately indicate the same nicotine dose (Fig. 1D). These genetic differences were consistent in both males and females (Supplementary fig. 1A-F). In agreement with the findings

between *CYP2A6* genotypes, statistically significant differences in  $f_{\text{NIC} \rightarrow \text{COT}}$ , cotinine clearance and  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$  were observed when comparing the faster versus the slower NMR strata (Supplementary fig. 1G-H). There was a significant inverse correlation between NMR (i.e. a continuous measure of *CYP2A6* activity) and  $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ , suggesting the effect size is even larger when comparing the two extreme ends of *CYP2A6* activity ( $\text{Rho}=-0.57$ ,  $P<0.0001$ ).

***Different relationships between plasma cotinine and tobacco exposure were observed between smokers with different CYP2A6 genotype groups (Study 2)***

Next, the quantitative relationship between plasma cotinine and nicotine intake in smokers was investigated. Demographic variable comparisons are shown in supplementary table 1. Independent regression lines between urinary TNE and plasma cotinine were constructed in *CYP2A6* RM and NM ( $n=74$  and  $n=89$ , respectively). As illustrated by fig. 2, the slope of the regression line in *CYP2A6* RM was significantly lower compared to the slope in *CYP2A6* NM (slope: 0.33 in *CYP2A6* RM versus 0.42 in *CYP2A6* NM,  $P=0.001$ , Fig. 2). Statistical significance is demonstrated by a significant interaction between *CYP2A6* genotype and cotinine levels in the linear regression analysis presented in supplementary table 2A. A similar difference in regression line slopes was observed between the NMR strata (Supplementary fig. 2). There was a significant inverse correlation between NMR, a continuous measure of *CYP2A6* activity, and the cotinine to TNE ratio ( $\text{Rho}=-0.46$ ,  $P<0.0001$ ). These observations indicate that cotinine predicts nicotine intake, and therefore tobacco consumption, differently in smokers with reduced compared to normal *CYP2A6* activity. As illustrated by fig. 2, a 300 ng/mL (which is equivalent to 1702 nmol/L) cotinine level was indicative of a 100 nmol nicotine equivalents exposure in *CYP2A6* NM, whereas the same cotinine level was indicative of roughly 125 nmol exposure in *CYP2A6* RM. Alternatively, at 100 nmol tobacco exposure, *CYP2A6* RM would have 300 ng/mL plasma

cotinine levels, whereas the *CYP2A6* NM would have only 240 ng/mL. This is a 25% difference (60 ng/mL or 340 nmol/L) in cotinine levels between *CYP2A6* genotype groups. Since reduced metabolizers include those with a range of decreased activity combined together, the 25% reflects an average difference (Fig. 3A). As illustrated by some specific genotypes, or quartiles of NMR, these differences in cotinine estimates of dose can be  $\approx 2$  fold when comparing between the extremes of *CYP2A6* activity or among different genotypes (fig. 3B and fig. 3C).

***Different relationships between plasma cotinine and tobacco specific nitrosamine exposures were observed in smokers with different CYP2A6 genotype groups (Study 2)***

Next, we investigated the quantitative relationship between plasma cotinine and tobacco specific nitrosamines exposure in smokers. As seen with TNE, the slope of the regression line between urinary NNAL and plasma cotinine in *CYP2A6* RM was significantly lower compared to the slope in *CYP2A6* NM (slope: 1.279 in *CYP2A6* RM versus 1.687 in *CYP2A6* NM,  $P=0.003$ , fig. 4. Statistical significance demonstrated by a significant interaction term in the linear regression analysis presented in supplementary table 2B). These results demonstrated that that cotinine levels predict NNAL levels, and therefore nitrosamine exposure, differently in smokers with reduced compared to normal *CYP2A6* activity. As illustrated by fig. 4, a 300 ng/mL (which is equivalent to 1702 nmol/L) cotinine level was indicative of a 510 pg/mg creatinine NNAL exposure in *CYP2A6* NM, whereas the same cotinine level was indicative of roughly 375 pg/mg creatinine NNAL exposure in *CYP2A6* RM. Alternatively, at 375 pg/mg creatinine NNAL exposure, *CYP2A6* RM would have 300 ng/mL plasma cotinine levels, whereas the *CYP2A6* NM would have only 225 ng/mL. The magnitude of the *CYP2A6* genotype effect (25%) on plasma cotinine levels was similar to that observed with TNE as the exposure marker; as with the TNE

biomarker this is an underestimation of effect size for some genotype or phenotype comparisons (see figure 3).

To determine whether the above observations were unique to plasma cotinine, independent regression lines between urinary NNAL and urinary TNE levels were constructed in *CYP2A6* RM and NM. The slope of the regression lines did not differ between *CYP2A6* genotypes (Supplementary fig 3, and supplementary table 2C), indicating that as expected TNE's relationship to tobacco specific nitrosamines exposure in smokers was independent of *CYP2A6* genotype. Together this demonstrates that the impact of *CYP2A6* genotype on plasma cotinine levels was specific to cotinine as an exposure biomarker.

