Sources of Variation in Nicotine Metabolism and Associations with Smoking Abstinence in Adolescents and Adults

by

Meghan Jo-Ann Chenoweth

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Pharmacology and Toxicology
University of Toronto

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Abstract

Smoking remains a major public health concern; worldwide, approximately one billion people smoke. Despite the fact that many smokers are motivated to quit, only a small minority of those making quit attempts successfully quit each year. Variation in the rate of nicotine metabolic inactivation influences a number of smoking behaviours, including cessation. Thus, we sought to characterize genetic, environmental, and demographic sources of variability in the rate of nicotine metabolism, and resultant influences on smoking behaviours. Variation in the activity of the major nicotine-metabolizing enzyme, cytochrome P450 2A6 (CYP2A6) changes nicotine clearance and is associated with altered smoking behaviours, including cessation, in adults. We demonstrate here that slow (versus normal) nicotine metabolizers are more likely to achieve prolonged abstinence in adolescence, as in adulthood. We further demonstrate that in clinical trials, adult slow (versus normal) nicotine metabolizers are more likely to achieve early abstinence. We also investigated additional sources of genetic variability in the rate of nicotine
metabolism and their potential influences on smoking. We demonstrate that genetic variation in an additional nicotine-metabolizing enzyme (i.e., \textit{FMO3}), and a cytochrome P450 co-enzyme (i.e., \textit{POR}), does not substantially alter nicotine metabolism, CYP2A6 activity, or tobacco consumption. We further demonstrate that environmental and demographic sources of variability in CYP2A6 activity, such as gender and ethnicity, explain only a small proportion of the total variation in CYP2A6 activity; however, these factors may have unique impacts on smoking behaviours and thus should be further investigated in studies of smoking. Overall, our findings provide additional information regarding the role of variation in nicotine metabolism rate in cessation outcomes in both adolescents and adults. A greater understanding of the factors that influence smoking cessation will help optimize treatment outcomes and reduce the burden of tobacco-related disease.
ACKNOWLEDGMENTS

There are so many people I would like to thank for supporting me, encouraging me, and putting up with my (hopefully) occasional melt downs during the past five years.

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Thank you to my supervisory committee members, Drs. Denis Grant and Bernard Le Foll, for their helpful insights and suggestions, and for their encouragement to pursue projects outside of my comfort zone.

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Finally, I would like to thank Alex. If my attempt at trying to sort out what to write for this acknowledgment section is any indication, I’m going to be a blubering mess at our wedding. I am so grateful to you for your patience and calm demeanor that serves to offset my more spirited moments. We are an incredible team. Thank you so much for choosing me.
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OTHER RESEARCH ARTICLES


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Conference and seminar presentations

Oral Presentation: CHENOWETH, M. J., et al., “Role of genetically variable CYP2A6 in smoking cessation in adolescents.” June 2012 – Visions in Pharmacology, Toronto, ON, Canada

Oral Presentation: CHENOWETH, M. J., et al., “Impact of nicotine metabolism genetics on adolescent and adult smoking behaviours.” April 2015 – Campbell Family Research Institute Trainees Seminar Series, Centre for Addiction and Mental Health, Toronto, ON, Canada


Abstracts presented

Poster Presentation: CHENOWETH, M. J., et al. “Slow CYP2A6 nicotine metabolism increases quit rates in adolescent smokers.” March 2012 – Society for Research in Nicotine and Tobacco Annual Meeting, Houston, TX, USA; April 2012 – Canadian Human and Statistical Genetics Meeting, Niagara Falls, ON, Canada


