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Effects of Nicotine Metabolic Rate on withdrawal symptoms and response to cigarette smoking following abstinence

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Clinical Trials Registry: NCT01627392

Key words: Nicotine, nicotine metabolite ratio, NMR, withdrawal, craving

Abstract

This study investigated the influence of the rate of nicotine metabolism, as indicated by the nicotine metabolite ratio (NMR) on tobacco dependence. We stratified 136 smokers based on saliva NMR as fast (n=65) and slow (n=71) metabolizers. Two "loading cigarettes" were smoked after overnight, and a "reward cigarette" after six hours daytime abstinence. Blood nicotine concentrations, expired carbon monoxide, withdrawal/craving and reward questionnaires were collected before/after smoking and during daytime abstinence. Compared to slow, fast metabolizers had shorter nicotine elimination half-life ($p<0.001$), lower plasma nicotine concentrations ($p<0.001$) and higher withdrawal/craving scores ($p<0.05$) for most times during daytime abstinence, indicating that fast metabolizers are likely smoking more to relieve withdrawal symptoms (negative reinforcement). Reward/satisfaction scores were similar in fast and slow metabolizers, suggesting that faster nicotine metabolism assessed by NMR is not associated with greater positive reinforcement. *CYP2A6* normal (n=82) and reduced (n=42) genotype predicted plasma nicotine concentrations but not withdrawal symptoms.

Introduction

Nicotine dependence underlies tobacco dependence and sustains cigarette smoking, which remains a major cause of premature death [1]. Nicotine dependence is motivated by seeking rewarding effects (e.g. stimulation, pleasure), also termed positive reinforcement, and reversing aversive effects of nicotine withdrawal (irritability, anxiety, difficulty concentrating, etc), also called negative reinforcement.

Nicotine is metabolized primarily by the hepatic cytochrome P450 enzyme CYP2A6, with approximately 80% of nicotine converted to cotinine (COT), which is further metabolized by the same enzyme to 3'-hydroxycotinine (3HC) [1]. There is wide individual variability in the

clearance of nicotine, due both to genetic variation and environmental and hormonal factors. The ratio of 3HC/COT, also called the nicotine metabolite ratio (NMR), is a phenotypic biomarker that can be measured in plasma, urine and saliva and is correlated with the rate of nicotine clearance [2]. The NMR accounts for both genetic and non-genetic influences of CYP2A6 activity, is reproducible within subjects, and independent of the time since last cigarette smoked [3-5].

The rate of nicotine metabolism is an important determinant of tobacco and nicotine dependence. Faster nicotine metabolism is associated with greater dependence/higher tobacco consumption and lower rates of quitting without pharmacotherapy and with transdermal nicotine patch compared to slower metabolizers [6-11]. One potential mechanism for this association is that fast metabolizers experience more severe craving/withdrawal and thus are more likely to smoke to relieve such symptoms, i.e. for negative reinforcement [7,12]. This hypothesis is supported by findings showing that smokers with higher NMR experience more anxiety, insomnia, difficulty concentrating, anger and impatience during abstinence [6,13]. Another possible mechanism is that due to the faster elimination of nicotine, tolerance to psychoactive effects dissipates more rapidly, and therefore subsequent nicotine exposures (i.e. cigarette smoking) are more rewarding, that is, positive reinforcement may be greater among faster metabolizers. In support of this idea, brain imaging studies show that fast metabolizers exhibit greater reactivity in dopamine-dependent reward circuitry when given visual smoking cues than slow metabolizers (defined here as the highest and lowest NMR quartile, respectively) [14].

Smoking behavior, severity of dependence and the rate of nicotine metabolism vary by race [15,16]. On average Blacks metabolize nicotine more slowly, due to higher frequency of several slow metabolism variants of *CYP2A6* and *UGT2B10* genes, the latter of which codes the UDP-glucuronosyltransferase (UGT) isoform mainly responsible for nicotine glucuronidation [10,17-21] and smoke fewer cigarettes per day (CPD) [22]. Although one may expect that their slower nicotine metabolism and reduced CPD may be associated with less severe nicotine dependence, Blacks report greater difficulty quitting than Whites [15], suggesting higher nicotine dependence. The paradox between slower metabolism and higher dependence may be related to greater smoking intensity observed previously in Blacks compared to Whites [22], but also other factors unrelated to nicotine metabolism.

Sex differences also present some paradoxes; women metabolize nicotine faster than men due to estrogen-mediated induction of CYP2A6 [23,24] but smoke on average fewer CPD [22], while the nicotine intake per cigarette is on average the same in both sexes [25]. Most clinical trials studying NMR do not find a sex difference in quit rates, indicating that the nicotine metabolism rate predicts cessation success in women as well as in men [7-10].

Aim of the present study was to investigate the association of NMR with withdrawal/craving symptoms after abstinence from smoking and the response to smoking a cigarette as assessed by questionnaire scores, nicotine plasma concentration, expired carbon monoxide (CO) and heart rate (HR) changes after two different abstinence periods, i.e. overnight and six hours during the day. We hypothesized that fast, relative to slow, metabolizers will demonstrate

more severe withdrawal symptoms and greater craving to smoke during abstinence and more reward after subsequent smoking. Furthermore, we aimed to investigate possible racial differences of these mechanistic relationships, and also to compare NMR to *CYP2A6* genotype as a biomarker of withdrawal effects.

Results

A total of 552 potential participants were scheduled for an in-person screening visit. Among them, 275 did not meet eligibility (e.g. COT< 50 ng/mL, vital signs not within normal range) and 106 did not meet the study's NMR cut points. From the remaining 171, 34 declined/didn't complete the study. A total of 137 participants completed the study, but one participant with nicotine concentrations below limit of quantification (LOQ) and very low CO was excluded from analysis as it is assumed that this participant did not inhale smoke from the cigarettes during the study. Finally, 136 participants were included in the final analysis, 71 slow metabolizers and 65 fast metabolizers by NMR.