***Different relationships between plasma cotinine and polycyclic aromatic hydrocarbon exposure were observed in smokers with different CYP2A6 genotype groups (Study 2)***

Next, we investigated the quantitative relationship between plasma cotinine and polycyclic aromatic hydrocarbon exposure in smokers. As illustrated by supplementary fig. 4 and fig. 5, the slopes of the regression lines in *CYP2A6* RM were lower compared to the slope in *CYP2A6* NM (1-hydroxyfluorene slopes: 0.024 in *CYP2A6* RM vs. 0.027 in *CYP2A6* NM,  $P=0.013$ , supplementary fig. 4 and supplementary table 2D; 1-hydroxypyrene slopes: 0.012 in *CYP2A6* RM versus 0.010 in *CYP2A6* NM,  $P=0.079$ , Fig. 5 and supplementary table 2E).

***Males had a greater reduction in cotinine clearance than in cotinine formation (Study 1)***

Because *CYP2A6* activity differs between the sexes, we investigated the impact of sex on cotinine formation and cotinine clearance. The  $f_{\text{NIC} \rightarrow \text{COT}}$  did not differ between the sexes (74% in males compared to 75% in females, non-significant, Fig. 6A). However males had a significantly lower cotinine clearance compared to females (0.58 versus 0.81 ml/min/kg in males and females,

respectively,  $P < 0.001$ , Fig. 6B). A significantly higher  $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$  ratio, which is the conversion factor between plasma cotinine levels and the nicotine dose (Fig. 1D), was observed in males compared to females (1.39 versus 1.12 in males and females, respectively,  $P = 0.001$ , Fig. 6C). As observed with the entire study population, similar impacts of sex on  $f_{\text{NIC} \rightarrow \text{COT}}$ , cotinine clearance and  $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$  could be observed when examined only within the subgroup of *CYP2A6* NM (i.e. excluding the *CYP2A6* RM, supplementary fig. 5).

### ***Different relationships between plasma cotinine and tobacco exposure were observed in male smokers compared to female smokers (Study 2)***

Independent regression lines between urinary TNE and plasma cotinine were constructed by sex. The slope of the regression line in males was significantly lower compared to the slope in females (0.34 in males versus 0.42 in females,  $P = 0.01$ , Fig 7 and supplementary table 2F). The magnitude of the sex effect on cotinine levels was similar to the effect of *CYP2A6* genotype. At 100 nmol tobacco exposure, there is a 25% difference in plasma cotinine levels (60 ng/mL or 340 nmol/L) between the sexes. As observed with the entire study population, similar impacts of sex could be observed when examined within just the *CYP2A6* NM (slopes:  $0.36 \pm 0.02$  in males versus  $0.54 \pm 0.03$  in females,  $P = 0.001$ )

## ***DISCUSSION***

Our analyses demonstrate that variation in *CYP2A6* metabolic activity, such as those that exist between *CYP2A6* genotypes or the sexes, may alter cotinine removal (i.e. cotinine clearance) to a different extent than cotinine formation (i.e.  $f_{\text{NIC} \rightarrow \text{COT}}$ ). Individuals with lower *CYP2A6* activities, such as *CYP2A6* RM and males, have higher ratios of cotinine formation to cotinine removal (i.e.  $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ ) compared to individuals with faster *CYP2A6* activity, such as

*CYP2A6* NM and females. Thus, the quantitative relationship between plasma cotinine and tobacco and tobacco derived carcinogen exposures in smokers varies between those with different *CYP2A6* activity.

### **The pharmacokinetic mechanism**

The greater effect of altered *CYP2A6* activity on cotinine clearance compared to formation is likely related to differences in nicotine and cotinine's hepatic metabolism. Nicotine exhibits a higher hepatic extraction ratio compared to cotinine, which is largely due to differences in their intrinsic clearances. The intrinsic clearance ( $V_{\max}/K_m$ ) of nicotine is 1.69  $\mu\text{L}/\text{min}/\text{mg}$  protein in human liver microsomes, which is 10 times faster than the 0.16  $\mu\text{L}/\text{min}/\text{mg}$  protein of cotinine (1, 2, 44, 45). For drugs like nicotine, with high extraction ratios, clearance is determined in large part by liver blood flow and is less affected by changes in liver enzymatic activity. In contrast for low extraction drugs like cotinine clearance is largely affected by changes in liver enzymatic activity. This differential impact of variation in *CYP2A6* activity is consistent with previous observations on the effects of *CYP2A6* induction. Estrogen induces *CYP2A6* protein expression in humans (46). During pregnancy, when estrogen levels are elevated, the clearance of nicotine increases by 60% whereas the clearance of cotinine increased by 140% (47). Thus, higher *CYP2A6* activity during pregnancy has a greater effect on cotinine clearance than nicotine clearance.

### ***CYP2A6*, sex and race**

Consistent with a higher  $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$  ratio predicting higher cotinine levels for the same tobacco and tobacco-derived carcinogen exposure (equation 1), we observed different quantitative relationships between cotinine and tobacco and tobacco-derived carcinogen exposure in smokers with reduced *CYP2A6* activities (*CYP2A6* RM and males). These observations provide

a mechanistic explanation for the systematic variation in plasma cotinine levels previously observed between *CYP2A6* genotypes, the sexes and possibly races (9, 10, 12).

Our observations explain the directional inconsistencies between cotinine and other indicators of tobacco consumption in genetic association studies involving *CYP2A6* (9, 10). Due to the higher  $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$  ratio, *CYP2A6* RM would have higher plasma cotinine levels upon the same tobacco exposure compared to the *CYP2A6* NM. This masks, or reduces, the true size of the effect of *CYP2A6* genotype on tobacco consumption based on plasma cotinine (i.e. *CYP2A6* RM have higher cotinine levels with lower tobacco consumption). These results could also explain why the same *CYP2A6* genetic variants have been associated with lower lung cancer risk while having paradoxically higher plasma cotinine levels (10).