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<tr>
<td>3HC</td>
<td>3’hydroxycotinine</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BC</td>
<td>Birth control</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAMH</td>
<td>Centre for Addiction and Mental Health</td>
</tr>
<tr>
<td>CCS</td>
<td>Canadian Cancer Society</td>
</tr>
<tr>
<td>CCSA</td>
<td>Canadian Centre on Substance Abuse</td>
</tr>
<tr>
<td>CDC</td>
<td>US Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COT</td>
<td>Cotinine</td>
</tr>
<tr>
<td>CPD</td>
<td>Cigarettes per day</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>e-cigarette</td>
<td>Electronic cigarette</td>
</tr>
<tr>
<td>ER-α</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FMO3</td>
<td>Flavin-containing monooxygenase-3</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalized estimating equation</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IM</td>
<td>Intermediate metabolizer</td>
</tr>
<tr>
<td>ITT</td>
<td>Intent-to-treat</td>
</tr>
<tr>
<td>LC/MS-MS</td>
<td>Liquid chromatography–tandem mass spectrometry</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NDIT</td>
<td>Nicotine Dependence in Teens</td>
</tr>
<tr>
<td>NIC</td>
<td>Nicotine</td>
</tr>
<tr>
<td>NM</td>
<td>Normal metabolizer</td>
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<tr>
<td>NMR</td>
<td>Nicotine metabolite ratio</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>NRT</td>
<td>Nicotine replacement therapy</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>POR</td>
<td>NADPH-cytochrome P450 oxidoreductase</td>
</tr>
<tr>
<td>QSU-B</td>
<td>Questionnaire on smoking urges- brief</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>SAMHSA</td>
<td>Substance Abuse and Mental Health Services Administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SM</td>
<td>Slow metabolizer</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylamine</td>
</tr>
<tr>
<td>TNE</td>
<td>Total nicotine equivalents</td>
</tr>
<tr>
<td>TPP</td>
<td>Tegmental pedunculopontine nucleus</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>USDHHS</td>
<td>US Department of Health and Human Services</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Appendix A: Description of the Nicotine Dependence in Teens (NDIT) cohort.

Appendix B: Descriptions of the Kick it at Swope (KIS) III clinical trial and the nicotine pharmacokinetic study.

Appendix C: Description of the Pharmacogenetics of Nicotine Addiction Treatment (PNAT) 2 clinical trial.

Appendix D: Associations between variation in CYP2A6 and CYP2B6 and tobacco dependence throughout adolescence and in young adult smokers.
STATEMENT OF RESEARCH PROBLEM

Despite years of tobacco control efforts to reduce the prevalence of smoking, tobacco use remains a leading cause of morbidity and mortality worldwide. The decrease in smoking prevalence has largely plateaued in North America, and more people than ever before are smoking in low- and middle-income countries (Giovino, Mirza et al. 2012; Physicians for a Smoke-Free Canada 2012; Health Canada 2013). The vast majority of smokers begin smoking in adolescence, with 99% of first use occurring by age 26 (CDC 2012a). Numerous and diverse factors influence the risk for smoking, and include psychological, sociodemographic, lifestyle, environmental, and genetic determinants. Understanding the role of these risk factors in smoking in adolescence and adulthood is paramount to developing new strategies to improve quit rates and reduce the economic and health care burden stemming from tobacco use.

Nicotine is the primary psychoactive alkaloid in tobacco smoke, responsible for the reinforcing effects derived from cigarette smoking (Benowitz 2010). Heritability estimates indicate that the contribution of genetic factors to smoking range from ~40-50% for smoking initiation and cessation, to up to ~80% for smoking quantity and nicotine dependence (Koopmans, Slutske et al. 1999; Xian, Scherrer et al. 2003; Maes, Sullivan et al. 2004; Vink, Willemsen et al. 2005; Broms, Silventoinen et al. 2006). Nicotine is principally inactivated to cotinine, and further to 3'hydroxycotinine, by the hepatic CYP2A6 enzyme (Hukkanen, Jacob et al. 2005). The CYP2A6 gene is highly polymorphic, with over 40 CYP2A6 alleles discovered and characterized to date, many of which alter the rate of nicotine metabolism (http://www.cypalleles.ki.se/cyp2a6.htm). A phenotypic measure of CYP2A6 genotype is the nicotine metabolite ratio (NMR), which is the ratio of 3’hydroxycotinine to cotinine. The NMR is strongly correlated with the rate of nicotine clearance (Dempsey, Tutka et al. 2004). Variation in CYP2A6 and NMR is associated with a number of smoking behaviours, including cessation (Gu, Hinks et al. 2000; Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Schnoll, Patterson et al. 2009). The vast majority of the work characterizing the impact of nicotine metabolism variation on smoking behaviours has been conducted in adult smokers. In adolescents, a handful of studies have been performed; these show that CYP2A6 slow metabolism increases the risk for acquiring tobacco dependence, but slows the progression in dependence and reduces cigarette consumption (O'Loughlin, Paradis et al. 2004; Audrain-
McGovern, Al Koudsi et al. 2007). The influence of CYP2A6 variation on smoking cessation outcomes in youth, however, has not been assessed.

