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Nicotine Metabolite Ratio: Comparison of the Three Urinary Versions to the Plasma Version and Nicotine Clearance in Three Clinical Studies

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Abstract

Background: Variation in CYP2A6 activity influences tobacco smoking behaviors and smoking-related health outcomes. Plasma Nicotine Metabolite Ratio (NMR) is a robust phenotypic biomarker of CYP2A6 activity and nicotine clearance. In urine, the NMR has been calculated as a ratio of free trans-3'-hydroxycotinine to free cotinine ($NMR_{F/F}$), total trans-3'-hydroxycotinine to

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RF Tyndale has consulted for Quinn Emanuel and Ethismos Research Inc (unrelated to this work). N.L.B. serves as a paid consultant to pharmaceutical companies that are developing or that market smoking cessation medications. He also has been a paid expert witness in litigation against tobacco companies, including on issues related to light cigarettes. T.P.G. has consulted to Novartis, Frutarom and Pfizer, as serves as Deputy Editor, *Neuropsychopharmacology* (unrelated to this work). No potential conflicts of interest were disclosed by the other authors.

free cotinine ($NMR_{T/F}$), or total trans-3'-hydroxycotinine to total cotinine ($NMR_{T/T}$). We evaluated these three urinary NMR versions relative to plasma NMR and nicotine clearance and elucidated mechanisms of discrepancies among them.

Methods: Baseline plasma and urine biomarker data were available from two smoking cessation clinical trials and one nicotine pharmacokinetic study (total N=768). NMRs were compared using Pearson correlations, linear regressions and ANOVA analyses. *UGT2B10* and *UGT2B17* were genotyped.

Results: Urinary $NMR_{T/F}$ was the most highly related to plasma NMR (R^2=0.70, P<2.2e-16) followed by $NMR_{F/F}$ (R^2=0.68, P<2.2e-16), while $NMR_{T/T}$ was less strongly related (R^2=0.60, P<2.2e-16); consistent across study, ethnicity, sex, heaviness of smoking, and analyte analysis. Controlling for cotinine glucuronidation, as a phenotype or *UGT2B10* genotype, corrected the $NMR_{T/T}$ discordance with plasma NMR (P_{anova}<0.001). Similar findings were obtained for relationships of nicotine clearance with plasma NMR > urinary $NMR_{T/F}$ > $NMR_{F/F}$ > $NMR_{T/T}$ (R^2=0.41>0.37>0.35>0.25 respectively).

Conclusion: Urinary $NMR_{T/F}$ followed by $NMR_{F/F}$ are the best urinary alternatives to plasma NMR or nicotine clearance. $NMR_{T/T}$ has the least utility as it is influenced substantially by variation in cotinine glucuronidation.

Impact: This work highlighted the variation in urinary NMRs, and identified mechanisms for disparities among them, which facilitates their use in predicting smoking-related outcomes.

Keywords

Nicotine Metabolite Ratio/NMR; glucuronidation; cotinine; nicotine clearance; Total Nicotine Equivalents

1. INTRODUCTION

CYP2A6 is a genetically polymorphic enzyme that inactivates nicotine (the major psychoactive compound in cigarettes) and metabolically activates tobacco-specific carcinogens(Nakajima et al., 1996a; Nakajima et al., 1996b). Individual variation in CYP2A6 activity influences numerous smoking behaviors, including heaviness of smoking and smoking cessation(Lerman et al., 2015; Lerman et al., 2006; Schnoll et al., 2009). Faster CYP2A6 metabolizers, determined by genetics or by a phenotypic marker, the nicotine metabolite ratio (NMR), have a greater risk for tobacco-related diseases (e.g. lung cancer and chronic obstructive pulmonary disease)(Dempsey et al., 2004; Lerman et al., 2006; Tanner et al., 2018). The NMR, which can be measured in a number of biological matrices, is a ratio between nicotine's main metabolites cotinine (COT) and trans-3'-hydroxycotinine (3HC)(Helen et al., 2012). The plasma NMR (3HC/COT) is a biomarker of CYP2A6 activity as CYP2A6 exclusively metabolizes COT to 3HC, and is a surrogate for nicotine clearance due to the major role of CYP2A6 in metabolic nicotine clearance(Dempsey et al., 2004). Both COT and 3HC can be further metabolized via glucuronidation(Kuehl and Murphy, 2003). Although CYP2A6 activity and nicotine clearance are best approximated by the NMR measured in plasma(Helen et al., 2012; Tanner et al., 2015), urine samples have also been used for NMR determination(Kandel et al., 2007). In urine, CYP2A6 enzyme activity

is predicted to be most accurately phenotyped by an NMR with the substrate in the denominator (i.e. free COT) and total product, 3HC and its consequent metabolites (i.e. total 3HC: 3HC + 3HC-glucuronide), in the numerator. In addition to the enzymatically logical ratio (i.e. total 3HC to free COT ($NMR_{T/F}$)), urinary NMR has been also calculated as two analytically pragmatic versions: free 3HC to free COT ($NMR_{F/F}$), and total 3HC to total COT ($NMR_{T/T}$)(Arger et al., 2019; Benowitz et al., 2003; Derby et al., 2008; Jain; Taghavi et al., 2018). Total values represent the sum of the free (unconjugated) and glucuronide (conjugated) form(Derby et al., 2008; Swan et al., 2009). Compared to plasma NMR, urinary NMR measurements show greater variability, due to individual variation in renal clearance of COT and 3HC. Moreover, the different versions of the urinary NMR have potentially different relationships with plasma NMR, CYP2A6 activity and nicotine clearance(Helen et al., 2012; Tanner et al., 2015). Together the interchangeable use of these different urinary NMRs has led to some confusion in the literature about their relation to plasma NMR and their relevance for use in associating with smoking behaviours(Jain). The aims of the current study were to identify differences in the relationship between the three urinary NMRs and plasma NMR, to examine potential influences on these relationships (e.g. heaviness of smoking), to identify potential mechanisms contributing to discordances, and to assess the relative performance of these urinary NMRs in predicting nicotine clearance. We also assessed urinary NMRs following acute bolus nicotine administration(Benowitz et al., 2009a; Dempsey et al., 2002; Dempsey et al., 2004; Rubinstein et al., 2013a; Rubinstein et al., 2013b).