Baseline characteristics

The NMR frequency histogram can be found as supplementary information (Figure S1). Table 1 shows the baseline characteristics, as well as comparisons based on NMR, race and sex. Significant correlations were found between saliva and plasma NMR ($r=0.708$, $p<0.001$), saliva NMR and nicotine half-life ($r= -0.432$, $p<0.001$), and saliva NMR and saliva COT ($r=-0.349$, $p<0.001$). Comparisons within NMR groups by race and by sex can be found as supplementary information (Table S1 and Table S2). Complete *CYP2A6* genotyping was available for 124 participants (91.2%; not available in 12 cases due to incomplete genotype results (n=11) or DNA not available (n=1)). Among them, 82 (66.1%) were normal metabolizers (NM) (median NMR 0.47, range 0.08-1.1) and 42 (33.9%) reduced metabolizers (RM) (median NMR 0.18, range 0.06-0.72). Thirty-three of 42 RM (78.6%) were NMR slow metabolizers, while for 50 of the 82 NM (61%) the NMR was indicative for fast metabolism (Fig. 1). Analysis of the baseline characteristics using the genotype (i.e. NM vs RM) showed significant higher NMR in the NM group ($p<0.001$), but no significant differences regarding other parameters.

Association of nicotine metabolism as assessed by NMR with withdrawal/craving

Table 2 shows the association of NMR with withdrawal/craving outcomes after the two abstinence periods. In a within-subject comparison, the PANAS (Positive and Negative Affect Schedule) negative score was significantly higher after overnight compared to daytime abstinence ($p<0.001$), while the Tiffany Questionnaire on Smoking Urges-Brief (QSU) Global Craving Score neared significance ($p=0.05$). Compared to slow, fast metabolizers did not demonstrate more severe craving/withdrawal symptoms after overnight abstinence;

however, significantly higher craving and withdrawal and PANAS negative scores nearing significance were seen after six hours daytime abstinence. When comparing only Whites (n=98), significant differences were seen regarding nicotine concentrations (lower in fast compared to slow metabolizers both after overnight and daytime abstinence ($p<0.001$)), but not for HR, CO or craving scores. When analyzing only Blacks (n=38), fast metabolizers had significantly lower nicotine concentrations ($p=0.008$) and expired CO ($p=0.022$) after overnight abstinence compared to slow metabolizers, as well as after daytime abstinence ($p=0.01$ and 0.003, respectively). Using the genotypes, significant differences were seen only regarding nicotine concentrations (lower in NM compared to RM both after overnight ($p=0.011$) and after six hours daytime abstinence ($p=0.003$)).

Association of nicotine metabolism as assessed by NMR with rewarding effects

Table 3 shows the association of NMR with the response to smoking after abstinence. Significant score differences (higher craving and withdrawal, and lower satisfaction in fast metabolizers compared to slow metabolizers) were seen after the first cigarette of the day (Cig 1) but not after Cig 3.When analyzing only Whites or using the genotypes, no significant differences were found. Analysis of Blacks only revealed significantly lower nicotine concentrations ($p=0.04$), expired CO ($p=0.003$) and higher craving ($p=0.019$) after Cig 1, as well as lower expired CO ($p=0.024$) after Cig 3 in fast compared to slow metabolizers.

Nicotine concentrations and scores over time

Figure 2 shows the nicotine plasma concentrations over time, by NMR and genotype. Fast metabolizers had significantly lower area under the nicotine concentration-time curve (AUC) compared to slow metabolizers by NMR (34.1 (8.4-68.6) vs 48.8 (15.0-125.4), $p<0.001$) and by genotype (37.0 (8.4-79.4) vs 46.2 (18.0-125.4), $p=0.001$). When analyzing the two races separately, these differences remained among Whites by NMR (34.9 (10.9-68.6) vs 49.0 (15.0-91.2), $p<0.001$) and by genotype (36.6 (10.9-79.4) vs 45.4 (18.2-91.2), $p=0.012$), and among Blacks by NMR (30.2 (8.4-50.5) vs 46.6 (20.2-125.4), $p=0.003$) but not by genotype ($p=0.058$). Whites had significant differences (fast lower concentrations compared to slow NMR) for every time point except post-Cig 1, post-Cig 2 and post-Cig 3, while Blacks for every time point except post-Cig 3.

Figures 3 and 4 show the Minnesota Nicotine Withdrawal Scale (MNWS) and QSU, respectively, over time, by NMR and genotype. Significant differences between fast and slow metabolizers were seen for the MNWS and QSU craving scores at various time points using the NMR but not with the genotype. Using the area under the effect-time curve (AUEC), fast metabolizers had significantly higher MNWS-AUEC (39.3 (10.9-185.3) vs 35.9 (0.32-112.3), $p=0.02$) and QSU-AUEC (20.8 (8.4-42.0) vs 16.6 (6.7-36.5), $p=0.005$) compared to slow metabolizers by NMR, but not by genotype. No significant MNWS differences were seen between fast and slow metabolizers when analyzing only Whites or Blacks. Analysis of the

QSU craving over time in Whites showed significantly higher scores among fast compared to slow metabolizers at 1, 2 and 3 hours, and in Blacks post-Cig 1. In Whites, the QSU-AUEC was higher among fast compared to slow metabolizers by NMR (20.8 (8.4-42.0) vs 17.6 (8.4-36.5), p=0.029) but not by genotype, while no significant differences were found in Blacks. Regarding other scores (i.e. PANAS, modified Cigarette Evaluation Scale (mCES)), no significant differences were seen by NMR except higher mCES satisfaction in slow compared to fast metabolizers post-Cig 1 (Table 3). Analysis of only Blacks or Whites showed no significant differences by NMR regarding those scores. Using the genotypes, a significant difference was seen for the 4h PANAS negative score (higher among NM compared to RM, p=0.036).

