

Application of a pharmacokinetic/pharmacogenetic approach to assess the nicotine metabolic profile of smokers in the real-life setting

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Running title: Nicotine metabolism and CYP2A6 genotype in smokers

Highlights

- Simultaneous analysis of nicotine metabolite ratio and CYP2A6 genotype in smokers
- Fast and sensitive method validated in subjects with different smoking status
- Identification of smokers who inherit metabolically deficient CYP2A6 alleles
- Optimization of smoking cessation pharmacotherapy
- To be used in the real life setting as part of smoking cessation programs

Abstract

The nicotine metabolite ratio, i.e., the ratio 3-hydroxycotinine/cotinine, is used to assess the nicotine metabolic status and has been proven to predict the response to smoking cessation treatments in randomized clinical trials. In the current study, a pharmacokinetic-pharmacogenetic integrated approach is described, based on the development of a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for nicotine metabolite ratio assay in plasma and a real-time PCR analysis for fast genotyping of CYP2A6. The pharmacokinetic-pharmacogenetic approach was validated in 66 subjects with different smoking status. The LC/MS/MS assay was rapid and sensitive enough to detect plasma cotinine levels also in second-hand exposed abstainers. In the cohort of patients of the present study the following results were obtained: (i) the frequencies of CYP2A6 genetic variants were comparable with those from clinical trials carried out in Caucasian populations; (ii) all the subjects carrying the CYP2A6 deficient allele also had a slow metabolizer phenotype; (iii) slow metabolizers had mean nicotine metabolite ratio approximately 50% of that of the normal/fast metabolizers; (iv) women had higher nicotine metabolite ratio than men; and (v) salivary nicotine metabolite ratio measures were comparable to plasma levels. Overall, the findings of the current study demonstrate that the simultaneous assessment of nicotine metabolite ratio and CYP2A6 genotype from human blood samples is feasible and accurate and could be used in a smoking cessation program to optimize treatments and identify those smokers who inherit metabolically deficient CYP2A6 alleles.

Key words: smoking cessation; nicotine metabolite ratio; CYP2A6; mass spectrometry; genotyping.

1. Introduction

Nicotine dependence is recognized as a medical condition and active and secondhand smoking have been associated to a myriad of human diseases [1]. For these reasons, smoking cessation is a prominent health issue; however, despite the efficacy of currently available pharmacotherapy, only 5-35% of treated subjects continue to abstain from smoking [2]. One possible explanation of the high percentage of treatment failures is the inter-individual variability of nicotine metabolism that may cause inadequate drug exposure after transdermal nicotine replacement therapy (tNRT) in combination with bupropion and varenicline [3].

Nicotine is metabolized to its major active metabolite cotinine (COT) by the hepatic enzyme CYP2A6; COT is subsequently converted into *trans*-3-hydroxycotinine (3HC), almost exclusively by CYP2A6 [4]. Several genetic variants of CYP2A6 have been identified and associated with inter-individual variability in nicotine metabolism with effects on smoking behaviour [5]. CYP2A6 genotypes display different frequencies among ethnic groups [6]. A prospective nicotine patch trial was aimed to stratify smokers on the basis of serum COT levels. It was demonstrated that smokers with low baseline COT plasma concentrations (<250 ng/ml) responded better to tNRT than those with high COT levels [7]. These results have been subsequently confirmed by others using the plasma 3HC/COT ratio (also termed as “nicotine metabolite ratio”), as a phenotypic marker for CYP2A6 activity that correlates with nicotine metabolic clearance [8-10]. Specifically, slow metabolizers (baseline mean 3HC/COT ratio < or = 0.26) were considered good candidates for standard tNRT, whereas fast metabolizers could instead benefit from bupropion [10], which is not a substrate of CYP2A6. More recently, a randomized, double blind, placebo-controlled clinical trial demonstrated the usefulness of a nicotine metabolite ratio cut-off of 0.31 to guide treatment decision between nicotine patch or varenicline [11].

It is worth mentioning that the results of this type of studies cannot always be translated into the real-world setting due to a number of factors, including strict patient inclusion criteria, standardized treatments and patient compliance. For these reasons, it is important to

validate robust biomarkers of smoke exposures. The current study describes a pharmacokinetic-pharmacogenetic integrated approach to accurately assess the nicotine metabolic profile of smokers in the real-life setting. These findings could be easily translated into clinical recommendations within smoking cessation programs and could help physicians to select the most appropriate drug for each smoker.