Similarly, during transdermal nicotine patch treatment (i.e. one week after starting treatment) steady state plasma cotinine levels in biochemically confirmed abstinent individuals (38) were inversely related to the pre-treatment *CYP2A6* activity (supplementary fig. 6). The 25% (50 ng/mL) average differences in cotinine levels between participants with faster and slower *CYP2A6* activity (NMR) was in agreement with the effect size observed in smokers in study 2. The difference double to 50% (100 ng/mL) when comparing those in the first versus fourth quartile of *CYP2A6* activity. Thus for the same systemic nicotine exposure (delivered by 21mg patch for all participants), the individuals with slower *CYP2A6* activity had higher steady state cotinine levels than the individuals with faster *CYP2A6* activity (supplementary fig. 6).

Our results could explain the systematic variation in plasma cotinine levels between the sexes (48). Due to the lower average estrogen levels, males have lower *CYP2A6* protein expression and activity compared to females (49). Thus, males have higher  $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$  ratios,

which suggest that males would have higher plasma cotinine levels compared to females with similar tobacco exposure.

Finally, our observation could also explain the differences in plasma cotinine levels between Caucasians and African Americans even when controlling for tobacco exposure. About 50% of African Americans are *CYP2A6* RM compared to the 20% in Caucasians (50). Therefore, African Americans have, on average, higher  $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$  ratios compared to Caucasians, resulting in higher plasma cotinine levels for similar tobacco exposure.

### **Implications for cancer research**

Plasma cotinine is often used as an objective, non-self-report, indicator of tobacco exposure in smoking-related cancer research. However, there could be a 25% or greater variation in plasma cotinine levels following the same tobacco and tobacco-derived carcinogen exposure depending on *CYP2A6* genotype or in the presence of other inducers/inhibitors of *CYP2A6* activity. To put this difference in perspective, Munafò et al. reported that each risk allele in rs16969968, a single nucleotide polymorphism in the  $\alpha 5$  nicotinic acetylcholine receptor, is associated with a 138.4 nmol/L (or 24 ng/mL) increase in plasma cotinine levels, which in turn was predicted to increase lung cancer risk by 1.3 times (5). Our data suggest that the difference between *CYP2A6* genotypes (or sex) would be approximately 340 nmol/L (or 60 ng/mL) at 100 nmol tobacco exposure (or roughly 375 pg/mg Cre NNAL). Hence, cotinine levels alone, without considering *CYP2A6* activity, may not provide the most accurate information regarding tobacco and tobacco-derived carcinogen exposure in smokers, particularly when comparing groups with different frequencies of *CYP2A6* genotypes (or race) or sex. Biomarkers of nicotine intake such as TNE that are less dependent on individual differences in nicotine metabolism or biomarkers of



toxicants that are more directly involved in the carcinogenic process, will enhance our understanding of the contribution of variable levels of tobacco consumption on lung cancer risk.

In conclusion, the quantitative relationship between plasma cotinine level and tobacco and tobacco-derived carcinogen exposure differs among groups with different levels of CYP2A6 activity. These comparison groups include the different *CYP2A6* genotype groups within a race (e.g. *CYP2A6* RM versus *CYP2A6* NM among Caucasians) and different races with distinct prevalence of *CYP2A6* RM (e.g. Caucasians with 20% *CYP2A6* RM versus African Americans with 50% *CYP2A6* RM). Furthermore, groups with different estrogen levels may also have variation in cotinine levels unrelated to differences in tobacco exposure. Those include different sexes, or those with varying pregnancy and menopause statuses. Therefore cotinine levels, as a quantitative marker of tobacco and tobacco-derived carcinogen exposure, are not directly comparable between *CYP2A6* genotypes, sexes, and races.

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## FIGURE LEGEND:

**Figure 1.** Reduced CYP2A6 activity had a greater impact on cotinine removal than cotinine formation (Study 1), which would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure. **A.** CYP2A6 reduced metabolizers metabolized (n=33) a significantly lower amount of nicotine to cotinine compared to CYP2A6 normal metabolizers (n=148). **B.** CYP2A6 reduced metabolizers (n=33) had lower cotinine clearance ( $CL_{COT}$ ) compared to CYP2A6 normal metabolizers (n=148). **C.** The  $f_{NIC \rightarrow COT}/CL_{COT}$  ratio was higher in CYP2A6 reduced metabolizers (n=33) compared to CYP2A6 normal metabolizers (n=148). Data presented as mean  $\pm$  95% confidence interval. **D.** The  $f_{NIC \rightarrow COT}/CL_{COT}$  ratio is the conversion factor between plasma cotinine levels and the actual nicotine dose.

**Figure 2.** Cotinine's ability to predict tobacco exposure was different between CYP2A6 genotypes (Study 2). The slope between urinary TNE and plasma cotinine was significantly lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, supplementary table 2A), suggesting the quantitative relationship between cotinine and tobacco exposure (i.e. TNE) differed between CYP2A6 genotypes. # indicates statistical significant difference in Spearman's Rho compared to the CYP2A6 normal metabolizers. The numbers after the slopes are standard error. Of note, the strength of correlations between plasma cotinine and urinary TNE in the CYP2A6 RM was significantly weaker than in CYP2A6 NM (Fisher's  $r$  to  $Z$  transformation:  $P < 0.01$ ).