2. Materials and methods

2.1 Participants and Studies

2.1.1 Study 1 (Kick It at Swope III)—The first study (see Table 1) included African American light smokers (≤10 cigarettes/day) (trial described elsewhere (NCT00666978)) (Cox et al., 2011; Cox et al., 2012) (see Table 1). Baseline plasma and urinary samples (N=429) were collected when participants were smoking *ad libitum*. Three individuals were missing plasma NMR and seven had plasma COT levels below <10 ng/ml indicative of NMRs which were considered unstable (not regular smokers)(Benowitz, 1983; Scheidweiler et al., 2011). One individual was missing 5 out of 10 urinary analytes. Thus, the final sample size with urinary and plasma data available for analysis was N=418. For the genetic sub-study, *UGT2B10* and *UGT2B17* genotype data were available for N=377 and N=328 participants, respectively.

2.1.2 Study 2 (Pharmacogenetics of Nicotine Addiction Treatment-2)—The second study (see Table 1) included primarily European ancestry heavy smokers (≥10 cigarettes/day) (trial described elsewhere (NCT01314001))(Chenoweth et al., 2014; Lerman et al., 2015). Baseline plasma and urinary samples (N=139 subset from the CAMH recruitment site) were collected when participants were smoking *ad libitum*. Three individuals were missing plasma NMR data. Thus, the final sample size with urinary and plasma data available for analysis was N=136. For the genetic sub-study, *UGT2B10* and *UGT2B17* genotype data were available for N=79 and N=99 participants, respectively.

2.1.3 Study 3 (Pharmacokinetics of Nicotine and Cotinine)—

The third study included smokers and non-smokers; study procedures are described elsewhere(Dempsey et al., 2004). *Smokers:* Plasma (N=94) and urinary (N=101) samples were collected at baseline during *ad libitum* smoking as well as following the administration of an acute oral solution of deuterium-labeled nicotine. Smokers with no blood samples drawn (N=2), missing plasma NMR data at steady-state (i.e. unlabeled analytes) (N=3), missing nicotine clearance data (N=5), or plasma COT level <10 ng/ml (N=10) were excluded. The final sample size of smokers with urinary and plasma data available from *ad libitum* smoking was N=79. *Non-smokers:* Plasma (N=133) and urinary (N=143) samples from non-smokers were collected following the acute administration of an oral solution of labeled nicotine. The full dataset with labeled acute oral nicotine data (available for N=89 smokers and for N=125 non-smokers) was used for additional assessments of metabolic profile (N=214).

All three studies were approved by institutional review boards at the respective study sites and at the University of Toronto.

2.2. Analytical Chemistry

Liquid chromatography-tandem mass spectrometry was used to determine plasma and urinary analyte concentrations(Benowitz et al., 1994; Dempsey et al., 2004). The ratio of plasma concentrations of free 3HC over free COT was used to determine plasma NMR(Dempsey et al., 2004; Helen et al., 2012; Tanner et al., 2015). The limit of quantification (LOQ) for plasma samples was 1 ng/mL. Urinary NMR was calculated using three different published approaches: a ratio of free 3HC to free COT ($NMR_{F/F}$), total 3HC to free COT ($NMR_{T/F}$), or total 3HC to total COT ($NMR_{T/T}$)(Arger et al., 2019; Benowitz et al., 2003; Derby et al., 2008; Taghavi et al., 2018). In studies 1 and 3, urinary glucuronides were determined indirectly, as the difference between free concentrations before and after enzymatic de-conjugation(Dempsey et al., 2004; Taghavi et al., 2018). Urinary LOQs for the indirect method were 10 ng/mL for COT, 3HC, nicotine, and nicotine-N-oxide (NNO), and 1 ng/mL for cotinine-N-oxide (CNO), norcotinine (NCOT), and nornicotine (NNIC). In COT-verified smokers (i.e. with plasma COT >10 ng/mL), analytes with resulting negative glucuronide values, or those below the LOQ, were imputed as LOQ/sqrt(2) (i.e. 7.07 ng/mL) as per convention(Chenoweth et al., 2016). Negative glucuronides generated from labeled acute oral nicotine data were dummied in as zeroes. In Study 2, a direct method was used to measure glucuronide metabolite levels in urine with LOQs of 5 ng/mL(Taghavi et al., 2018).

For studies 1 and 2, total nicotine equivalents (TNE) was determined as the molar sum of urinary concentrations of nicotine and nine of its metabolites (i.e. TNE-10): COT, cotinine glucuronide (COT-gluc), 3HC, 3HC glucuronide (3HC-gluc), nicotine glucuronide (nicotine-gluc), NNO, CNO, NCOT, and NNIC. In study 3, nicotine and six of its metabolites (COT, COT-gluc, 3HC, 3HC-gluc, nicotine-gluc, and NNO) were available (i.e. TNE-7). TNE-10 and TNE-7 account for about 90% and 80% of nicotine dose, respectively(Dempsey et al., 2004). Study 3 also included measures of nicotine clearance(Dempsey et al., 2004).