2. Materials and methods

2.1 Subjects and samples collection

The study was submitted and approved by the Ethics Committee of Pisa University Hospital and conducted in accordance to the principles of the Declaration of Helsinki. All subjects gave their signed informed consent before their blood samples were obtained.

Participants were 66 subjects attending the Smoking Cessation Centre of the University Hospital (Pisa, Italy), treated as per current clinical practice with nicotine replacement therapy (NRT – as transdermal patches, lozenges, inhalers or gums, varenicline or bupropion. The following clinical informations were collected by administering standard questionnaires: smoking status (including date and hour of the last cigarette/cigar smoked; number of cigarettes smoked from awakening in the day of the blood sampling; number of cigarettes smoked per day in the last 7 days); level of nicotine dependence (according to Fagerström Test for Nicotine Dependence score); number of pack-years; use of electronic cigarettes (E-cig); health status; current use of pharmacotherapy for smoking cessation (including NRT or other medicines).

Paired plasma and saliva samples were obtained from a subset of participants (n=9) to confirm whether saliva could be used to reliably assess the nicotine metabolite ratio. An aliquot of blood samples were centrifuged at 1,000×g for 10 min to obtain plasma; plasma and whole blood were stored at 4°C and used within 72 h to measure plasma levels of COT and 3HC and analyze CYP2A6 polymorphisms, respectively. For saliva collection, the Salivette® device (Sarsted AG & Co., Nümbrecht, Germany) was used. Saliva samples were centrifuged for 2 min at 1,000×g to yield clear supernatant in a conical tube and stored as for

blood samples. A heterogeneous sample of subjects was enrolled, regardless of the pharmacological treatment, health or smoking status (i.e., current smokers or abstainers). Cigarette or cigar tobacco smokers and/or E-cig users were enrolled. Carbon monoxide level in expired air (CO_{exp}) was measured after a deep inspiration and an apnoea of 10 sec by using the Mycro 4 Smokerlyzer (Bedfont Scientific Ltd, Rochester, England). “Abstainers” were defined as those subjects who reported not having smoked tobacco cigarettes or cigars in the last seven days and had a $\text{CO}_{\text{exp}} < 5$ ppm.

2.2 LC/MS/MS analyses and definition of nicotine metabolic ratio

Stock solutions of COT and 3HC, and their internal standards, COT-D₃ and 3HC-D₃, were prepared at a concentration of 1 mg/ml in methanol and stored at -20°C. Sample preparation was performed as follows: 100 µl of plasma or saliva and 300 µl acetonitrile plus 1% formic acid (1:1) were added into the Phree 96-Well Plate (Phenomenex, Torrance, CA, USA) and vortexed for 2 min at 500×g. Filtration was carried out by placing the Phree 96-Well Plate onto a sample manifold with a collection plate positioned inside, at a vacuum of 15 mmHg until filtrate was collected. Eluate was dried by a gentle stream of nitrogen, reconstituted with 1 ml of a methanol/water (40/60, v/v) mixture, and injected into the HPLC-MS/MS system. The HPLC separation was carried out using a 100 x 2.0 mm, 4µm Phenomenex Synergy Polar-RP column. A binary, linear gradient elution with 8 mM ammonium acetate in water and acetonitrile containing 0.1% formic acid was used at a flow rate of 0.4 ml/min. Analyses were carried out by an AB/Sciex (Concord, ON, Canada) API4000 triple quadrupole mass spectrometer equipped with an ESI source and coupled to an Agilent 1290 UPLC system. The mass spectrometer was operated in SRM mode, monitoring three transitions each compound, including the following quantifiers: 177→80 (COT), 193→80 (3HC), 180→80(COT-D₃), and 196→80 (3HC-D₃). Calibration curves were carried out by 4 calibration solutions, with concentration range from 4 to 1,000 ng/ml for both analytes.

The nicotine metabolite ratio cut-off used to define the metabolic phenotype was selected on the basis of the most recent clinical trial of nicotine replacement therapy [11]. In particular,

we stratified slow and normal/fast metabolizers as those with as nicotine metabolite ratio <0.31 and ≥ 0.31 , respectively.

2.3 Genotype analysis

Two common, low-activity variants of CYP2A6 were analysed: the CYP2A6*9A and the CYP2A6*2. DNA was extracted from 200 μ l of peripheral blood, using the EZ1 QiaRobot (Qiagen) according to manufacturer protocol. Single nucleotide polymorphisms analysis of CYP2A6*9A and CYP2A6*2 was performed by a Real Time PCR 7900 HT (Applied Biosystem) with a TaqMan Genotyping assay technology (Applied Biosystem), as per manufacturer protocol.