**Figure 3. A.** The cotinine to TNE ratio (i.e. cotinine levels per nicotine intake) was significantly lower in CYP2A6 NM compared to the CYP2A6 RM. (Study 2) **B.** The cotinine to TNE ratio decreased with CYP2A6 genotypes with increasing activity (Study 2). As illustrated using some different CYP2A6 genotypes, containing reduced function (\*9) or loss of function (\*4) allele compared to the wild type individuals (\*1/\*1). Of note, individuals who are fully null for CYP2A6 (i.e. these with two copies of gene deletions, CYP2A6\*4/\*4) had unexpectedly lower COT to TNE ratio compared to the wild-type individuals (\*1/\*1, data not shown), suggesting the minor remaining cotinine formation pathway (likely CYP2B6 or CYP2A13) is considerably slower than the low affinity cotinine removal pathway (likely UGT2B10 glucuronidation or renal clearance). **C.** The cotinine to TNE ratio decreased by NMR quartiles (Study 2). Data presented as mean  $\pm$  95% confident intervals. Statistical comparisons were performed by Mann Whitney or Kruskal-Wallis tests.

**Figure 4.** Cotinine's ability to predict tobacco specific nitrosamines exposure (as indicated by NNAL levels) was different between CYP2A6 genotypes (Study 2). The slope between urinary NNAL levels and plasma cotinine was significantly lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, supplementary table 2B). This suggested the quantitative relationship between cotinine and tobacco specific nitrosamines exposure differed between CYP2A6 genotypes. The numbers after the slopes are standard error.

**Figure 5.** Cotinine's ability to predict polycyclic aromatic hydrocarbon exposure (as indicated by 1-hydroxypyrene) was different between *CYP2A6* genotypes (Study 2). The slope between 1-hydroxypyrene levels and plasma cotinine was lower in *CYP2A6* reduced metabolizers (n=74) compared to that of *CYP2A6* normal metabolizers (n=89, supplementary table 2E). The numbers after the slopes are standard error.

**Figure 6.** Males (n=54), who have reduced *CYP2A6* activity compared to females (n=127), had a greater ratio of cotinine formation over cotinine removal, which would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure in male smokers (Study 1). **A.** The  $f_{\text{NIC} \rightarrow \text{COT}}$  did not differ between the sexes. **B.** Males had significantly lower  $\text{CL}_{\text{COT}}$  compared to the females. **C.** Males had a higher  $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$  compared to females indicating an over-estimation of nicotine dose for their cotinine levels (see Fig 1D). Data presented as mean  $\pm$  95% confidence interval.

**Figure 7.** Cotinine's ability to predict tobacco exposure was different between the sexes. The slope between urinary TNE and plasma cotinine was significantly lower in males (n=71) compared to that of females (n=92, supplementary table 2F), suggesting cotinine over-estimates tobacco exposure (i.e. TNE) in males compared to females (Study 2). The numbers after the slopes are standard error.