Several additional and genetically polymorphic enzymes (including CYP2B6 and flavin-containing monooxygenase-3 (FMO3)) play a minor role in nicotine metabolism (Benowitz, Hukkanen et al. 2009; Al Koudsi and Tyndale 2010). While CYP2B6 may play a role in nicotine metabolism in the brain, it is not thought to contribute substantially to peripheral nicotine metabolism (Lee, Jepson et al. 2007b; Al Koudsi and Tyndale 2010). FMO3-mediated nicotine N-oxidation accounts for 4-7% of urinary nicotine recovery, but may be higher in individuals with deletion of the CYP2A6 gene (Benowitz et al., 2009; Yamanaka et al., 2004). Common FMO3 gene variants that reduce FMO3 activity may result in altered nicotine metabolism rates, with consequent effects on smoking behaviours, especially in individuals with reduced CYP2A6 activity. Furthermore, altered activity of enzymes that support the function of CYP enzymes, including NADPH-cytochrome P450 oxidoreductase (Miller, Agrawal et al. 2011), may represent an additional source of variation in nicotine metabolism and smoking behaviours.

In addition to CYP2A6 genotype and potential contributions from other genes, a number of sociodemographic (e.g., ethnicity and sex) and lifestyle (e.g., cigarette consumption) factors, as well as medications (e.g., estrogen-containing birth control pills and hormone replacement therapy), are individually associated with variation in CYP2A6 activity/NMR (Benowitz, Pomerleau et al. 2003; Benowitz, Lessov-Schlaggar et al. 2006; Kandel, Hu et al. 2007; Derby, Cuthrell et al. 2008). No study has yet been undertaken to investigate all of these sources of NMR variation simultaneously, or to quantify their individual and overall impact on NMR. The potential of NMR as a genetically-informed predictive biomarker of smoking cessation outcomes was recently assessed in a clinical trial, where a treatment-by-NMR interaction was observed. In those with higher NMR (i.e., normal metabolizers), varenicline was a superior treatment to nicotine patch, whereas the efficacy of varenicline and patch was similar in those with lower NMR (i.e., slow metabolizers) (Lerman, Schnoll et al. 2015).

To date, the Food and Drug Administration (FDA) has approved only three types of pharmacotherapies to treat nicotine dependence: nicotine replacement therapy, bupropion, and varenicline. These existing treatments modestly improve cessation success, with varenicline providing the greatest efficacy (Jorenby, Hays et al. 2006; Aubin, Bobak et al. 2008; Eisenberg, Filion et al. 2008). The current paradigm of drug development, from preclinical screening to
gaining approval, takes upwards of 15 years and costs $2 billion (Perkins and Lerman 2011). Despite these enormous costs, less than two-thirds of drugs are efficacious in phase III trials (Perkins and Lerman 2011; Perkins and Lerman 2014). An improved drug development approach that includes short-term (i.e., one-week) efficacy screening of medications earlier in the development process has recently been proposed (Perkins and Lerman 2011; Perkins and Lerman 2014). The incorporation of pharmacogenetic information, especially that which influences cessation outcomes, may improve the interpretation of results from early efficacy studies. While NMR predicted smoking cessation outcomes at end-of-treatment, whether NMR predicts one-week abstinence is currently unknown. In addition, it is not known if the factors associated with NMR alter its ability to predict one-week abstinence.

Collectively, these studies will provide more comprehensive insight into the genetic and demographic sources of variability in the rate of nicotine metabolism, and how this in turn is likely to influence smoking discontinuation in adolescents and short-term smoking abstinence in treatment-seeking adults. In addition, by examining associations between CYP2A6 and smoking cessation in novice smokers, we will add to the growing literature demonstrating the importance of genetic factors in adolescent smoking behaviours, which has not been emphasized traditionally.
MAIN RESEARCH OBJECTIVES

The decrease in tobacco smoking prevalence has slowed in North America, underscoring the need to improve understanding of the predisposing factors to cigarette smoking, as well as to create new strategies to promote cessation. Previous studies have highlighted the role of variability in \textit{CYP2A6} in interindividual differences in smoking cessation in adults. However, it is not known whether variation in nicotine metabolism also influences abstinence in adolescence. Therefore, \textbf{the first objective of this thesis was to determine whether CYP2A6 variation is associated with smoking discontinuation in adolescent smokers, as it is in adult smokers.}