2.4 Statistical analysis

Data were expressed as mean \pm standard error of the mean. The Mann-Whitney and chi-square tests were used to compare differences between two independent groups. Correlation was established by linear regression or Spearman's rho. Statistical significance was determined as a p values less than 5%. Results were plotted by Prism software (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

In the current study an integrated approach was developed for the simultaneous assessment of CYP2A6 polymorphisms and nicotine metabolite ratio, to assess the nicotine metabolic phenotype and genotype of smokers in the real-life setting.

The clinical characteristics of the study subjects with measurable nicotine metabolites, by group of nicotine metabolizers, are shown in Table 1. Thirteen more males than females were studied and the proportion of fast/normal metabolizers were higher than that of slow metabolizer within both sexes. Fast/normal metabolizers reported a higher daily and lifetime (i.e. number of pack-years) cigarettes consumption and similar level of nicotine dependence (i.e. FTND score) and COexp, as compared to slow metabolizers. No statistically significant

differences were observed between slow and fast/normal metabolizers for all the examined clinical characteristics.

The LC/MS/MS assay allows the analysis of plasma COT and 3HC and calculation of the nicotine metabolite ratio on a large number of biological samples by a high-throughput separation system coupled to a fast liquid chromatography and mass specific detection. The analysis was rapid (5-min running time), sensitive enough to detect plasma COT levels also in second-hand exposed abstainers, and remained linear across the analyte levels in active smokers. In particular, the lower limit of detection of the solid-phase extraction analysis was found to be 0.2 ng/ml for both COT and 3HC on triplicate samples and retention times were 2.59 ± 0.01 and 2.26 ± 0.01 (n=3) for COT and 3HC, respectively. The between-day and within-day coefficients of variation were 8.4 and 6.8% and 4.2 and 6.3 % (n=3) for COT and 3HC, respectively. Linearity was in the range of 0.4-1,000 ng/ml with the following parameters: COT ($y = 0.005x + 0.013$, $r = 0.997$), 3HC ($y = 0.005x + 0.003$, $r = 0.996$). Recovery was 91.4 and 93.9 % (n=3) for COT for 3HC, respectively. Accuracy was 107.8 and 111.4% (n=3) for COT and 3HC, respectively. Representative chromatograms of an active smoker and an ex-smoker are shown in Fig. 1.

With regards to biomarkers of smoking exposure, CO_{exp} levels in the examined subjects ranged from 0 to 35 ppm with a mean value of 12.2 ppm. Abstainers, i.e. those with $CO_{exp} < 5$ ppm, were 36% (Fig. 2A). Plasma concentrations of COT and 3HC varied widely from 94.6 to 340 ng/ml and from 30.6 to 151 ng/ml, with mean values of 227 and 74.9 ng/ml, respectively (Fig. 2B). We assessed the relationship between CO_{exp} and plasma COT levels (Spearman $r = 0.82$, $P < 0.0001$; Fig. 3A), with findings similar to those previously reported in the Caucasian population [12]. Abstainers who reported to be (n=5) or not to be (n=5) exposed to second-hand smoke had very low CO_{exp} levels (i.e., 2.2 ± 0.37 and 1.6 ± 0.25 ppm, respectively), with no statistically significant difference between groups (Fig. 3B). Instead, abstainers who claimed to be exposed to second-hand smoke had a 20-fold higher plasma COT levels than those not exposed (i.e., 32.3 ± 24.5 and 1.5 ± 0.6 ng/ml, respectively; Fig. 3C). Although such an apparent discrepancy may be caused by the limited number of subjects analysed, caution

is required when using CO_{exp} as biomarker for both active and second-hand smoking exposure, due to the short half-life of CO and the confounding effect from environmental sources of CO [13].

The nicotine metabolite ratio (i.e. 3HC to COT ratio) is widely considered a better predictor of CYP2A6 activity than the metabolic ratio of COT to nicotine because of the long elimination half-life of metabolites, which makes nicotine metabolite ratio independent from the timing of the last exposure to nicotine [14,15]. In the current study slow metabolizers are defined as nicotine metabolite ratio <0.31 and normal metabolizers (including fast metabolizers) as those with nicotine metabolite ratio ≥ 0.31 , on the basis of the most recent clinical trial [11]. The nicotine metabolite ratio distribution is showed in Fig. 4A and the values ranged from 0.16 to 0.94. Calculation of nicotine metabolite ratio was possible for 90% of participants, while the remaining 10% were abstinent smokers with undetectable COT and/or 3HC plasma levels. Thirty eight percent of the examined subjects had a slow metabolizer phenotype and their mean nicotine metabolite ratio was approximately 50% of that of the normal/fast metabolizers (mean values of 0.23 ± 0.01 and 0.49 ± 0.03 , respectively; Fig. 4B). These values were almost superimposable to those obtained in 394 Caucasian smokers within an open-label nicotine replacement therapy clinical trial (0.23 ± 0.17 and 0.45 ± 0.22 , respectively) [16], confirming nicotine metabolite ratio as an accurate biomarker in the real-life setting.