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Figure 1

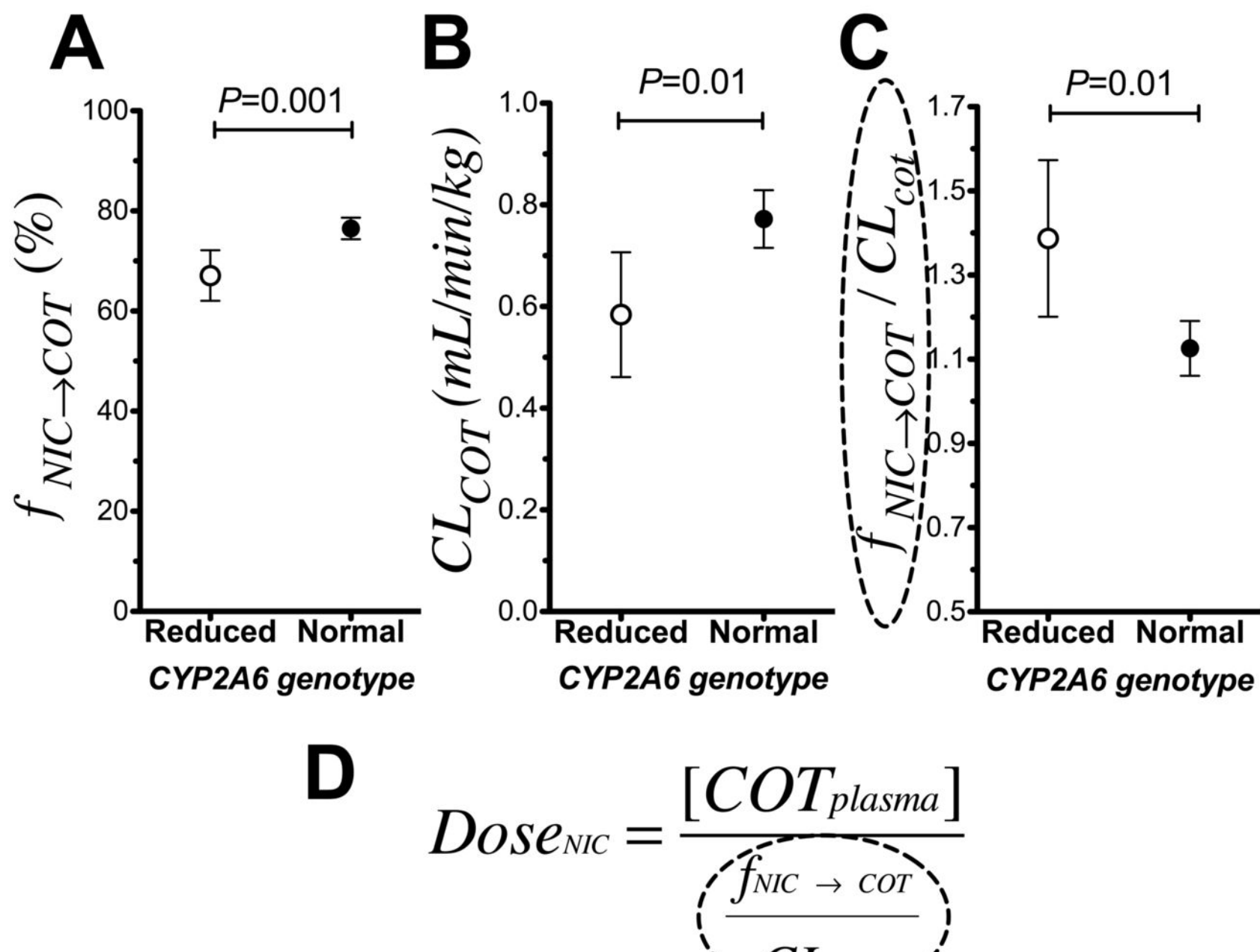


Figure 2

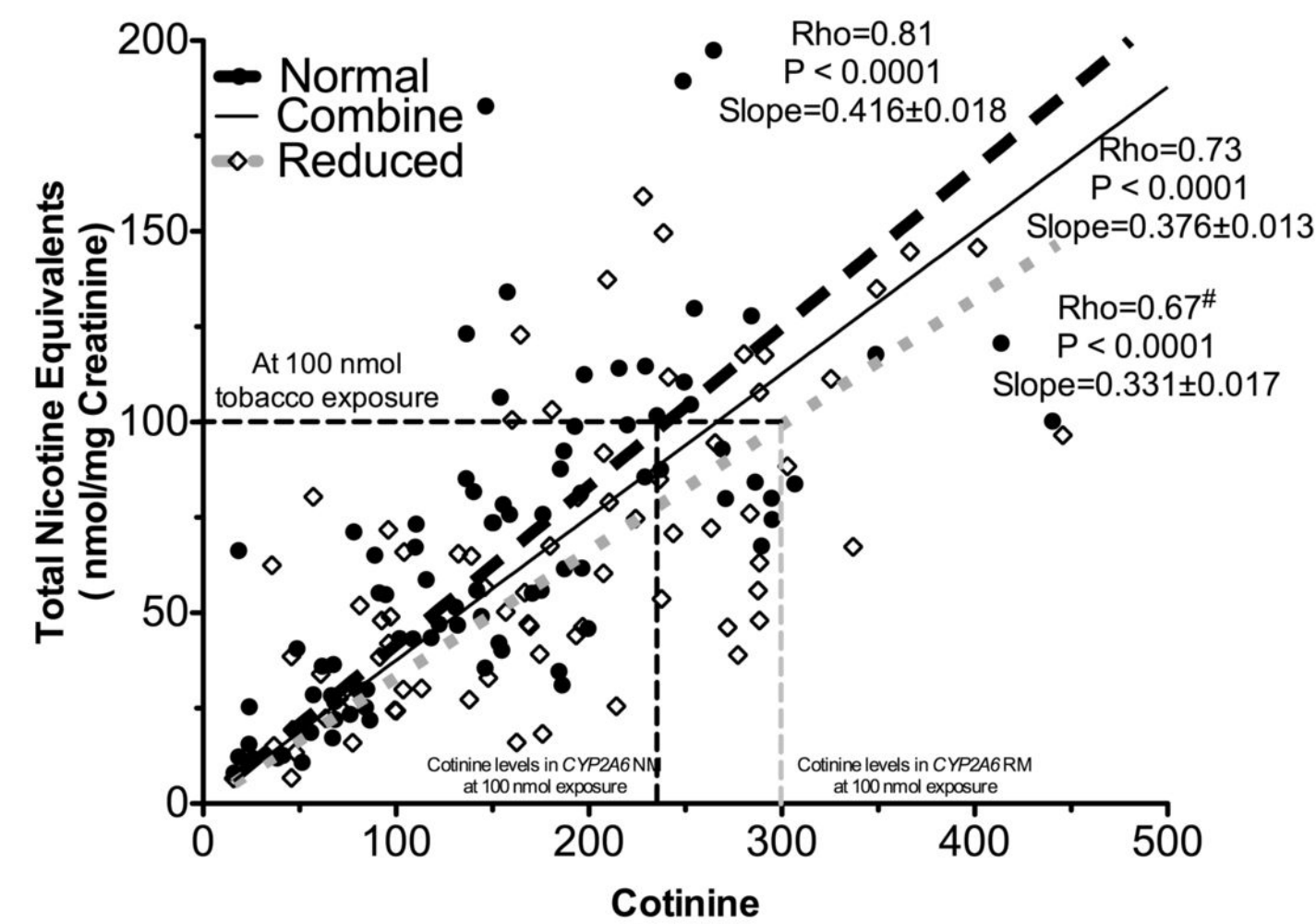