Boosts and changes

Investigation of pre- to post-smoking boosts and changes of nicotine, CO, HR and MNWS, QSU craving and PANAS showed no significant differences between NMR groups. Similar results were found when analyzing only Whites or Blacks, while analysis based on genotypes showed greater CO boost after Cig 3 in RM compared to NM (p=0.009). Cig 3 CO and nicotine boosts were significantly higher than Cig 1 (p<0.001), while Cig 3 HR changes were significantly lower than Cig 1 (p=0.003).

Explorative general linear model (GLM) analysis

After individual exploration of age, body mass index (BMI), and urine total nicotine equivalents (TNE), age emerged as potentially significant covariate and was included in the GLM analysis together with sex and race. NMR emerged as a significant covariate for mCES respiratory tract sensations (p=0.047) but not for other responses. Sex emerged as significant covariate for MNWS pre-Cig 1 (p=0.022), MNWS and craving during daytime abstinence (p=0.011 and p=0.007, respectively, higher scores in females), and PANAS positive post-Cig 3 (p=0.027, higher scores in males). Age was significant for PANAS negative during daytime abstinence (p=0.047, higher scores among older participants), PANAS positive post-Cig 3 (p=0.026 and p=0.022, respectively), mCES reward (Cig 1 p=0.007, Cig 2 p=0.045, Cig 3 p=0.045) and respiratory tract sensations (Cig 1 p=0.001, Cig 2 p=0.002, Cig 3 p=0.004) (higher scores among younger participants). Race emerged as statistically significant covariate for mCES satisfaction (post-Cig1 p=0.017, post-Cig 2 p=0.021) (higher scores in Whites).

Discussion

We found that fast metabolizers by the phenotypic biomarker NMR had shorter nicotine elimination half-lives, lower plasma nicotine concentrations and greater craving/withdrawal (as assessed by significant higher AUEC and withdrawal/craving scores for most times during abstinence and PANAS negative scores nearing significance at six hours) compared to slow metabolizers over six hours daytime cigarette abstinence. This supports the hypothesis that the NMR is associated with physical dependence and the idea that fast metabolizers are likely smoking more for negative reinforcement. The differences in withdrawal symptoms appear relative quickly after smoking the last cigarette. Many of the effects persisted when analyzing Blacks and Whites separately, thus indicating that associations with NMR were not due to confounding by race.

We did not confirm the hypothesis of positive reinforcement, as indicated by the absence of significant NMR group differences in satisfaction/reward and PANAS positive questionnaires and physiological measurements (e.g. HR changes). Furthermore, despite significant differences in nicotine concentration and the longer abstinence duration, withdrawal/craving scores were higher but not significantly different in fast compared to slow metabolizers after the overnight abstinence, possibly because the latter usually represents a normality in smokers' daily routine, thus triggering less withdrawal/craving effects than during six hours daytime abstinence. Based on our study, the non-invasive NMR appears to be a better biomarker of withdrawal effects than the *CYP2A6* genotype. This is consistent with the idea that nicotine metabolism is also mediated by other genes and environmental factors.

Previous studies have shown an association of fast NMR with lower nicotine levels and stronger craving one week into a quit attempt with transdermal nicotine [7] and higher withdrawal after 24 hour abstinence in adolescent smokers [6]. Smokers in the top NMR

quartile had greater craving compared to those in the lowest quartile following overnight abstinence [12], while in a study with community-based samples fast metabolizers experienced significantly higher anxiety compared to slow metabolizers with transdermal nicotine treatment [13]. Despite differences and limitations of those studies (e.g. small sample size in the adolescent study), these findings all support that fast metabolizers experience more severe withdrawal/craving during abstinence associated with differences in the rate of nicotine metabolism.

The observation that faster nicotine metabolism is associated with more severe withdrawal symptoms has important clinical implications. Withdrawal symptoms can present a major obstacle to smoking cessation and increase relapse risk [26,27]. Therefore, specific supporting measures (e.g. higher than standard nicotine replacement doses [7], behavioral intervention [13], use of varenicline to relieve craving/withdrawal symptoms [11,28]), should be considered for smokers with high NMR attempting to quit. However, other studies [8,9] failed to find an association between high NMR and craving/withdrawal, possibly due to methodological and sample differences (e.g. use of bupropion and not nicotine replacement therapy [8]).

Positive reinforcement is another suggested mechanism for the greater dependence and lower quitting rates in fast metabolizers. This is supported by a brain imaging study in which fast metabolizers exhibited greater reactivity in dopamine-dependent reward circuitry after visual smoking cues [14] and a study with administration of nicotine intravenously following overnight abstinence [12]. Possible reasons for the different findings in our study might be stronger reward effects after intravenous administration of nicotine compared to smoking, and differences between neural responses in functional magnetic resonance imaging (fMRI) and subjective feelings of reward.

Our data are consistent with previous findings that Blacks have on average slower nicotine metabolism compared to Whites [10,17-21]. Blacks also smoke fewer CPD [29], which could be related to slower metabolism [30], but also more intensive smoking [31] and higher nicotine intake per cigarette [22]. Blacks and slow NMR participants had higher COT levels despite lower TNE, consistent with other pharmacokinetic observations on effects of reduced CYP2A6 activity on COT levels [32,33]. Separate analysis of Blacks showed significant

differences in nicotine concentration and expired CO between fast and slow NMR at more time points compared to Whites, but less significant differences in craving scores, possibly due to the smaller sample size. Our findings further indicate possible higher reward effects in Whites, which might be associated with racial or NMR differences.

Regarding sex differences, similar to previous studies [23], female participants were significantly faster metabolizers than men. Our findings suggest that women might experience more negative effects during abstinence. The higher NMR and withdrawal do not offer an explanation regarding the fewer CPD reported in women, which may be associated with non-nicotine related behavior factors.