In addition to \textit{CYP2A6}, genetic variability in other nicotine metabolizing enzymes (i.e., FMO3) or CYP-supportive enzymes (i.e., NADPH-cytochrome P450 oxidoreductase; POR) may also affect the rate of nicotine metabolism and influence smoking behaviours. These effects may be more or less pronounced depending on the level of \textit{CYP2A6} activity. Polymorphisms in \textit{FMO3} and \textit{POR} have been shown to functionally impact FMO3 and POR activity, respectively, toward a variety of substrates \textit{in vitro}. \textbf{The second objective of this thesis was to assess the potential influence of genetic variability in FMO3 and POR on nicotine metabolism and smoking behaviours, and to determine their relative impact in CYP2A6 normal and slow metabolizers.}

In addition to genetics, environmental factors contribute to variation in \textit{CYP2A6} activity and resulting NMR. To date, a comprehensive analysis of the demographic and lifestyle factors associated with NMR variation has not been undertaken. Thus, \textbf{the third objective of this thesis was to characterize these additional sources of variability in NMR, and to determine their independent contribution to, as well as their overall impact, on NMR.}

The NMR was recently shown prospectively to predict smoking cessation outcomes in a clinical trial involving the nicotine patch and varenicline. Despite the availability of these treatments, long-term abstinence rates remain low. Short-term screening approaches that incorporate assessments of one-week abstinence may help expedite the process of finding new treatments for nicotine dependence. Therefore, \textbf{the fourth and final objective of this thesis was to determine whether NMR predicts one-week abstinence, and whether NMR prediction of early abstinence is mitigated by demographic and lifestyle factors that influence the NMR.}
1 GENERAL INTRODUCTION

1.1. Epidemiology of cigarette smoking

1.1.1. Global smoking prevalence

Today, over one billion people worldwide smoke cigarettes (Jha, Ramasundarahettige et al. 2013). In Canada, the prevalence of smoking has largely plateaued at 16% (Fig. 1) (Physicians for a Smoke-Free Canada 2012; Health Canada 2013). In contrast, smoking prevalence is increasing in developing countries (Giovino, Mirza et al. 2012). Recent estimates suggest nearly 50% and 11% of young men and women, respectively, use tobacco in low- and middle-income countries (Giovino, Mirza et al. 2012). Due to population growth, there has been an overall increase in the number of daily smokers (41% in men and 7% in women), with a 26% increase in the number of cigarettes smoked worldwide between 1980 and 2012 (Ng, Freeman et al. 2014). Despite extensive tobacco control efforts in many nations, these data suggest the worldwide tobacco market is increasing (Ng, Freeman et al. 2014). Far more people smoke than use illicit drugs. In 2013, the combined prevalence of past-month illicit drug use among United States adults aged 18 years and older was half that of smoking, at ~9% (SAMHSA 2014).

Figure 1 | The prevalence of current smoking among Canadians aged 15 years and older is shown from 1965 to 2012. ‘Current smoking’ comprises both daily and non-daily, or occasional, smokers. Note that the years for which smoking data are included are not evenly distributed. Source: Health Canada (2012 Canadian Tobacco Use Monitoring Survey), and Physicians for a Smoke-Free Canada, 2012.
1.1.2. Smoking-related diseases

1.1.2.1. General trends in prevalence and health care system burden

If current smoking trends continue, tobacco use is projected to kill one billion people during the 21st century (Jha, Ramasundarahettige et al. 2013). Tobacco use remains the leading cause of death from non-communicable diseases (Jha and Peto 2014). Tobacco use contributes to deaths from six of the eight leading causes of mortality, including lung and bronchial cancers, tuberculosis, chronic obstructive pulmonary disease (COPD), lung infection, stroke, and heart disease (Fig. 2) (WHO 2008). Smoking increases the risk of dying from bronchitis, emphysema, and airway cancer 17- and 12-fold in men and women, respectively (USDHHS 2014). Each year, cigarette smoking results in ~$100 billion in medical care costs. Smoking also results in substantial productivity losses, costing an additional ~$100 billion annually in the United States alone (CDC 2008; CDC 2012b). In Canada, it is estimated that the total economic cost of tobacco use is ~$17 billion per year (CCSA 2006).