2.3 Genotyping

The *UGT2B10*2* (rs61750900) and *UGT2B17*2* deletion allele were genotyped using a TaqMan genotyping (custom order) and gene expression (HS00854486_sH) assay(Helen et al., 2012; Zhu et al., 2013), respectively, and an ABI Viia 7 real-time PCR machine according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The *UGT2B10* splice variant (rs2942857) was imputed following genome-wide SNP genotyping, as described(Chenoweth et al., 2018). All genotype frequencies were in Hardy Weinberg equilibrium.

A recently derived *UGT2B10* genetic risk score (GRS) comprised of rs61750900 and rs2942857(Murphy et al., 2014) was used to assess the impact of UGT2B10 activity on the relationship between urinary NMR and plasma NMR. A score of 0 was given to those homozygous for the reference allele for both variants (i.e. G/G and A/A, respectively), a score of 1 was given to those with a heterozygous genotype for one of the variants (i.e. G/T or A/C, respectively) and a score of 2 was given to those homozygous for either of the variant alleles, or heterozygous for both variants (i.e. rs61750900 T/T or rs2942857 C/C, or G/T and A/C, respectively).

2.4 Statistical analyses

Demographic characteristics were compared using Chi-Square or Mann-Whitney U tests for categorical and continuous variables respectively. Relationships between log-transformed plasma NMR and the three urinary NMRs were assessed by Pearson r correlations, then compared using Steiger's equation on the Fisher r-to-z transformed values (using the "cocor" package v1.1–3 in R)(Diedenhofen and Musch, 2015; Steiger, 1980). Linear regression models were used to fit a line between NMRs before and after glucuronidation correction and to identify outliers with the poorest fit (i.e. highest residuals). To determine whether the change in fit was deemed significant, two conditions needed to be satisfied: 1) the change in the regression estimates of the urinary NMRs had to be greater than 10%, and 2) the adjusted R-squared value of the model after correction showed a statistically significant increase at a 0.05 significance level determined by ANOVA chi-squares tests for nested models. *UGT2B10* and *UGT2B17* analyses were restricted to African Americans in Study 1 and European Americans in Study 2 to reduce potential confounding effects of population stratification. All statistical analyses were performed in R version 3.6.0 or RStudio version 1.1.456. Plot 1 was generated in R using "ggplot2" library(Wickham, 2009).

3. Results

3.1 Nicotine Metabolite Ratio (NMR) characteristics in urine compared to plasma: Study 1

Participant demographics and baseline characteristics are shown in Table 1 and Online supplementary Table S1. The strongest positive Pearson correlation between log-transformed plasma and urinary NMR was observed for urinary $NMR_{T/F}$ (R^2=0.70, P<2.2e-16), followed by urinary $NMR_{F/F}$ (R^2=0.68, P<2.2e-16) and urinary $NMR_{T/T}$ (R^2=0.60, P<2.2e-16) (Figure 1). The difference in the plasma-to-urinary correlation observed for urinary $NMR_{T/F}$ and $NMR_{F/F}$ was significant (z=−2.51, P=0.012), as was that

for urinary $NMR_{T/T}$ compared to $NMR_{T/F}$ and $NMR_{F/F}$ ($NMR_{T/F}$ z=3.74, P<0.001; $NMR_{F/F}$ z=3.41, P<0.001). Consistent with the strength of the correlation, the linear regression line fit was best between plasma NMR and urinary $NMR_{T/F}$, where the fewest poorly predicted outlying points were observed (6.7% of values had absolute residuals >0.3). In comparison, the $NMR_{T/T}$ regression line fit displayed the most predicted outlying points (10.5% of values had absolute residuals >0.3).

Because females have higher CYP2A6 activity resulting in faster nicotine and COT metabolism and NMR(Cox et al., 2011), we examined relationships between plasma and urinary NMRs stratified by sex. The observed rank order of plasma to urinary NMR relationships remained the same (i.e. $NMR_{T/F}$ > $NMR_{F/F}$ > $NMR_{T/T}$) in females and males when analyzed separately (Online supplementary Figure S1).

3.2 Replication and extension of the NMR characteristics: Study 2

Study 2 included heavier smoking participants that were predominantly males of European ancestry (Table 1) with a urinary metabolic profile that differed from Study 1 (Figure 2A–B), whose participants were predominantly African American. Our data were consistent with the slower N-glucuronidation among African Americans(Murphy et al., 2014), resulting in metabolic profile differences in the portion of nicotine excreted as products of N-glucuronidation, with COT-gluc at 15.3% and 4.6%, and nicotine-gluc at 6.3% and 1.2%, for Study 2 and Study 1, respectively (Figure 2A–B). Despite these differences in N-glucuronidation and resulting urinary metabolic profiles, and despite additional differences in levels of smoking (P<1e-5), method of analysis, and other characteristics (i.e. sex (P<1e-5), age (P=0.004), and BMI (P<1e-5)), the rank order of plasma to urinary NMR relationships (i.e. $NMR_{T/F}$ (R^2=0.54, P<2.2e-16) > $NMR_{F/F}$ (R^2=0.53, P<2.2e-16) > $NMR_{T/T}$ (R^2=0.37, P=1.7e-15)) were replicated in Study 2 (Online supplementary Figure S2, Online supplementary Table S2). Similar to Study 1, there was also a significant difference in the plasma-to-urinary correlation observed for urinary $NMR_{T/T}$ compared to $NMR_{T/F}$ and to $NMR_{F/F}$ ($NMR_{T/F}$ z=2.55, P=0.01; $NMR_{F/F}$ z=2.30, P=0.02) in Study 2. Consistent with the strength of the correlation, the linear regression line fit was best between plasma NMR and urinary $NMR_{T/F}$, where the fewest poorly predicted outlying points were observed (6.6% of values had absolute residuals >0.3) while the $NMR_{T/T}$ regression line fit displayed the most predicted outlying points (11.0% of values had absolute residuals >0.3). This suggests the robust nature of the strength of the relationship between plasma NMR and two of the urinary NMRs, $NMR_{T/F}$ and $NMR_{F/F}$, relative to that found for $NMR_{T/T}$.