Concordance between genotype and NMR was not complete. Although several *CYP2A6* gene variants have been shown to have an effect on smoking behavior [21,34], the currently identified variants explain only a small percentage of the variation in nicotine metabolism [7,35]. In a recent genome wide association study of NMR, over 700 significantly associated variants were identified on chromosome 19q13 (the loci of *CYP2A6)* [36]; further characterization will improve utility of the *CYP2A6* genotype. In addition, there are likely unaccounted for effects of UGT and other genetic variations and hormonal and environmental effects. Next to estrogen, other substances (e.g. phenobarbital, rifampin, broccoli) can induce CYP2A6, while others (e.g. grapefruit, menthol) can inhibit its activity [21]. It is therefore not surprising that the *CYP2A6* genotype did not predict outcomes as well as the NMR.

Limitations of our study include that, despite our intention, achieved plasma nicotine baseline after the loading cigarettes was not equal in the two NMR groups. The number of Blacks and women were relatively small. Furthermore, we studied only six hours abstinence, while longer abstinence periods would be associated with more intense withdrawal/craving, and factors other than nicotine dependence might also affect the desire to smoke/craving for tobacco. Despite these limitations, the present study clearly demonstrates in a prospectively stratified design that high NMR is associated with lower blood nicotine levels and higher craving/withdrawal during brief smoking abstinence.

In conclusion, our study provides evidence that fast metabolizers by NMR are likely smoking more for negative reinforcement, i.e. to relieve craving/withdrawal symptoms and to a lesser extent if at all due to positive reinforcement, i.e. greater reward effects after smoking. The non-invasive saliva NMR appears to be a better biomarker of withdrawal/craving effects than the *CYP2A6* genotype.

Methods

Participants and recruitment: Participants were healthy or with stable medical or psychiatric conditions volunteers, between the ages of 18 and 70, of self-reported African American or Caucasian descent (both parents and grandparents of same race), who smoked at least five CPD regularly for the last year. Participants were recruited through Craigslist, flyers, and newspaper ads. At an initial screening visit, medical history was provided by a questionnaire. A physical exam was performed upon entry on the research ward. Participants provided a saliva sample for COT to verify smoking status (i.e. COT ≥ 50 ng/mL) and to assess NMR, and they completed a demographics, the Fagerstrom Test for Cigarette Dependence (FTCD) (including TFC and CPD) [37], and questions regarding amount and frequency of alcohol use. Based on the saliva NMR, participants were stratified as fast or slow metabolizers. Based on prior studies [38], cut points were ≤ 0.20 and ≥ 0.37 in Blacks, and ≤ 0.26 and ≥ 0.45 in Whites (all within 0.01). The study was approved by the University of California San Francisco Institutional Review Board.

Study procedures: Eligible participants were admitted to the research ward at Zuckerberg San Francisco General Hospital the evening before the study to enforce 12 hours overnight abstinence (last cigarette at 9 PM). In the evening, a urine sample was collected for TNE. In the morning of the study day, participants had an intravenous catheter inserted for blood drawing and a light breakfast was served. One hour later (~9 AM) participants smoked two cigarettes (Marlboro Regular for non-menthol smokers and Marlboro Menthol for menthol smokers) with a standardized puffing protocol (i.e. one puff of two seconds duration every 38 seconds for a total of ten puffs from each cigarette). The second cigarette (Cig 2) was smoked approximately 26 minutes after Cig 1. These “loading cigarettes” were intended to relieve overnight withdrawal symptoms and place participants in a similar nicotine-satiated baseline state across the study groups. Participants then abstained from smoking for six hours, which

represents on average three half-lives of nicotine, allowing an estimate of elimination half-life, and also adequate time for the development of significant withdrawal/craving symptoms [39-41]. After six hours daytime abstinence, a third cigarette (Cig 3) of the smoker's own brand was smoked in their usual way. This "reward cigarette" was followed by a 90-minute period of monitored "free" ad libitum smoking (these results will be described in another publication). A modified version of the MNWS [42] was administered before Cig 1 and Cig 3, immediately after Cig 1, Cig 2, and Cig 3 and 2 and 4 hours after Cig 2. The QSU [43] was administered at the same time points with the MNWS, as well as 1, 3 and 5 hours after Cig 2. The mCES [44] was administered after each cigarette. The PANAS [45] was assessed before Cig 1 and Cig 3, as well as immediately, 2 and 4 hours after Cig 2 and immediately after Cig 3. Nicotine blood concentrations were measured before and 2 minutes after each loading cigarette, then 30 minutes, 1, 2, 3, 4 and 5 hours after Cig 2, and before and 2 minutes after Cig 3. One sample (baseline) was also used to calculate plasma NMR in order to validate saliva NMR. CO and HR were measured before each blood sample.

Study measures: The modified MNWS (excluding items relating to sleep disturbance and constipation) included eight items (angry/irritable/frustrated, anxious/nervous, depressed mood/sad, desire or craving to smoke, difficulty concentrating, increased appetite/hungry, restless and impatient), rated on a 0=none to 4=severe scale. The QSU total (global) craving score is the average of all responses (mean of ten items, rated on a 1=strongly disagree to 7=strongly agree scale). The mCES (total of 12 items) was used to assess satisfaction (three items), psychological reward (five items), aversion (two items), craving reduction (one item) and respiratory tract sensations as responses to smoking (one item). The mCES is rated on a 1=not at all to 7=extremely scale, and scoring is done by adding the item scores for each scale. The PANAS included items assigned as Positive or Negative Affect (each score is the

sum of ten items, rated on a 1=very slightly/not at all to 5=extremely scale). Plasma nicotine and CO boosts represent changes in levels between pre- and post-smoking.

Laboratory methods: Saliva 3HC and COT were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [46], plasma nicotine by gas chromatography-tandem mass spectrometry [47] (LOQ 1 ng/mL). TNE in urine was calculated by taking total (molar sum of free and glucuronide conjugate) of six metabolites of nicotine (nicotine, COT, 3-hydroxycotinine, nicotine-n-oxide, cotinine-n-oxide, nornicotine and norcotinine) assayed by LC-MS/MS and normalized by urine creatinine [2].