[Image: Figure 2 | Tobacco use contributes to deaths from six of the eight leading causes of death globally. The darker shade in each bar represents the proportion of mortality from that cause that is attributable to tobacco use. Modified from the World Health Organization (WHO) report on the global tobacco epidemic, 2008. TB, tuberculosis; COPD, chronic obstructive pulmonary disease.]
1.1.2.2. Lung cancer

Cigarette smoke contains over 4,000 chemicals, more than 60 of which are known or suspected carcinogens, including polycyclic aromatic hydrocarbons, N-nitrosamines, and aromatic amines (Hecht 2002). The causative link between cigarette smoking and lung cancer, the leading cause of cancer-related mortality (Hecht 2003), was first recognized in the 1950s (Doll and Hill 1954). Over 85% of lung cancer cases are attributable to smoking, and the risk for smoking-related lung cancer increases with a younger age at smoking initiation, longer smoking duration, and greater smoking quantity (Hegmann, Fraser et al. 1993). Each year in the United States, lung cancer causes >50,000 and >70,000 deaths in female and male smokers, respectively (USDHHS 2014). In Canada, the collective number of lung cancer deaths in men and women was estimated to be 20,500 in 2014 (CCS 2014). Never-smokers that are regularly exposed to second-hand smoke are also at elevated risk of lung cancer compared to never-smokers with minimal exposure to second-hand smoke (Humble, Samet et al. 1987; Hackshaw, Law et al. 1997). Lung cancer from second-hand smoke exposure results in >7,000 deaths in American women and men each year (USDHHS 2014). Smoking also increases the risk for developing other cancers, including cancers of the mouth, throat, larynx, esophagus, stomach, pancreas, bladder, cervix, colon, and rectum (Hecht 2002).

The relative risk for smoking-related lung cancer varies according to ethnicity, with the highest risk occurring in African Americans and Native Hawaiians relative to Caucasian, Japanese, and Hispanic Americans (Haiman, Stram et al. 2006). The elevated risk for lung cancer in African Americans was observed at a variety of levels of daily cigarette consumption, including ≤10 cigarettes, 11-20 cigarettes, and 21-30 cigarettes (Haiman, Stram et al. 2006). The differences in lung cancer risk are unlikely to be attributable to heavier smoking among African Americans, as African American smokers typically smoke fewer cigarettes compared to Caucasian smokers, and the elevated risk for lung cancer remained significant after controlling for cigarette consumption (Haiman, Stram et al. 2006). However, African Americans may smoke cigarettes more intensively (Perez-Stable, Herrera et al. 1998); whether this contributes to their elevated rates of lung cancer remains to be determined.
1.1.2.3. Cardiovascular disease

Cigarette smoking is associated with peripheral vascular disease, stroke, aortic aneurysm, and myocardial infarction (Bonita, Duncan et al. 1999; Burns 2003; Mahonen, McElduff et al. 2004). The risk of dying from coronary heart disease is increased ~5-fold in smokers, with coronary heart disease causing 100,000 smoking-related deaths each year in the United States alone (USDHHS 2014). An additional ~25,000 smokers die annually from other forms of heart disease including rheumatic and pulmonary heart disease (USDHHS 2014). As with lung cancer, the risk for cardiovascular disease is positively associated with the heaviness and duration of smoking (Miettinen, Neff et al. 1976; Al-Delaimy, Manson et al. 2002; Burns 2003; Bjartveit and Tverdal 2005); even smokers consuming fewer than five cigarettes per day appear to be at increased risk for coronary heart disease (Rosengren, Wilhelmsen et al. 1992). Passive exposure to cigarette smoke also increases the risk for coronary heart disease in a dose and duration-dependent manner (He, Vupputuri et al. 1999; Kavey, Daniels et al. 2003).