3.3 Glucuronidation as a possible mechanism explaining the poorer relationship between plasma NMR and urinary total 3HC/total COT (i.e. $NMR_{T/T}$)

There is substantial individual variation in the rate of COT glucuronidation and resulting levels of COT-gluc (of note, CYP2A6 cannot further metabolize COT-gluc); this variation was first hypothesized by Taghavi and colleagues to be responsible for the poor relationship between plasma NMR and urinary $NMR_{T/T}$(Taghavi et al., 2018). The addition of COT glucuronidation ratios (i.e. COT-gluc/TNE-10) to the $NMR_{T/T}$ model significantly improved the regression coefficients and the closeness of regression fit with plasma NMR (P<1e-8) (Figure 3A–B, Online supplementary Table S2). As expected (as COT-glucuronide is not

part of these NMRs), adding COT glucuronidation ratios to $NMR_{F/F}$ and $NMR_{T/F}$ (Online supplementary Table S2) did not alter the relationship to plasma NMR. The addition of 3HC glucuronidation ratios (i.e. 3HC-gluc/TNE-10) had a negligible and non-reproducible effect on the relationship between plasma NMR and urinary $NMR_{T/T}$ (Figure 3C, 3D). Thus, urinary $NMR_{T/T}$ appears to be a weaker predictor of plasma NMR, due at least in part to the inclusion of the highly variable COT glucuronide in the denominator. To further evaluate the role of COT and 3HC glucuronidation, *UGT2B10* and *UGT2B17* genotypes (the genes encoding the enzymes primarily responsible for COT and 3HC glucuronidation, respectively) were examined. Initially, we established that the recently published *UGT2B10* GRS(Murphy et al., 2014) explained 38.5% of the variation in the log-transformed UGT2B10 phenotype (COT-gluc/TNE-10) in study 1 (Online supplementary Figure S4). Consistent with the impact of the addition of COT glucuronidation ratios to the urinary NMR $_{T/T}$ model (above), the addition of the *UGT2B10* GRS enhanced the regression fit of plasma NMR and urinary $NMR_{T/T}$ in both studies 1 and 2 (Online supplementary Figure S3), while neither 3HC glucuronidation (above) nor *UGT2B17* genotypes did (Online supplementary Figure S3).

3.4 NMR prediction of nicotine clearance

Modelling nicotine clearance is important for understanding individual variability in nicotine intake, smoking behaviours and consequently risk for smoking-related diseases(Benowitz, 2008; Tyndale and Sellers, 2001). As one of the clinical applications of the NMR is as a proxy for nicotine clearance, we directly assessed how the different NMRs performed in predicting nicotine clearance in a small pharmacokinetic study (Study 3, Online supplementary Table S1; Demographics, Online supplementary Table S4). As expected, despite the relatively small sample size of smokers in the study, plasma NMR predicted nicotine clearance in *ad libitum* smokers, explaining 41.1% of the variance in nicotine clearance (P<0.0001). Urinary $NMR_{T/F}$ and $NMR_{F/F}$ explained 37% and 35% of the variance in nicotine clearance, respectively (both Ps<1e-08) while urinary $NMR_{T/T}$ predicted nicotine clearance to a lesser extent (25% of variation explained; P=1.36e-06). The addition of COT glucuronidation ratios to the $NMR_{T/T}$ model increased the proportion of variation explained to 33% (Figure 4). Together this indicates that $NMR_{T/T}$, compared to urinary $NMR_{T/F}$, and $NMR_{F/F}$, is more poorly related to both plasma NMR and nicotine clearance, and that this is likely due to the inclusion of COT-gluc in the denominator of this urinary $NMR_{T/T}$.

3.5 Urinary NMRs are substantially altered by route of nicotine administration

In study 3, a total of 97 European, 59 African, and 68 Asian American smokers and non-smokers received labeled oral nicotine acutely (Demographics, Online supplementary Table S4). Plasma NMR correlated with nicotine clearance as previously shown(Benowitz et al., 2009b; Dempsey et al., 2004). However, acute oral nicotine provided substantially different 8-hr urinary NMRs and composition of TNE components compared to *ad libitum* smoking (i.e. those derived potentially at steady state, using an inhaled (i.e. smoked) delivery), particularly for the components of the urinary NMRs (Figure 2C–E). For instance, in the total group (N=214), plasma NMR correlated relatively poorly with all the three urinary NMRs ($NMR_{T/F}$ R^2=0.56; $NMR_{F/F}$ R^2=0.56; $NMR_{T/T}$ R^2=0.57); this was also observed for

the non-smokers (N=125, Online supplementary Table S5). This large difference in urinary metabolite composition of the urinary NMRs was further illustrated when examining the composition of the TNE derived from smoking vs. acute oral drug delivery within smokers (Figure 2C–E); overall, the portion of TNE which is excreted as each metabolite is different within the smokers during *ad libitum* smoking (Fig 2C) compared to acute oral labeled nicotine (Figure 2D). Together these findings suggest that all three urinary NMRs derived from a non-steady-state acute nicotine dose are poor predictors of plasma NMR and CYP2A6 activity.