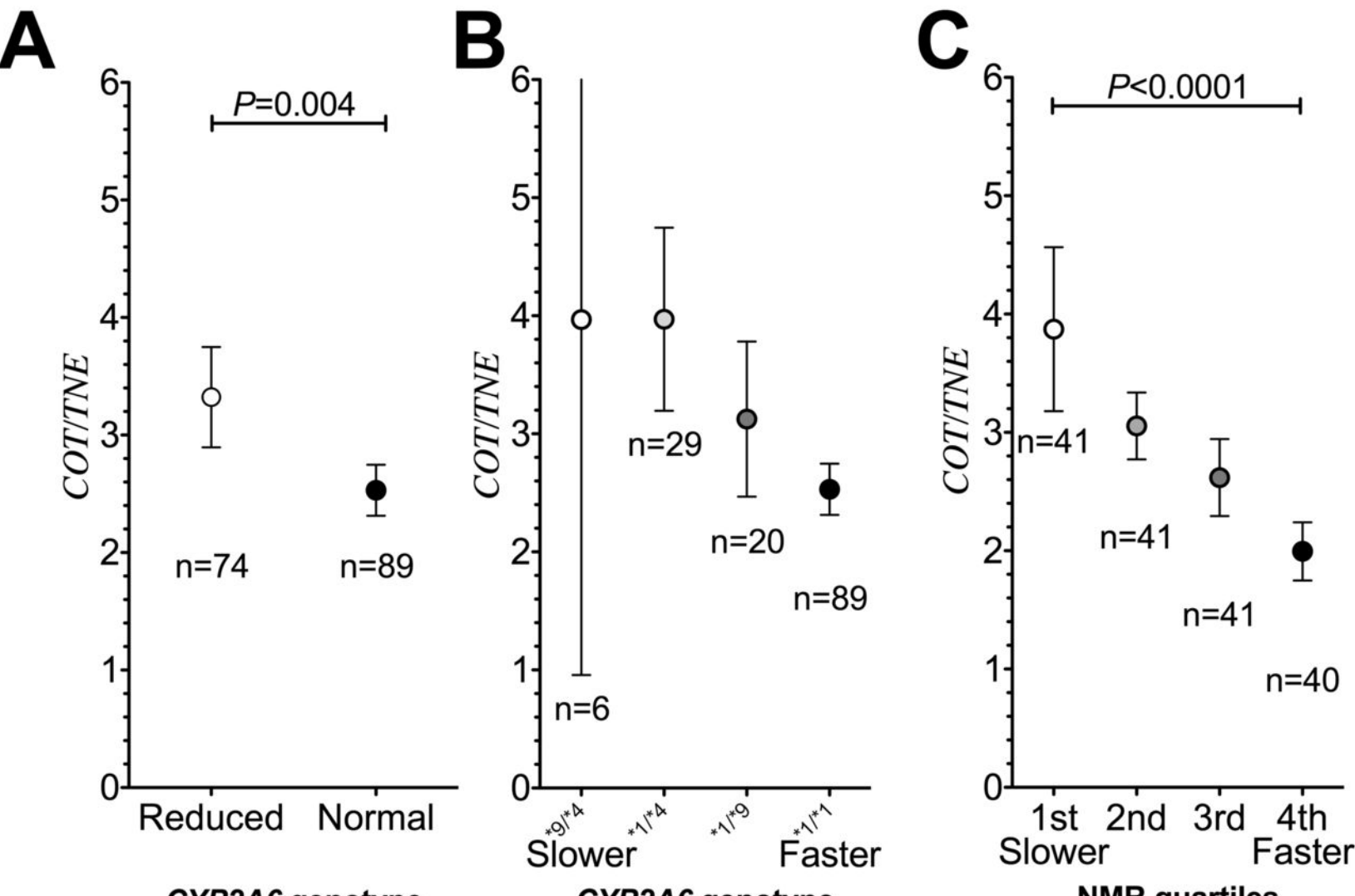
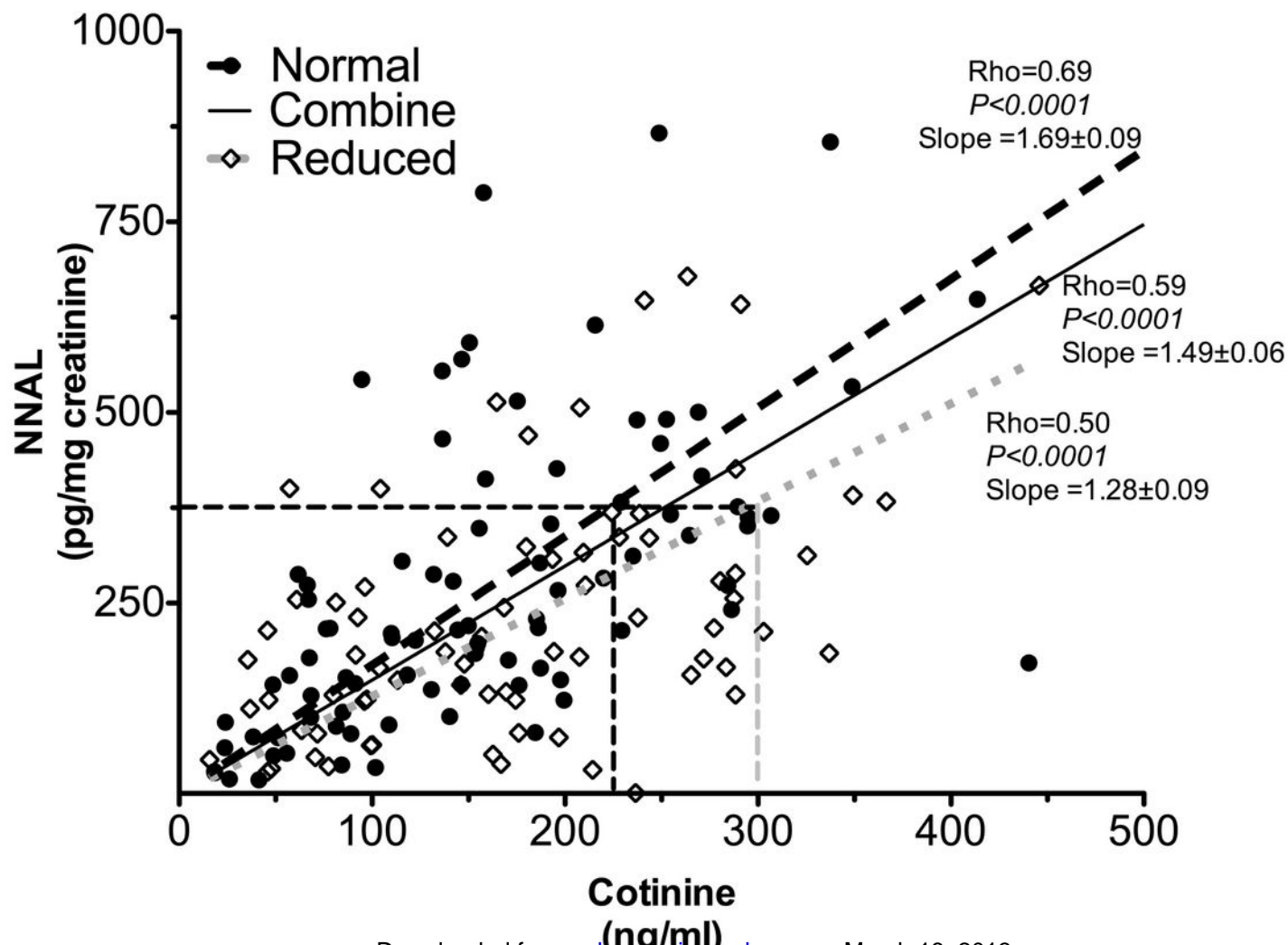