Cigarette smoke is implicated in vascular endothelial cell dysfunction and accelerates the process of atherosclerosis (Noronha-Dutra, Epperlein et al. 1993; Celermajer, Adams et al. 1996; Raij, DeMaster et al. 2001; Bernhard, Pfister et al. 2003). Cigarette smoking may also increase the risk for cardiovascular disease through its effect on increasing central adiposity. While smokers are typically leaner than non-smokers (Albanes, Jones et al. 1987), smoking is associated with higher waist circumference and waist-to-hip ratio (Bamia, Trichopoulou et al. 2004; Canoy, Wareham et al. 2005), which are measures of abdominal visceral adipose accumulation (Pouliot, Despres et al. 1994). The risk for smoking-related abdominal obesity appears to be dose-dependent, as heavier smokers (>15 cigarettes/day) displayed a greater risk of abdominal obesity compared to lighter smokers (≤15 cigarettes/day) (Liu, David et al. 2012). Abdominal obesity, in turn, increases the risk for adverse cardiovascular outcomes. Among women, having a higher waist-to-hip ratio (≥0.88) was associated with a greater risk (relative risk = 3.3) of developing coronary heart disease relative to a lower waist-to-hip ratio (<0.72), after controlling for BMI and other coronary heart disease risk factors (Rexrode, Carey et al. 1998). Thus, cigarette smoking is likely
to contribute to the risk for cardiovascular disease through at least two mechanisms, via its effects on vascular endothelial dysfunction and central adiposity.

1.1.2.4. Lung disease

Cigarette smoking is also an important risk factor for COPD (Buist, McBurnie et al. 2007), and is responsible for ~80% of deaths from COPD (CDC 2008). Each year in the United States, ~100,000 smokers, comprising approximately equal numbers of men and women, die from COPD (USDHHS 2014). Much of the variation in the national prevalence of COPD can be explained by differences in smoking (Buist, McBurnie et al. 2007). The global prevalence of COPD among adults aged 40 years and older is estimated to be 10%, and is expected to rise as the population ages and as the prevalence of smoking increases in developing countries (Halbert, Natoli et al. 2006; Buist, McBurnie et al. 2007). Smoking is also associated with the risk for bronchitis and pneumococcal disease. In a large cohort of American women followed for ten years, current smokers displayed a greater risk (relative risk = 2.9) for developing chronic bronchitis than never-smokers; the risk increased with increasing daily cigarette consumption (Troisi, Speizer et al. 1995). In a sample of immunocompetent adults in the United States, 58% of patients with invasive pneumococcal disease were current cigarette smokers, compared to only 24% of controls (Nuorti, Butler et al. 2000). Similar to the risk for developing chronic bronchitis (Troisi, Speizer et al. 1995), an increasing number of cigarettes smoked per day was positively associated with the risk for pneumococcal disease (Nuorti, Butler et al. 2000).

Passive smoke exposure also contributes to the development of lung disease, particularly in children. Children with one or more smoking parents are more likely to be diagnosed with asthma than children with parents who do not smoke (Coultas 1998). In addition, smoking by one or more parents is associated with an increased incidence of bronchitis and pneumonia in children in the first year of life (Colley, Holland et al. 1974). However, the recent increase in the prevalence of home smoking bans (Mills, White et al. 2011) will likely reduce the overall impact of passive smoke exposure on child and adolescent lung disease.
1.1.3. Adolescent smoking

1.1.3.1. Prevalence of adolescent smoking

Estimates of the global prevalence of youth smoking have been made possible by the Global Youth Tobacco Survey, a joint effort between the United States Centers for Disease Control and Prevention (CDC), the WHO, the Canadian Public Health Association, and other WHO members (Warren, Jones et al. 2006). Between 1999 and 2005, approximately 750,000 students aged 13 to 15 years participated (Warren, Jones et al. 2006). Overall, current cigarette smoking was reported by approximately 9% of respondents, with a higher percentage of boys reporting current smoking than girls (10.5% vs. 6.7%, respectively) (Warren, Jones et al. 2006). Current smoking prevalence was highest in the Americas and Europe (~18%), and lower in the African (9.2%), Eastern Mediterranean (5.0%), Southeast Asian (4.3%) and Western Pacific (6.5%) regions (Warren, Jones et al. 2006). Outside of the Americas and Europe, the prevalence of use of other tobacco products such as chewing tobacco and snuff was similar to or higher than cigarette smoking (Warren, Jones et al. 2006).

In the United States, 16% of high school students were current smokers in 2011 (CDC 2012c). Canadian data indicate that 11% of youth aged 15-19 years old were current smokers in 2012, down from a prevalence of 29% in 1996-1997 (Health Canada 2013). Among 20-24 year olds, the prevalence of current smoking was nearly double that in 15-19 year olds, at 20% (Health Canada 2013).