4. Discussion

The current study is the first to evaluate the three urinary versions of the nicotine metabolism ratio (NMR) in different ethnicities, and by sex and heaviness of smoking, compared to plasma NMR and to nicotine clearance. In addition, this is the first study to provide mechanistic elucidation for the discrepancies between urinary NMRs. Variation in CYP2A6 activity, captured phenotypically by the NMR, is an important source of individual variation in smoking behaviors, smoking cessation outcomes, and risk for tobacco-related diseases. Measurement of the NMR in plasma is not always available. In urinary assessments of the NMR, we found that urinary $NMR_{T/F}$ was routinely the most highly related to plasma NMR and to nicotine clearance. Urinary $NMR_{T/F}$ is also the most enzymatically relevant, as it includes only free COT (i.e. the CYP2A6 substrate) in the denominator and the products of CYP2A6-mediated COT metabolism and subsequent metabolism (i.e. 3HC and 3HC-glucuronide) in the numerator. Our secondary analyses showed that the strength and order of the relationship between the urinary NMRs (i.e. $NMR_{T/F} > NMR_{F/F} > NMR_{T/T}$) and plasma NMR remained consistent regardless of sex, ethnicity or heaviness of smoking.

Including COT-gluc in the denominator of the NMR (i.e. urinary $NMR_{T/T}$) decreased the correlation with plasma NMR, resulting in a poorer prediction of the CYP2A6 activity phenotype. This poorer prediction was expected given that COT-gluc is not a substrate of CYP2A6. TNE is sometimes analyzed only following deconjugation (e.g. not as free and deconjugated), to save costs; this provides total analytes only and thus can only be used to calculate urinary $NMR_{T/T}$; as described above, while useful, it is the least related to plasma NMR with the greatest possibility of further decline in utility as variation in conjugation increases. If using urinary $NMR_{T/T}$ considerable caution should be taken if the study investigates populations with known or unknown variation in COT glucuronidation or *UGT2B10* or compares directly two populations with and without variation in COT glucuronidation or *UGT2B10* (e.g. pregnant women to non-pregnant). For populations with rapid COT glucuronidation, such as pregnant women(Dempsey et al., 2002), it is likely that the ability of $NMR_{T/T}$ to accurately predict plasma NMR, CYP2A6 activity and nicotine clearance would be even poorer. The extent of COT glucuronidation, which accounts for up to 30% of recovered nicotine metabolites in urine(Benowitz et al., 1994; Byrd et al., 1992), also varies by ancestry: compared to individuals of European ancestry, African Americans have lower COT glucuronidation consistent with their higher frequency of non-functional variants in *UGT2B10* such as the splice variant rs2942857. In the current study, the impact of the *UGT2B10* GRS on the NMR relationships was apparent in both ethnicities. In

contrast, 3HC glucuronidation variation did not significantly alter the plasma to urinary NMR relationships, as expected, and consistent with previous studies(Zhu et al., 2013).

For urinary $NMR_{F/F}$, relative to $NMR_{T/F}$, the exclusion of 3HC-gluc from the numerator may have slightly underestimated total CYP2A6 activity, as 3HC can be further conjugated and excreted in urine as a glucuronide. The similarly high correlations between urinary $NMR_{T/F}$ and plasma NMR, and between urinary $NMR_{F/F}$ and plasma NMR, reflects the relatively small proportion of 3HC further metabolized to 3HC-glucuronide. However, caution should be used when utilizing urinary $NMR_{F/F}$ in populations known to have faster or more variable rates of 3HC glucuronidation, for example pregnant women(Dempsey et al., 2002).

The similarity of the plasma and urinary NMR relationship trends in Study 1 and 2 further supports that the observed effects are consistent regardless of ethnicity. The replication in Study 2 also provided additional confidence that regardless of how the analytes were measured (i.e. direct vs indirect LC/MS method), similar outcomes were observed. Moreover, consistent with prior data, glucuronidation ratios (represented here as COT-gluc/TNE-10 and 3HC-gluc/TNE-10) were not influenced by gender, age, BMI, creatinine, or TNE(Wassenaar et al., 2015). Thus, differences in smoking quantity (i.e. TNE or CPD), gender, age, or BMI likely do not explain how variation in glucuronidation alters urinary NMRs.

We also assessed the ability of urinary NMR to predict plasma NMR via non-smoking, non-steady-state routes involving acute nicotine administration. We found that all three urinary NMRs were relatively poorly related to plasma NMR following acute oral dosing. For the NMR to be stable and to reflect CYP2A6 activity, COT needs to be at steady state where 3HC becomes formation dependent(Helen et al., 2012; Lea et al., 2006). Thus, the observed urine-plasma NMR relationship existed only where COT and 3HC were at steady state (i.e. during regular smoking) and not following acute oral nicotine(Mooney et al., 2008; St Helen et al., 2013; Tanner et al., 2015). This is important to note as acute oral nicotine and COT have been used previously to determine NMR(Dempsey et al., 2004). Differences in metabolic profiles from the different route of administration of nicotine (i.e. regularly inhaled vs. acute oral bolus) contributes to this lack of utility of urinary NMRs following acute oral dosing.