Genotyping of *CYP2A6*1X2, CYP2A6*2, CYP2A6*4, CYP2A6*9, CYP2A6*12, CYP2A6*20, CYP2A6*23, CYP2A6*24, CYP2A6*25, CYP2A6*26, CYP2A6*27, CYP2A6*28* and *CYP2A6*35* was performed at the University of Toronto according to previously described protocols [18,48]. Those without variants (or with the duplication *CYP2A6*1x2* variant) were characterized as NM, those with a single copy of *CYP2A6*9* or *CYP2A6*12* as intermediate metabolizers and those with two copies, or any other reduced or loss of function variant as slow metabolizers. For analyses using the genotype, slow and intermediate metabolizers were grouped together as RM.

Data cleaning and analysis: Numerical data are presented as arithmetic mean and standard deviation if normally distributed or median and range if not normally distributed, nominal data as proportion (%). Measurements below LOQ were replaced by LOQ/$\sqrt{2}$ [49]. Since creatinine levels can vary by sex, age, BMI, and race, we also used a covariate-adjusted standardization method [50] to control measurement error bias. Skewed score values were log transformed before analysis. Differences were tested using the chi-square test for categorical variables, the *t* test for normally distributed continuous variables, and the Mann Whitney test for nonparametric variables. Missing data were not imputed. A $p<0.05$ was considered statistically significant. Subjective withdrawal (MNWS) and craving (QSU) effects were also determined as the AUEC using the trapezoidal method. Next to our main analysis investigating differences between slow and fast NMR, we also performed an additional analysis of Whites and Blacks only to investigate possible racial differences. Furthermore, in an additional explorative analysis we included sex and race that have been shown to influence NMR as covariates in a GLM, with fast and slow NMR entered as a categorical between-subject factor predicting withdrawal and reward outcomes. Other covariates (i.e. age, BMI, creatinine-corrected TNE, covariate-adjusted TNE) were also individually explored for their potential contributions on the NMR effects. For withdrawal after overnight abstinence and reward outcomes after each cigarette a univariate GLM was used, while for the six hour abstinence period a repeated measures GLM over time was used with the first time point (i.e. post-Cig 2) as covariate. Analyses were conducted using SPSS statistical software (IBM SPSS Statistics 23.0). Covariate adjustment of TNE was performed using SAS v. 9.4 (SAS Institute, Inc., Cary, NC, USA). Nicotine elimination half-lives were estimated from plasma nicotine concentrations using Phoenix WinNonlin 6.3 (Pharsight Corporation, Mountain View, CA).

Study highlights:

- *What is the current knowledge on the topic?*

  The nicotine metabolite ratio (NMR) is a phenotypic biomarker that is highly correlated with the rate of nicotine clearance, which is an important determinant of smoking behavior and nicotine dependence.

- *What question did this study address?*

  The present study aimed to examine the effect of NMR on nicotine withdrawal symptoms and the response to smoking a cigarette after overnight abstinence and six hours daytime abstinence.

- *What does this study add to our knowledge?*

  Fast metabolizers by NMR had lower blood nicotine concentrations and greater craving/withdrawal scores compared to slow metabolizers, but not greater reward after smoking, thus supporting the idea that fast metabolizers are likely smoking more to relieve craving/withdrawal symptoms.

- *How might this change clinical pharmacology or translational science?*

  Selection of medications and/or doses of medications based on the importance of relieving craving and nicotine withdrawal symptoms, guided by NMR, may be useful in optimizing smoking cessation therapy.

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Figure Legends

Figure 1. Frequency of slow and fast NMR in the *CYP2A6* genotype groups (n=124)

Figure 2. Average plasma nicotine concentrations over time based on a. NMR and b. Genotype (*=$p<0.05$, #=$p<0.001$ for differences between groups)

Figure 3. Average Minnesota Nicotine Withdrawal Scale (MNWS) score over time based on a. NMR and b. Genotype (*=$p<0.05$ for differences between groups)

Figure 4. Average Questionnaire on Smoking Urges (QSU) Global Craving Score over time based on a. NMR and b. Genotype (*=$p<0.05$ for differences between groups)

Supplementary Material

Figure S1. NMR frequency histogram

Table S1. Comparisons of slow and fast metabolizers in the two racial subgroups (mean (standard deviation), median (range) or n (%))

Table S2. Comparisons of slow and fast metabolizers in the two sex subgroups (mean (standard deviation), median (range) or n (%))

Table 1. Baseline characteristics (mean (standard deviation), median (range) or n (%))

	All (n=136)	**Slow NMR (n= 71)**	**Fast NMR (n=65)**	***p***	**Whites (n=98)**	**Blacks (n=38)**	***p***	**Male (n=83)**	**Female (n=53)**	***p***
Age (years)	36.3 (12.3)	34.4 (12.3)	38.3 (12)	0.065	35 (12.1)	39.6 (12.3)	0.05	34.7 (11.5)	38.8 (13.1)	0.054
Sex (female)	53 (39%)	21 (30%)	32 (49%)	**0.019**	35 (36%)	18 (47%)	0.211	na	na	na
Race (Blacks)	38 (28%)	22 (31%)	16 (25%)	0.408	na	na	na	20 (24%)	18 (34%)	0.211
BMI	25.7 (4.6)	25.6 (4.6)	25.8 (4.7)	0.793	25.1 (4.6)	27.2 (4.1)	**0.016**	25.5 (4.3)	25.9 (5.1)	0.649
FTCD	4.2 (2.1)	4.3 (2.1)	4.1 (2.2)	0.69	4.3 (2.1)	3.8 (2.2)	0.15	4 (2.1)	4.3 (2.2)	0.495
CPD	12 (5-40)	12 (5-40)	13 (5-35)	0.42	13 (5-40)	10 (5-20)	**0.001**	13 (5-40)	10 (5-30)	0.054
Years of smoking	14.5 (2-43)	13 (2-43)	15 (2-41)	0.231	13.5 (2-43)	16 (2-43)	0.515	15 (2-43)	14 (2-41)	0.561
TFC (minutes)	30 (0.5-480)	20 (0.5-180)	30 (2-480)	**0.033**	28 (1-480)	30 (0.5-240)	0.967	30 (0.5-480)	30 (2-240)	0.682
Alcohol grams per week (self-reported)	37 (0-486)	41 (0-486)	13 (0-235)	0.156	42.5 (0-486)	13 (0-333)	0.13	52 (0-390)	10 (0-486)	**<0.001**
Menthol cigarettes	38	20	18	0.95	14	24	**<0.001**	21	17	0.39