Gender differences in nicotine metabolism have been reported in several studies [17,18], in particular, women metabolize nicotine more rapidly than men. In the present study, women had higher nicotine metabolite ratio than men (Fig. 4C), although such a difference was just over the limits of statistical significance (mean values of 0.43 ± 0.04 and 0.36 ± 0.02 , respectively; $p=0.08$). This could be due the limited number of subjects together with the presence of confounding factors not analysed in the current study and reported to influence nicotine and cotinine metabolism among women, such as the use of oral contraceptives [17].

In the current study, the pharmacogenetic analyses demonstrated the presence of the two specific variant alleles in the CYP2A6 locus associated with decreased nicotine metabolism.

In particular, 1.7% and 10.2 % of subjects were heterozygous for the CYP2A6*2 (c.479T>A) and CYP2A6*9 (c.-48T>G) alleles, respectively, and the frequencies of the two polymorphisms investigated were comparable to those previously reported in the Caucasian population [6,16]. No homozygous variant subjects were identified.

Previously published evidences suggest that the genetic contribute of CYP2A6 to the slow metabolizer phenotype among Caucasians having any known CYP2A6 variant is about 15% [12]. Deficient variants of *CYP2A6* are associated with adult [19] and adolescent [20] smoking cessation rates, and may play a role in determining response to smoking cessation pharmacotherapy [16]. Therefore, the development of a CYP2A6 pharmacogenetic test aimed at predicting drug response could be a promising tool to select the most effective cessation treatment for an individual smoker. Finally, CYP2A6 pharmacogenetics may also be useful in determining the individual susceptibility to develop important chronic diseases, since it has been demonstrated that CYP2A6 reduced activity variants confer reduction in lung cancer risk in Asian [21,22], African American [23], and European [24] smokers.

A further confirmation of the nicotine metabolite ratio as an accurate biomarker was supported by the evidence that all subjects who had the CYP2A6 deficient allele had also a slow metabolizer phenotype (mean value of 0.20 ± 0.03 ; $n=8$). The CYP2A6 genotype analysis was thus sensitive and applicable in the daily clinical routine with a turnaround time of less than 24 hours.

As an additional finding of this study, it was demonstrated that nicotine metabolite ratio salivary values were as reliable as plasma levels (Fig. 4D). In particular, nicotine metabolite ratio was calculated by using COT and 3HC levels measured in 9-paired plasma and saliva samples. Linear regression analysis provides a slope of 0.96 ± 0.18 with a Y-intercept of 0.04 ± 0.01 and a correlation coefficient of 0.89. These findings are in agreement with those previously obtained [25], suggesting the usefulness of saliva test for measuring the phenotype for nicotine metabolic status in the real life setting.

In conclusion, we developed an integrated pharmacokinetic-pharmacogenetic approach able to simultaneously assess CYP2A6 activity and polymorphisms in the real-life setting. This test

could be used in physician-assisted smoking cessation programs for tailoring pharmacotherapy and identifying those smokers who inherit metabolically deficient CYP2A6 alleles. The possible future availability of a test that uses saliva instead of plasma samples could facilitate its applicability in clinical practice.

Conflict of interest statement

The authors have no conflict of interest of any kind related to the work presented in this publication.

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Tables

Table 1. Clinical characteristics of the study subjects with measurable nicotine metabolites by group of nicotine metabolizers.