In conclusion, our data suggest that urinary $NMR_{T/F}$ is the best alternative to plasma NMR and nicotine clearance, followed by $NMR_{F/F}$ which may have reduced the ability to predict CYP2A6 activity in instances where the rate of 3HC-glucuronidation is increased (e.g. pregnancy). Overall, $NMR_{T/T}$ has the least utility, as it least reflects plasma NMR, CYP2A6 activity, and nicotine clearance, and is biased substantially by variation in COT glucuronidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Faster (versus slower) nicotine metabolisers differ in their smoking behaviours, treatment responses, and smoking-related health problems. Nicotine metabolism, mediated by CYP2A6, can be proxied by the nicotine metabolite ratio (NMR, 3-hydroxycotinine/cotinine).
- This study evaluated 1) the three widely used versions of the urinary NMRs (i.e. using free versus glucuronidated metabolite ratios) compared to plasma NMR, and nicotine clearance, among different ethnicities, sexes, and levels of smoking, and 2) elucidated the mechanism explaining their differences.
- The urinary NMR version using total cotinine had the least utility; it is influenced by variation in cotinine glucuronidation which can differ substantially (e.g. by ancestry). Understanding the limitations of the different urinary NMRs has important implications for clinical, epidemiological and policy research of numerous smoking behaviors and diseases.

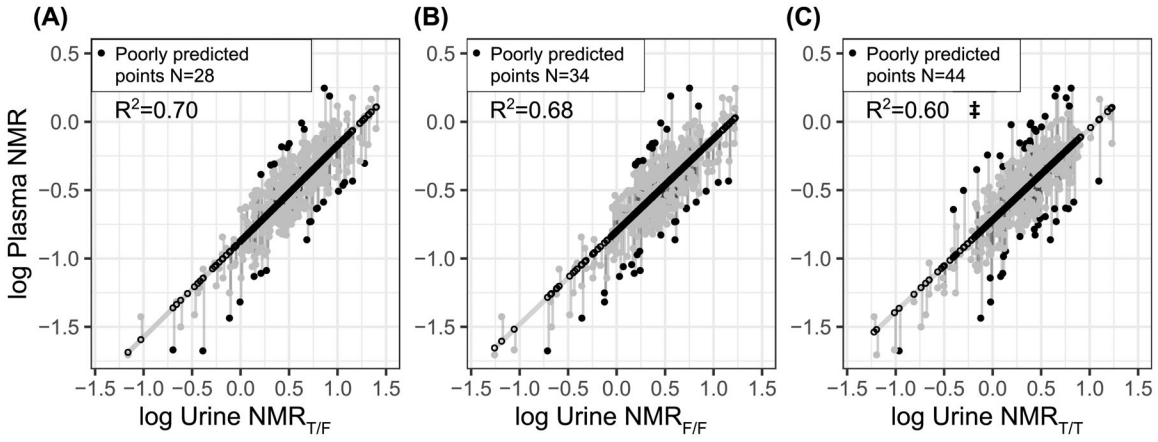

Figure 1. Plasma NMR correlates well with urinary $NMR_{T/F}$ (A) and $NMR_{F/F}$ (B), but not as well with $NMR_{T/T}$ (C) in light smoking African Americans of Study 1 (N=418).

Points with the poorest prediction, represented as higher absolute residuals (>0.3), shown in solid black circles. ‡ significant difference between Fisher's transformed Pearson r values ($P < 0.001$ in 2-tailed test) of the plasma NMR to $NMR_{T/T}$ compared to other urinary NMRs.

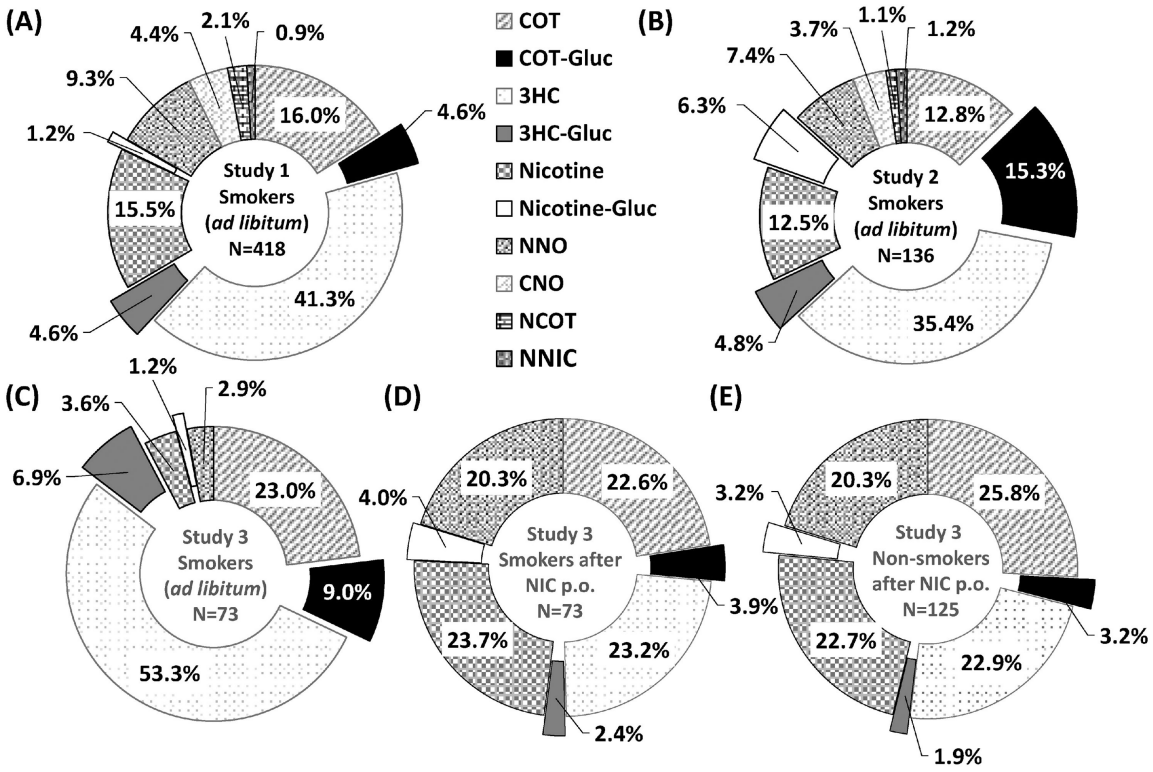

Figure 2. Nicotine metabolic profiles.