	(28%)	(28%)	(28%)		(14%)	(63%)		(25%)	(32%)	
Saliva COT (ng/mL)	145.2 (48.2-653.8)	189.8 (48.2-653.8)	119.3 (49.5-481.7)	**<0.001**	138.3 (48.2-653.8)	148.2 (74-610.3)	0.308	147.8 (48.2-653.8)	137.5 (52.4-481.7)	0.384
Saliva NMR	0.25 (0.06-1.32)	0.16 (0.06-0.27)	0.55 (0.37-1.32)	**<0.001**	0.36 (0.07-1.32)	0.19 (0.06-0.57)	**0.005**	0.21 (0.08-1.32)	0.46 (0.06-1.1)	**0.034**
Plasma NMR	0.39 (0-2)	0.27 (0-1)	0.66 (0-2)	**<0.001**	0.45 (0-2)	0.33 (0-2)	**0.007**	0.36 (0-2)	0.46 (0-2)	**0.044**
Nicotine elimination half-life (minutes)	111.3 (41.1-272.4)	130.4 (50.3-255.5)	93.0 (41.1-272.4)	**<0.001**	105.9 (41.1-272.4)	151.2 (50.3-255.5)	**0.001**	112.0 (50-272.4)	109.1 (41.1-255.5)	0.854
Urine TNE (nmol/mg creat)	53.3 (0.9-195.5)	51.6 (1-195.5)	55.0 (0.9-173.8)	0.812	62.1 (1-195.5)	36.8 (0.9-116.2)	**0.009**	45.0 (3.9-195.5)	67.4 (0.9-150.2)	0.098

NMR: nicotine metabolite ratio; BMI: body mass index; FTCD Fagerstrom Test for Cigarette Dependence; CPD: cigarettes per day; TFC: time to first cigarette after awakening in the morning; COT: cotinine; TNE: total nicotine equivalents

Table 2. Associations of NMR with withdrawal and physiological measurements (mean (standard deviation) or median (range))

	After overnight abstinence (i.e. before Cig 1)				*After six hours abstinence (i.e. before Cig 3)*			
	All (n=136)	**Slow NMR (n= 71)**	**Fast NMR (n=65)**	***p***	**All (n=136)**	**Slow NMR (n= 71)**	**Fast NMR (n=65)**	***p***
Plasma nicotine (ng/mL)	1.0 (0.7-6.7)	1.5 (0.7-6.7)	0.7 (0.7-6.6)	**<0.001**	1.4 (0.7-8.9)	2.25 (0.7-8.9)	1.2 (0.7-3.4)	**<0.001**
Expired CO (ppm)	9 (2-22)	9 (3-22)	8 (2-21)	0.31	8 (2-16)	9 (3-16)	8 (2-14)	**0.022**
HR	69.8 (9.1)	69.6 (9.4)	70 (8.9)	0.816	72.2 (10.2)	72.4 (10.2)	72 (10.2)	0.804
QSU global craving score	4 (1-7)	3.9 (1-6.4)	4.3 (1-7)	0.203	3.85 (1-7)	3.7 (1-7)	3.9 (1.5-7)	**0.016**
MNWS	7 (0-32)	6 (0-22)	8 (1-32)	0.087	8 (0-48)	8 (0-21)	9 (1-48)	0.057
PANAS negative	12 (10-35)	12 (10-24)	13 (10-35)	0.178	11 (10-30)	11 (10-20)	12 (10-30)	0.059
PANAS positive	26 (3-46)	26.5 (3-45)	26 (11-46)	0.790	24 (10-50)	24 (10-50)	22 (10-50)	0.493

NMR: nicotine metabolite ratio; CO: carbon monoxide; HR: heart rate; QSU: Questionnaire on Smoking Urges; MNWS: Minnesota Nicotine Withdrawal Scale; PANAS: Positive and Negative Affect Scale

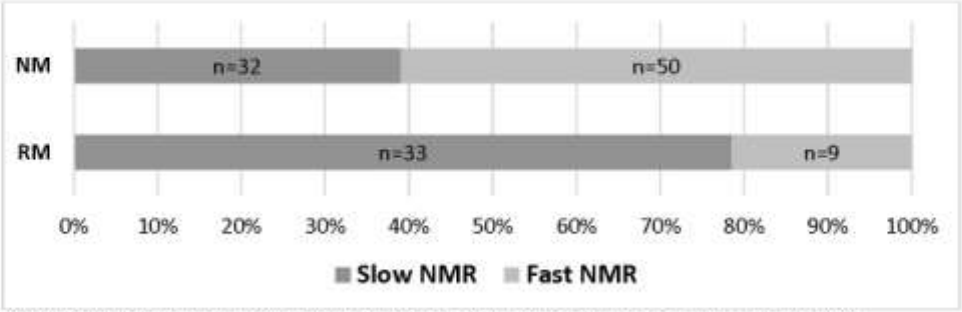
Table 3. Associations of NMR with the response to smoking after abstinence (mean (standard deviation) or median (range))