	Total	Slow metabolizers (NMR \leq 0.31)	Fast/Normal metabolizers (NMR $>$ 0.31)	p-value
Subjects, n	59	23	36	
Males, n (%)	33 (55.9)	13 (56.5)	20 (55.6)	0.94
Females, n (%)	26 (44.1)	10 (43.5)	16 (44.4)	
Abstainers, n (%)	10 (17.0)	3 (13.0)	7 (19.4)	0.72
Abstinence period:				
Days, median (IQR)	0.13 (0.07 – 0.81)	0.13 (0.06 – 0.8)	0.12 (0.07 – 0.78)	0.47
Hours/min, median (IQR)	3:00 (1:35 – 19:30)	3:00 (1:30 – 40:20)	3:00 (1:51 – 17:49)	0.47
Smokers/E-cig users:				
Only cigarettes, n (%)	43 (83.0)	19 (67.0)	24 (73.0)	0.18
Only cigars, n (%)	0 (0.0)	0	0	
Only E-cig, n (%)	2 (3.4)	1 (4.3)	1 (2.8)	
Cigarettes + cigars, n (%)	2 (3.4)	0	2 (5.6)	
Cigarettes + E-cig, n (%)	1 (1.7)	0	1 (2.8)	
Smokers in the last week, n (%)	48 (81.4)	20 (87.0)	28 (77.8)	
Cigarettes/day, median (IQR)	13.5 (6 – 20)	15 (6 – 18)	12.0 (5 – 20)	0.88
Smokers in the day of blood sample, n (%)	40 (67.8)	17 (73.9)	23 (63.9)	0.42
Cigarettes smoked from awakening in the day of blood sample, median (IQR)	4.0 (2 – 7)	4 (1 – 6)	4 (2 – 7)	0.58
Pack-years, median (IQR)*	32.5 (17 – 46)	23.0 (17 – 42)	34 (18 – 47)	0.37
FTND, median score (IQR)*	5 (4 – 7)	5.5 (4 – 7)	5 (4 – 7)	0.98
COexp, median ppm (IQR)	12 (4 – 19)	12 (4 – 16)	10.5 (3 – 19)	0.62
Use of NRT, n (%)	11 (18.6)	5 (21.7)	6 (16.7)	0.62

E-cig: electronic cigarette; NMR: nicotine metabolite ratio; FTND: Fagerström Test for Nicotine

Dependence; COexp: Carbon monoxide level in expired air; NRT: nicotine replacement therapy; IQR:

interquartile range. *n=3 missing values.

Figure legends

Fig. 1. Real sample chromatograms from (A) a smoker who smoked 18 cig/day, and (B) an ex-smoker, abstinent for 5 months. COT: cotinine; 3HC: 3-hydroxycotinine.

Fig. 2. (A) Distribution of carbon monoxide level in expired air (CO_{exp}) and (B) plasma levels of cotinine (COT) and *trans*-3-hydroxycotinine (3HC) in study population.

Fig. 3. (A) Correlation between carbon monoxide levels in expired air (CO_{exp}) and cotinine (COT) plasma concentrations. (B) CO_{exp} and COT plasma levels in abstainers who report to be (n=5) or not to be (n=5) exposed to second-hand smoke.

Fig. 4. (A) Nicotine metabolite ratio (NMR) distribution with the cut-off level for slow metabolizers. NMR means values (B) in slow and normal/fast metabolizers and (C) in males and females. (D) Correlation between plasma and saliva NMR values.