Compared to Study 1 (A), Study 2 (B) smokers had higher proportions of nicotine excreted as COT-gluc. Metabolic profile of nicotine differed between varying sources of nicotine in Study 3; *ad libitum* smokers (C), smokers (D) and non-smokers (E) after 2 mg oral dose of deuterium-labeled nicotine. Data shown as mean percent of each metabolite from the unadjusted total nicotine equivalents of nicotine + 9 metabolites (TNE-10) for Studies 1 (A) and 2 (B) while only nicotine + 6 metabolites (TNE-7) were available for Study 3 (C-E). Clockwise representation: cotinine (COT), cotinine glucuronide (COT-gluc), trans-3'-hydroxycotinine (3HC), trans-3'-hydroxycotinine glucuronide (3HC-gluc), Nicotine, Nicotine- glucuronide, nicotine N-oxide (NNO), cotinine N-oxide (CNO), norcotinine (NCOT), and nornicotine (NNIC).

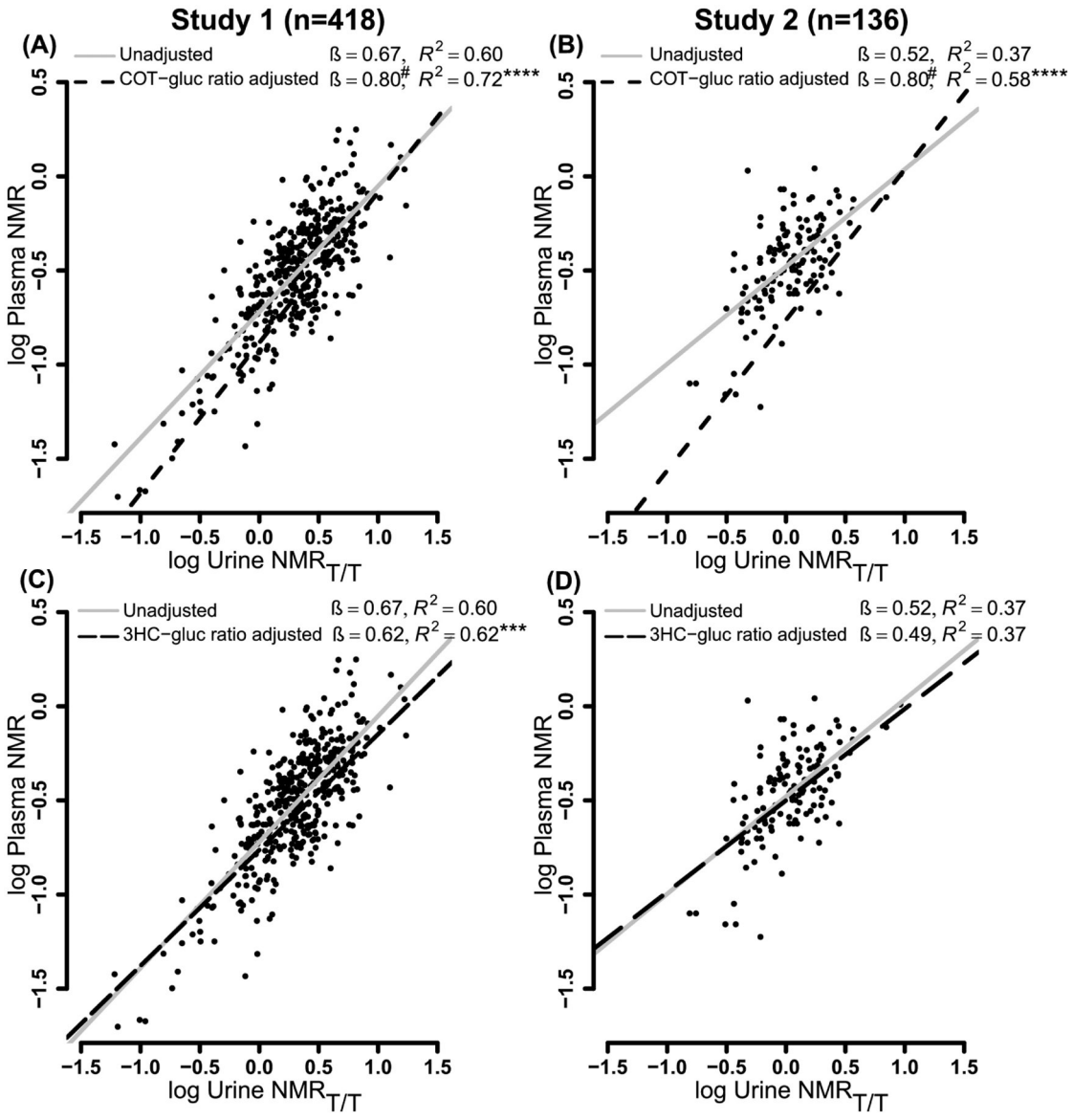

Figure 3. Variation in COT glucuronidation contributed to the poorer relationship of plasma NMR with urinary $NMR_{T/T}$.

Regressions of plasma NMR on urinary $NMR_{T/T}$ with and without addition of COT glucuronidation ratios in Study 1 (A), and replicated in Study 2 (B). Regressions of plasma NMR on urinary $NMR_{T/T}$ with and without addition of 3HC glucuronidation ratios in Study 1 (C), and Study 2 (D). Difference from base model was tested in ANOVA Chi-Square tests for nested models (P<1e-8 '****', <0.001 '***', <0.01 '**', <0.05 '*'). # indicates a >10% change in regression estimates.