Figure 3



**Figure 4**



**Figure 5**

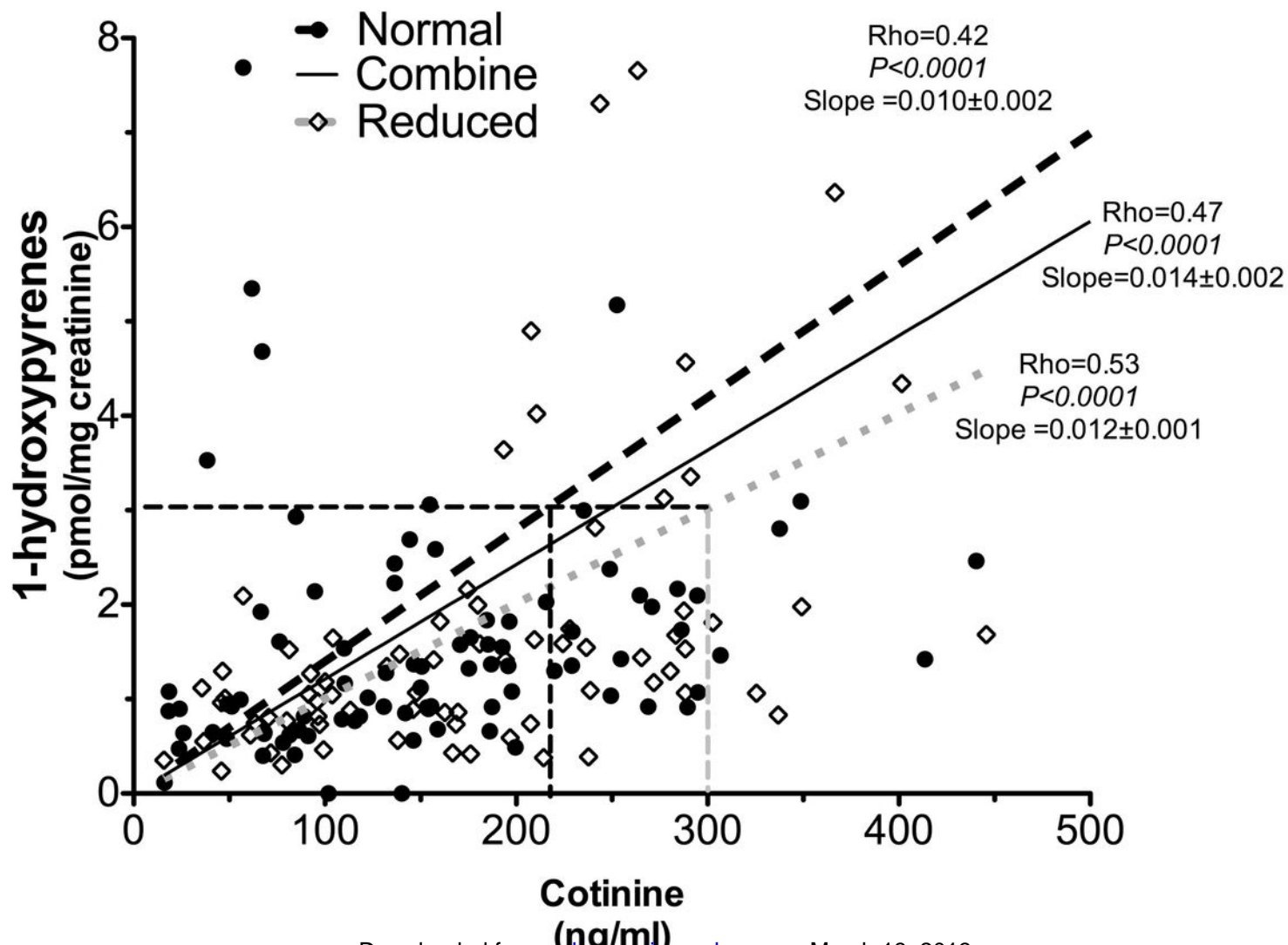
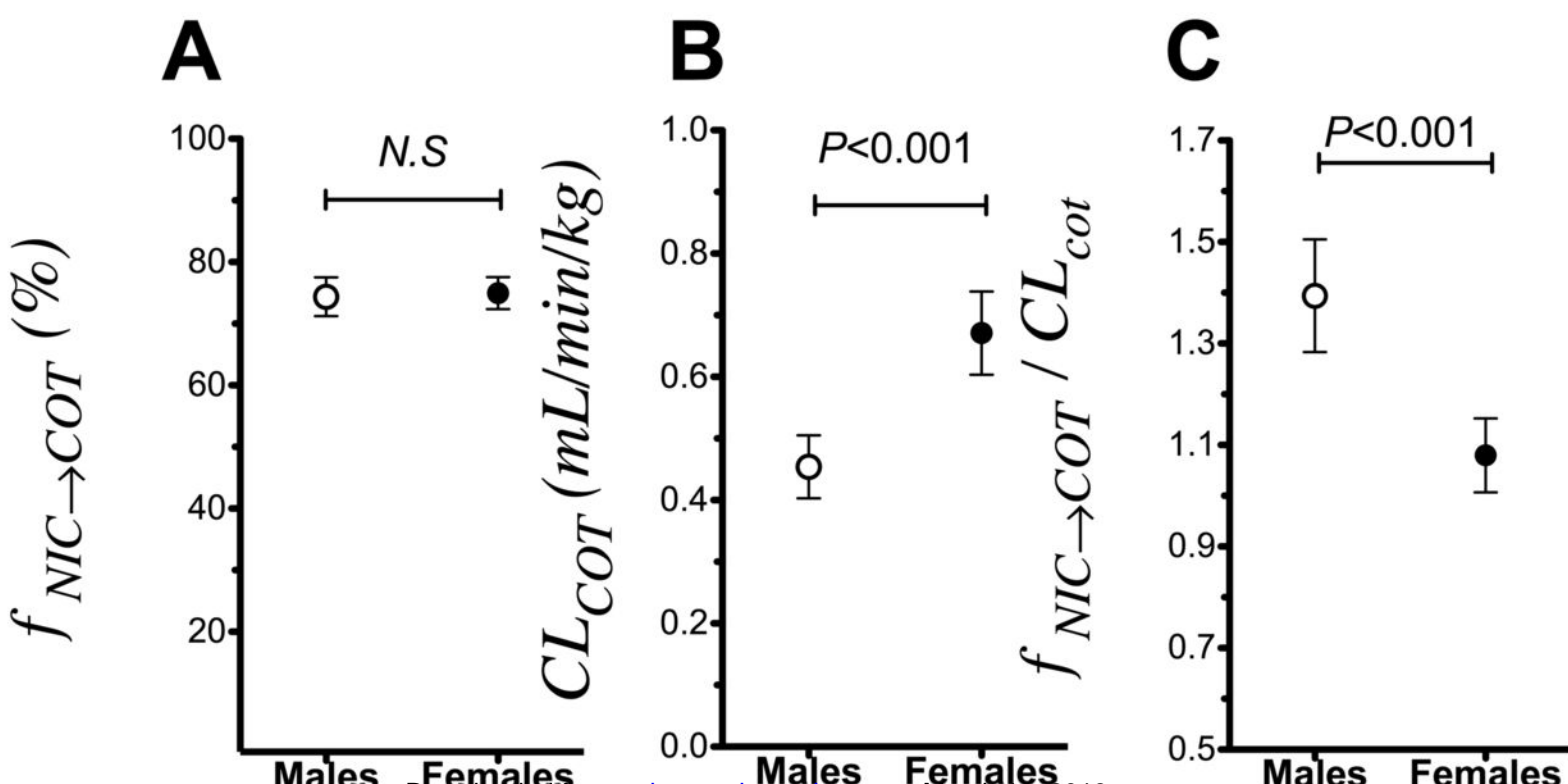


Figure 6



**Figure 7**