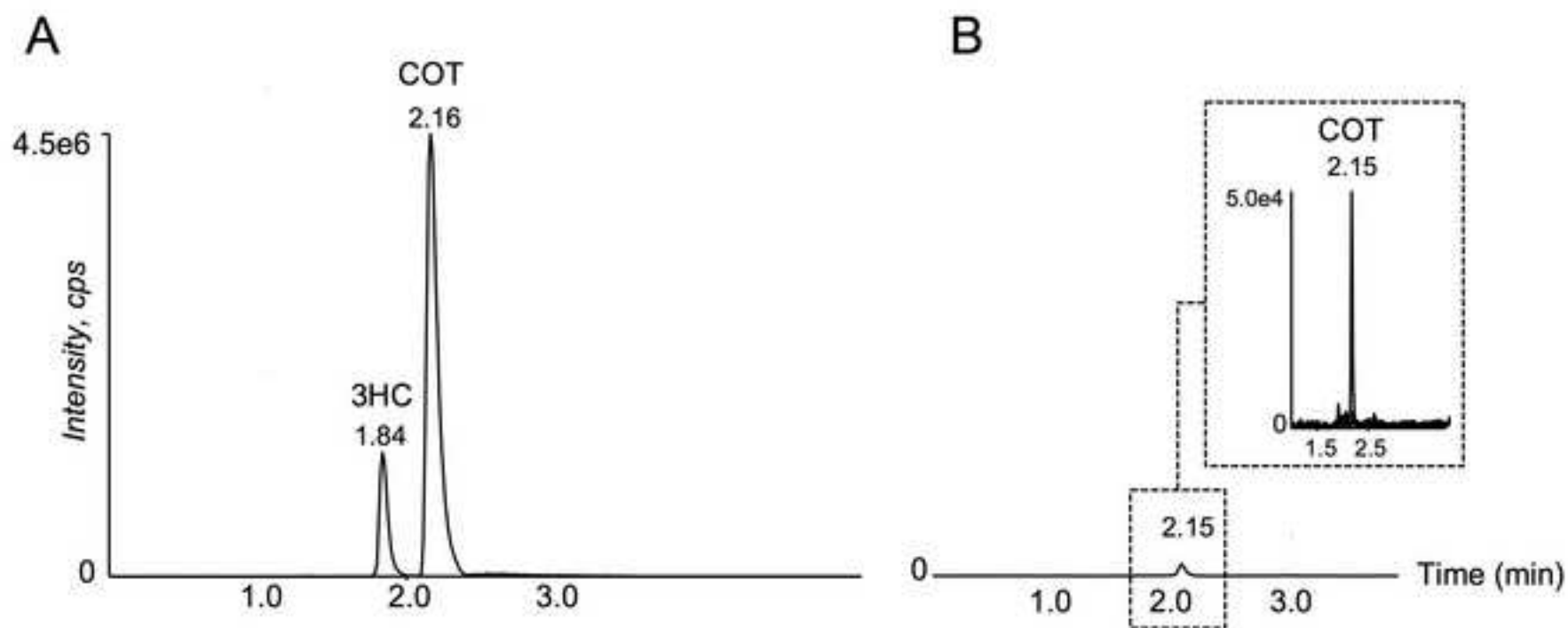


Figure 1