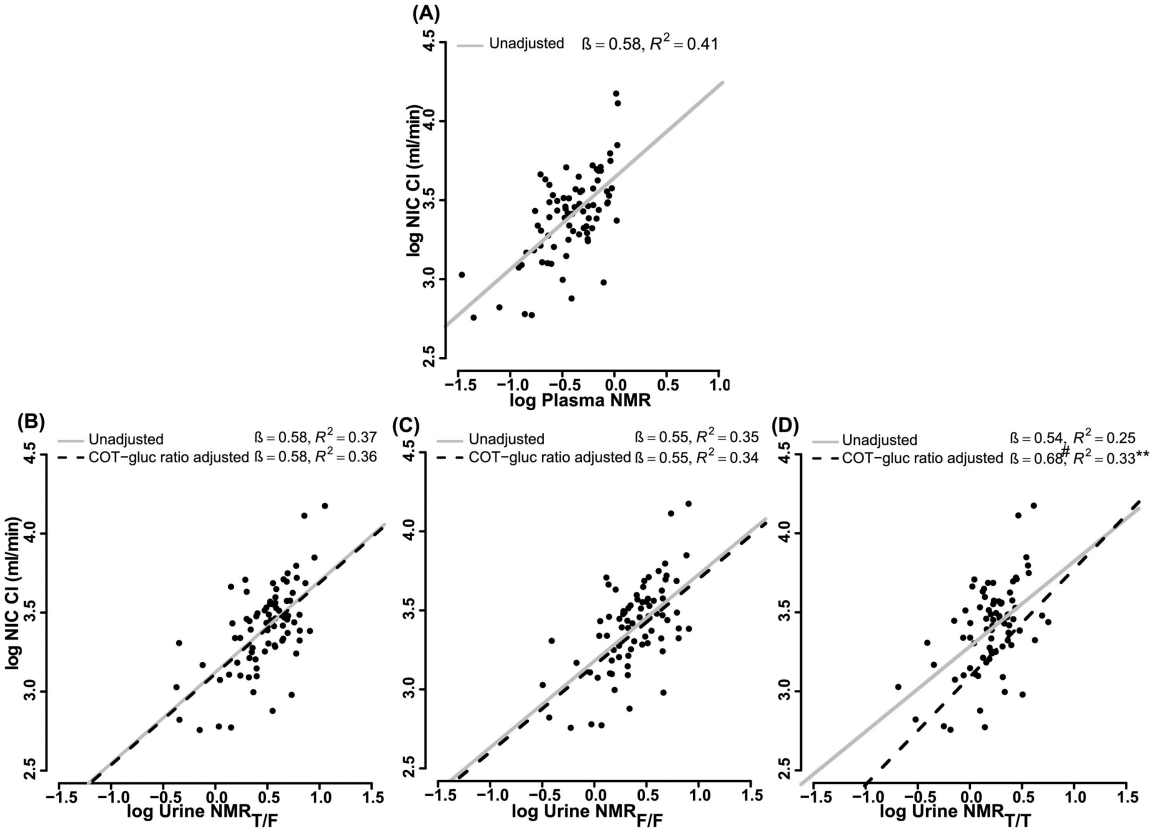

Figure 4. NMR relationship trends extend to nicotine clearance (*ad libitum* smokers in study 3).

Plasma NMR is a better predictor of nicotine clearance (A), followed by urinary $NMR_{T/F}$ (B), urinary $NMR_{F/F}$ (C), and urinary $NMR_{T/T}$ (D). COT glucuronidation effects significantly enhance the predictive ability of urinary $NMR_{T/T}$ for nicotine clearance in smokers in study 3 (N=79). Regression models were sequentially compared to base models in ANOVA Chi-Square tests for nested models (P<1E-8 '****', <0.001 '***', <0.01 '**', <0.05 '*'). # indicates a >10% change in regression estimates.

Table 1.

Demographics and baseline characteristics of smokers of Study 1 and 2 (shown as mean ± SD or n (%))

Characteristic	Study 1 (N=418)	Study 2 (N=136)
Age in years[a]	46.4 ± 11.6	42.8 ± 11.6
Body Mass Index (kg/m^2)[a]	31.5 ± 7.9	26.9 ± 4.8
Ethnicity[b]		
African Ancestry	415 (99.3)	3 (2.2)
European Ancestry	-	108 (79.4)
Other	3 (0.7)	25 (18.4)
Females[b]	281 (67.2)	48 (35.3)
Cigarettes/day (CPD)[a]	8.0 ± 2.2	19.3 ± 8.9
Plasma Nicotine Metabolite Ratio (NMR)	0.39 ± 0.26	0.40 ± 0.21
Urine NMR Total3HC/FreeCOT[c]	4.57 ± 3.98	5.22 ± 4.19
Urine NMR Free3HC/FreeCOT	3.72 ± 3.07	4.10 ± 3.13
Urine NMR Total3HC/TotalCOT[a]	2.76 ± 2.22	1.37 ± 1.13
TNE-10 (nmol/mg creatinine)[a]	53.6[d] ± 52.5	311.3 ± 246.3

SD = Standard Deviation; TNE-10 = Total Nicotine Equivalents of nicotine + 9 metabolites adjusted for creatinine

[a]Significant difference between studies (P<0.005) derived from the Mann-Whitney U test.

[b]Significant difference between studies (P<0.005) derived from the Chi-Square test.

[c]Significant difference between studies (P<0.05) derived from the Mann-Whitney U test.

[d]There was 1 sample missin creatinine.