	After first cigarette of the day (i.e. after Cig 1, except indicated otherwise)				**After Cig 3**			
	All (n=136)	**Slow NMR (n= 71)**	**Fast NMR (n=65)**	***p***	**All (n=136)**	**Slow NMR (n= 71)**	**Fast NMR (n=65)**	***p***
Plasma nicotine (ng/mL)	12.9 (0.7-40.8)	13.6 (2.2-40.8)	10.9 (0.7-32.3)	**0.038**	17.3 (3.6-51.8)	18.2 (6.1-51)	16.2 (3.6-51.8)	0.077
Expired CO (ppm)	15 (6-32)	16 (8-32)	15 (6-29)	0.107	16 (3-42)	16 (7-42)	16 (3-31)	0.097
HR	85.5 (12.7)	84 (12.4)	87.1 (12.9)	0.149	85.1 (11.2)	83.9 (11.4)	86.3 (10.9)	0.224
QSU global craving score	1.3 (1-7)	1.2 (1-4.8)	1.6 (1-7)	**0.022**	1.2 (1-5.5)	1.1 (1-4.9)	1.4 (1-5.5)	0.067
MNWS	4 (0-22)	3 (0-22)	5 (0-21)	**0.02**	3 (0-17)	3 (0-15)	4 (0-17)	0.512
PANAS negative	11 (10-35)[£]	11 (10-35)[£]	12 (10-28)[£]	0.508	11 (10-28)	11 (10-26)	11 (10-28)	0.393
PANAS positive	26 (10-47)[£]	25 (10-47)[£]	26 (10-43)[£]	0.613	25 (10-50)	26 (10-50)	24 (10-48)	0.732
mCES aversion	6	6	6	0.128	4	4	4	0.552

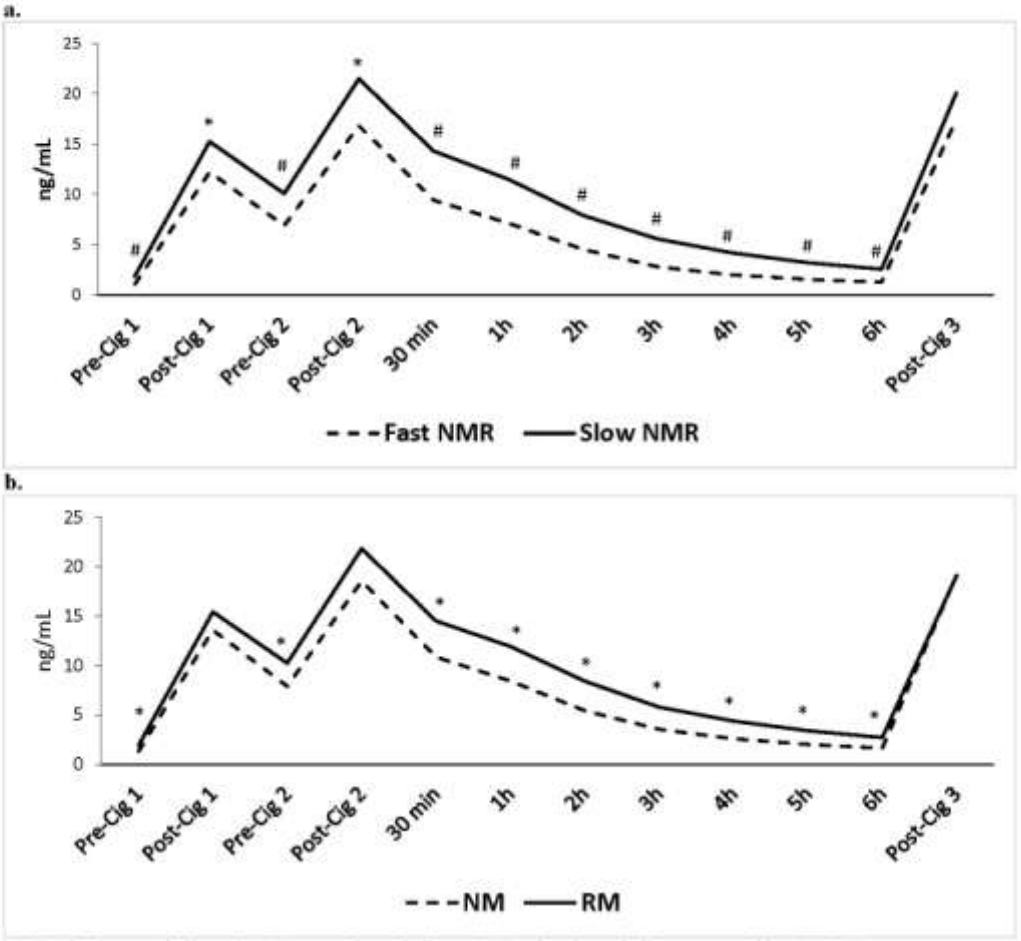
	(2-14)	(2-14)	(2-13)		(2-13)	(2-12)	(2-13)	
mCES craving	6 (1-7)	6 (1-7)	6 (1-7)	0.207	6 (1-7)	6 (1-7)	6 (1-7)	0.235
mCES reward	18 (5-35)	18 (5-35)	17 (5-35)	0.624	17 (5-35)	16 (5-35)	18 (5-35)	0.247
mCES satisfaction	14.5 (3-21)	16 (3-21)	12 (3-21)	**0.048**	15 (3-21)	15 (3-21)	15 (4-21)	0.482
mCES respiratory tract sensations	4 (1-7)	4 (1-7)	3 (1-7)	0.421	4 (1-7)	4 (1-7)	4 (1-7)	0.205

NMR: nicotine metabolite ratio; CO: carbon monoxide; HR: heart rate; QSU: Questionnaire on Smoking Urges; MNWS: Minnesota Nicotine Withdrawal Scale; PANAS: Positive and Negative Affect Scale; mCES: modified Cigarette Evaluation Scale

[£]: after Cig 2



NMR: nicotine metabolite ratio; NM: normal metabolizers; RM: reduced metabolizers, assessed by *CYP2A6* genotype