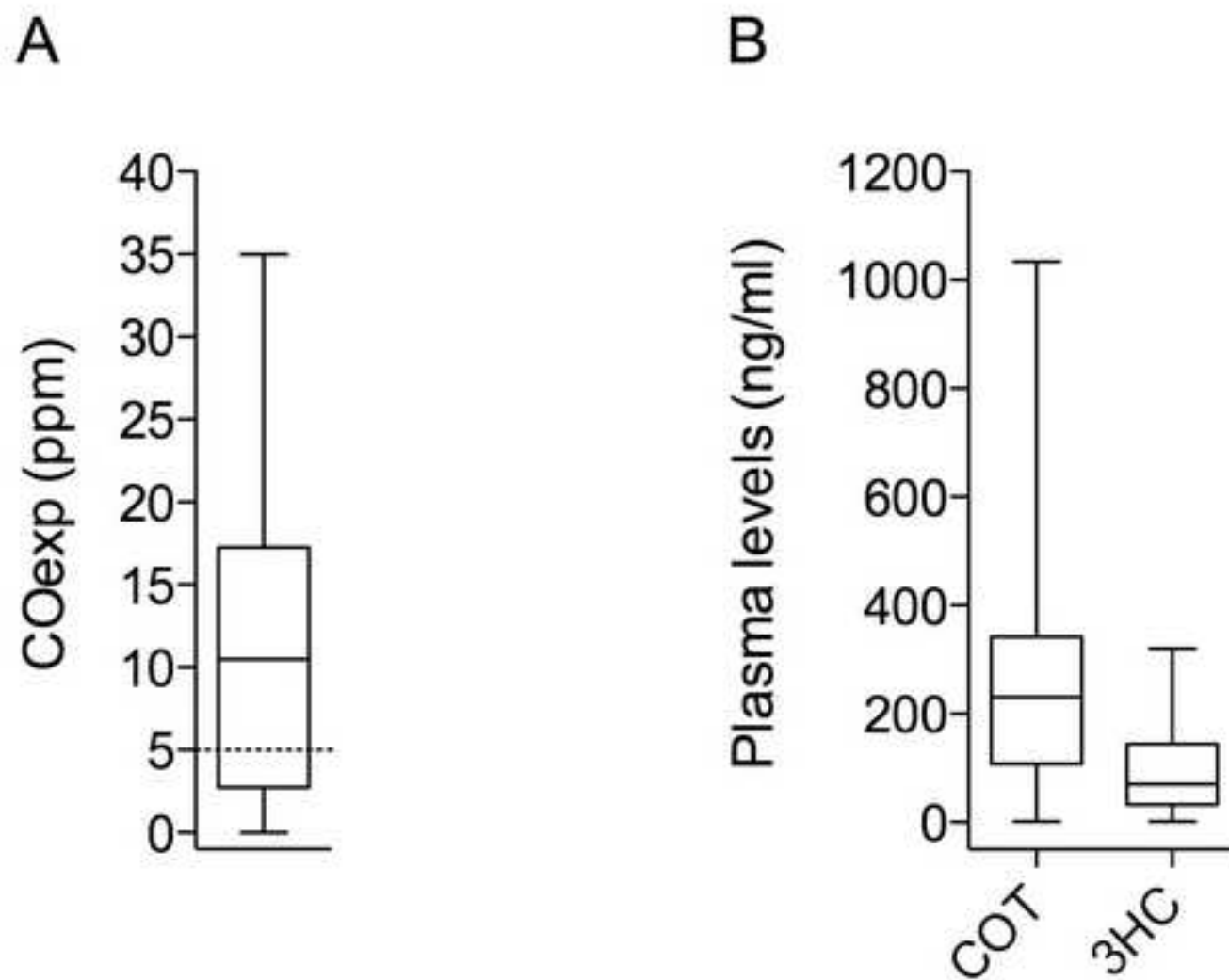


Figure 2

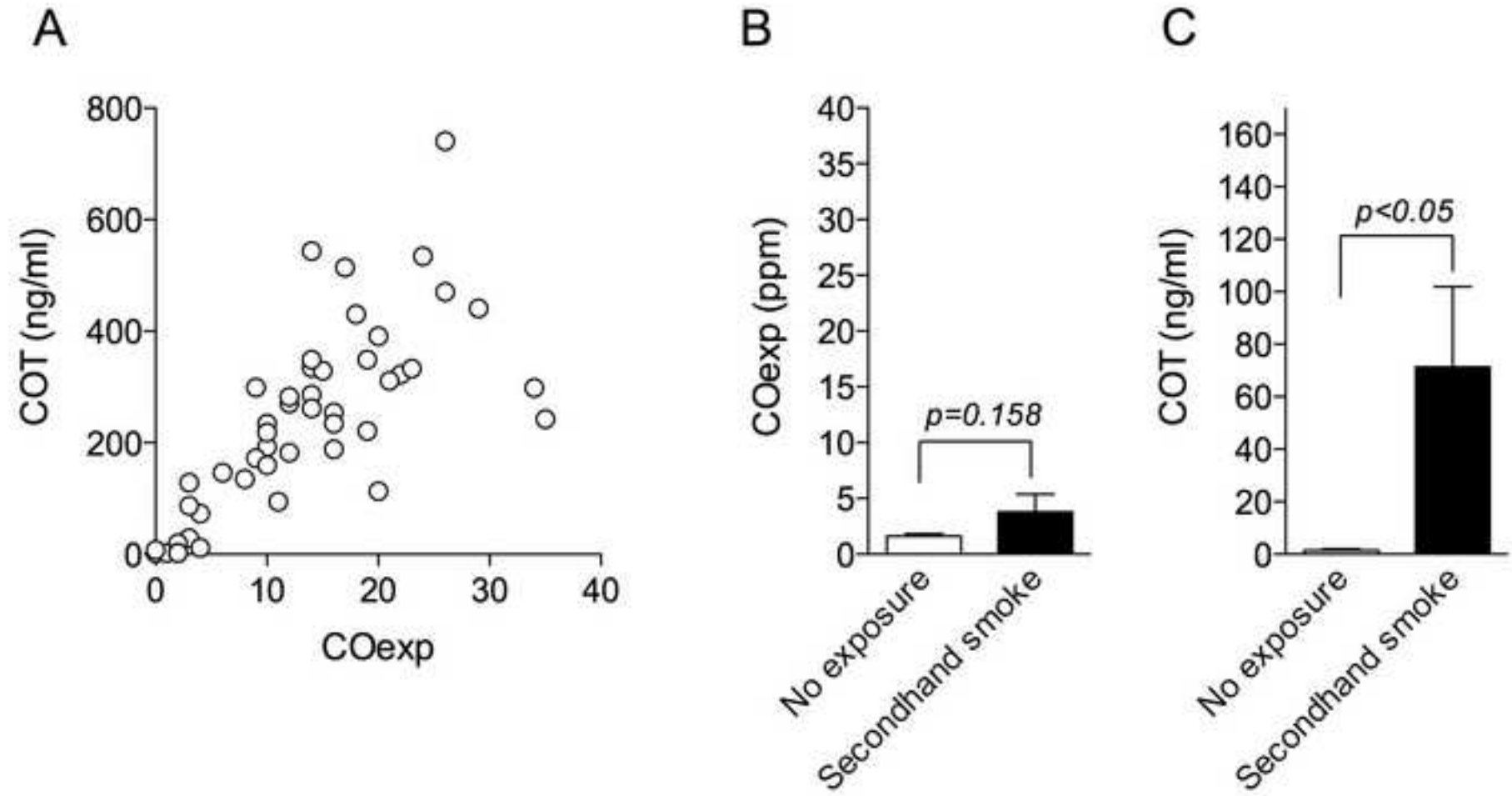