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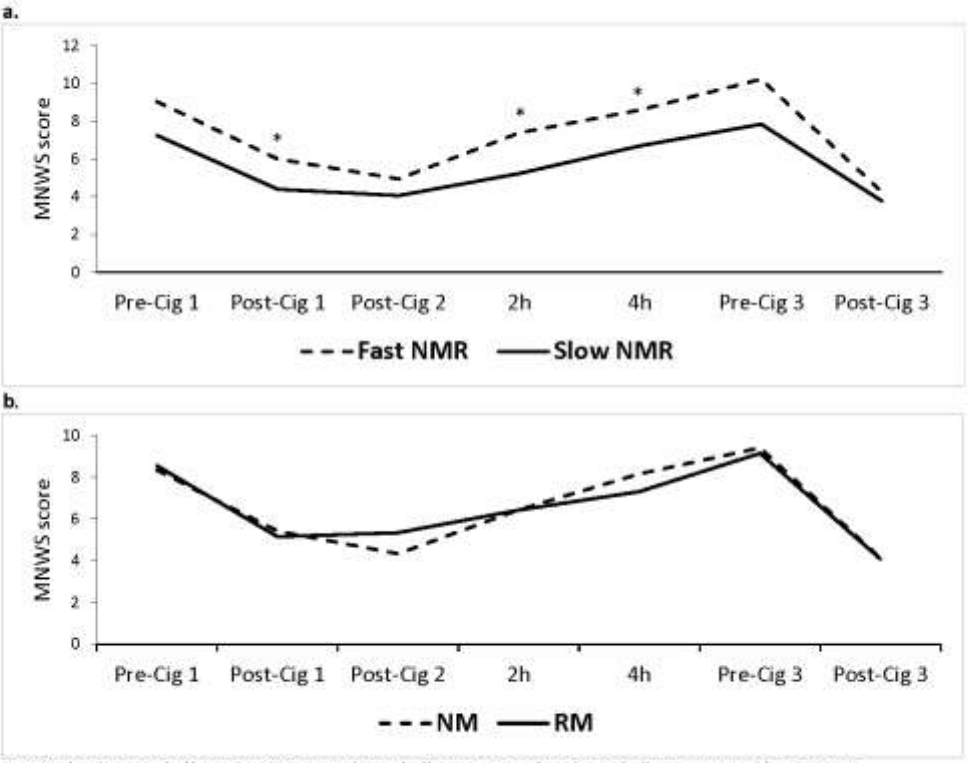

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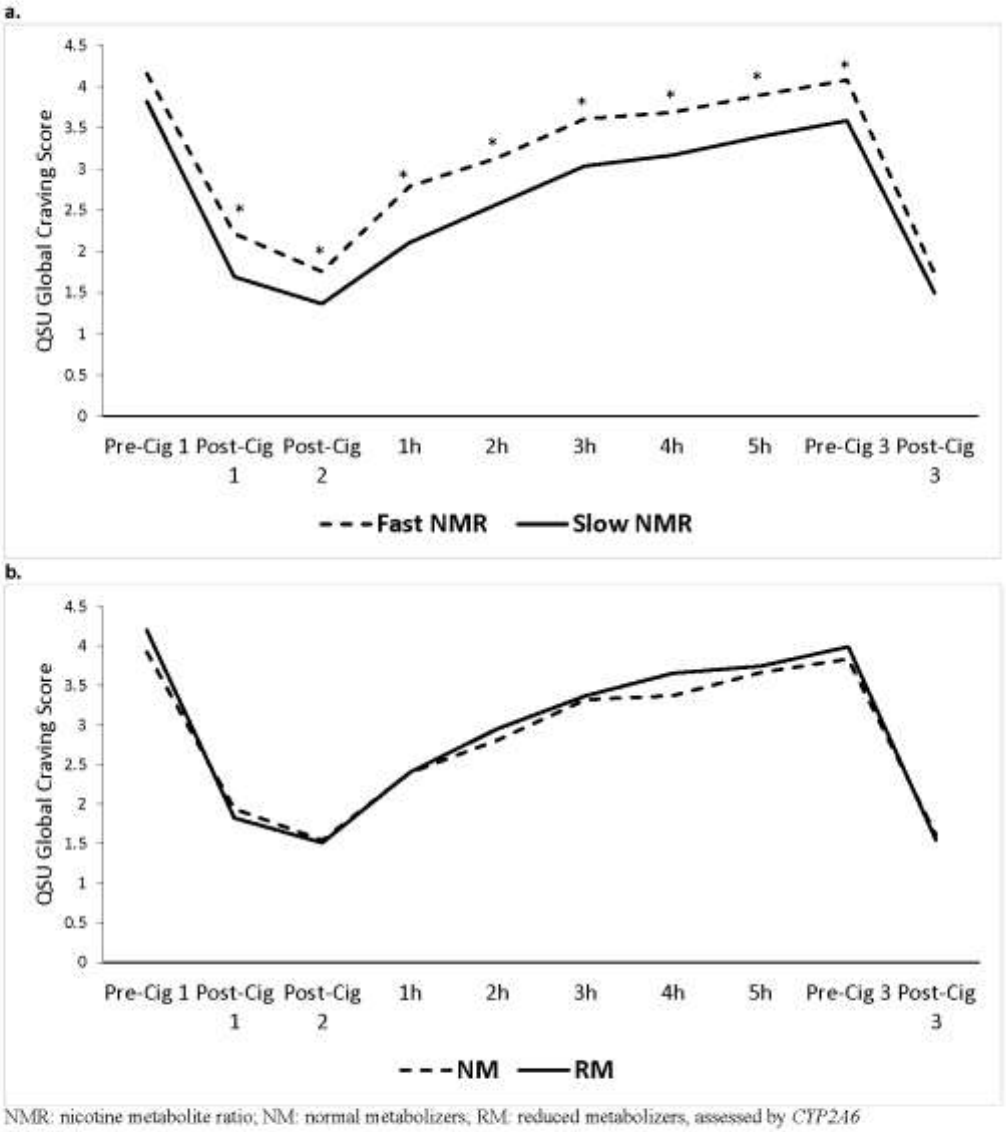

NMR: nicotine metabolite ratio; NM: normal metabolizers, RM: reduced metabolizers, assessed by *CYP2A6* genotype