Figure 3

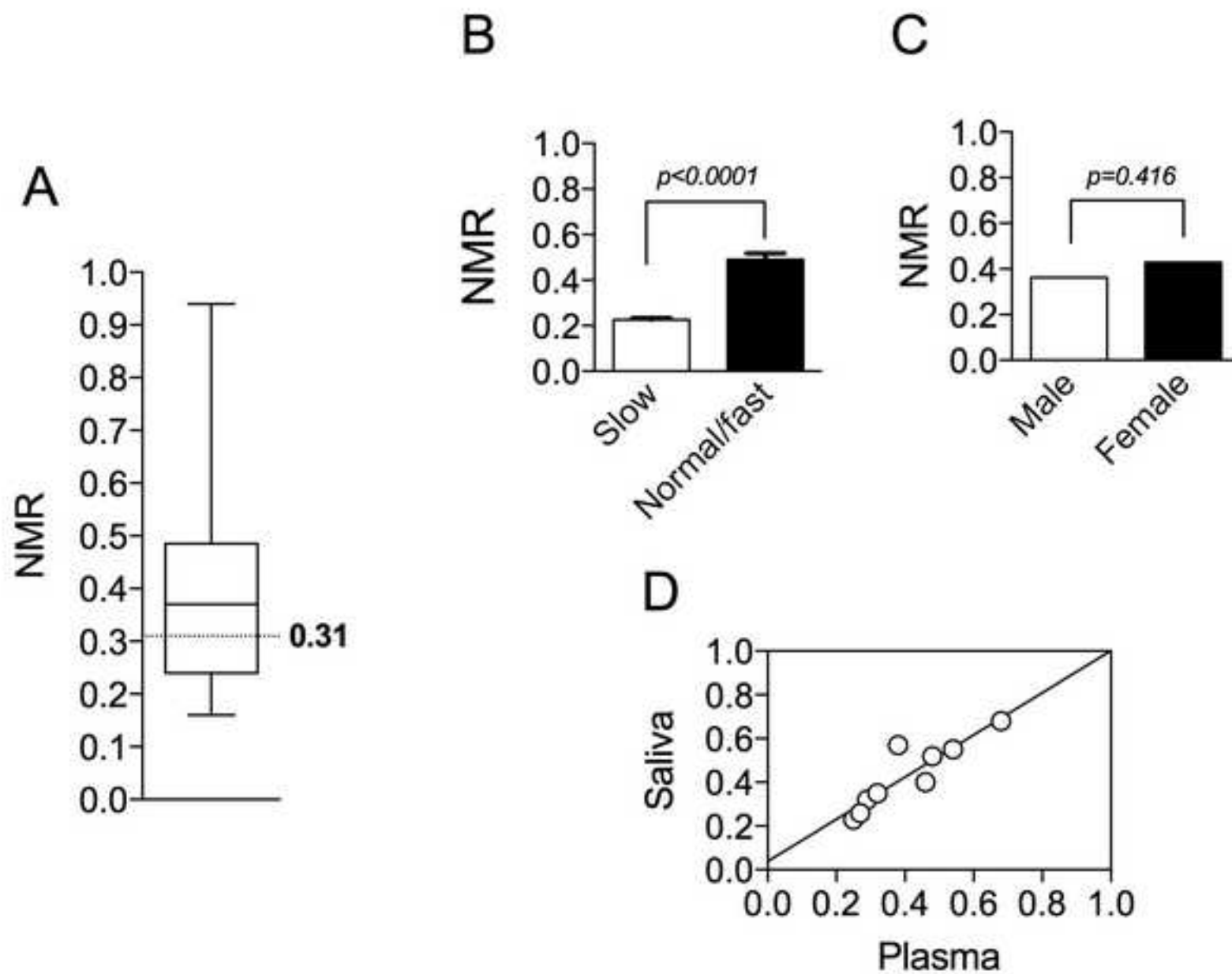


Figure 4