1.1.3.2. Proportion of smokers beginning to smoke in youth versus adolescence

The vast majority of smoking initiation begins in youth. More than a third of adult ever-smokers report trying their first cigarette by age 14 years (CDC 2012a), with over 80% and 99% initiating smoking by the age of 18 and 26 years, respectively (CDC 2012a).

1.1.3.3. Predictors of smoking initiation
Genetic, sociodemographic, psychological, environmental, and lifestyle factors play a role in smoking initiation in adolescence (O'Loughlin, Karp et al. 2009) (Fig. 3). Heritability studies in twins estimate that approximately 40% of the risk for smoking initiation is influenced by genetic factors (Koopmans, Slutske et al. 1999; Vink, Willemsen et al. 2005); for example, genetic variation in components or modulators of the dopaminergic, serotonergic, and opioid systems potentially influences the risk for smoking initiation (Sullivan, Jiang et al. 2001; Zhang, Kendler et al. 2006; Iordanidou, Tavridou et al. 2010; Doran, Schweizer et al. 2013). Among sociodemographic factors, low socioeconomic status, poor academic performance, and single-parent household status are associated with increased odds of initiating smoking (Ellickson, McGuigan et al. 2001; Fleming, Kim et al. 2002; Macleod, Hickman et al. 2008; O'Loughlin, Karp et al. 2009). Smoking in the environment by relatives, friends, and teachers also significantly influences smoking initiation (Flay, Hu et al. 1998; Kandel, Kiros et al. 2004; O'Loughlin, Karp et al. 2009), as do stress and impulsivity (O'Loughlin, Karp et al. 2009). For lifestyle-related factors, both the use of alcohol and other tobacco products have been shown to increase the odds of smoking initiation (Scal, Ireland et al. 2003; Tercyak, Rodriguez et al. 2007; O'Loughlin, Karp et al. 2009).

Figure 3 | A number of factors influence smoking initiation. Smoking initiation in adolescence is thought to be influenced by a number of genetic, psychological, demographic, and social/lifestyle factors; several examples from each of these categories are highlighted here.
1.1.3.4. Transition to regular smoking and nicotine dependence

For many adolescents, the use of cigarettes encompasses a brief period of experimentation. However, up to 25% of adolescent initiators become dependent on nicotine, the predominant psychoactive compound in cigarette smoke (Benowitz 2010). Nearly 50% of adolescent initiators report craving to smoke (DiFranza, Savageau et al. 2007; Doubeni, Reed et al. 2010); these and other symptoms of nicotine dependence often appear within the first month following initiation and before daily smoking (Difranza 2007). Genetic factors strongly contribute to the risk for nicotine dependence and regular smoking, with heritability estimates of up to 80% (Maes, Sullivan et al. 2004; Vink, Willemsen et al. 2005). Environmental factors, including smoking by peers, are also associated with an increased risk for transitioning to regular smoking (Audrain-McGovern, Al Koudsi et al. 2007). Future smoking and nicotine dependence are also predicted by having a pleasant or positive initial smoking experience, such as relaxation, ‘rush’, or ‘buzz’ (Rodriguez and Audrain-McGovern 2004; Audrain-McGovern, Al Koudsi et al. 2007; Hu, Griesler et al. 2011). In terms of ethnic differences, Caucasian youth demonstrate the highest rates of progression to daily smoking, compared to African American and Hispanic youth (Kandel, Kiros et al. 2004). Delinquency and concern about body weight may also contribute to daily smoking among adolescents (Voorhees, Schreiber et al. 2002; Fulkerson and French 2003; Kandel, Kiros et al. 2004).

1.1.3.5. Smoking prevention strategies and federal initiatives

Although the majority (>60%) of adolescents experiment with smoking (Kobus 2003), cognitive behavioural programs may provide some efficacy in preventing adolescent smoking initiation. A three-year school-based cognitive behavioural program called “Life Skills Training” implemented in seventh graders resulted in significantly less cigarette smoking (Botvin, Baker et al. 1990). The program focuses on personal and social development, including boosting self-esteem, effective communication skills, asserting one’s rights, and managing social influences that encourage drug use (Botvin, Baker et al. 1990). Students receiving training also displayed weaker normative beliefs concerning adult smoking, and higher interpersonal skills scores (Botvin, Baker et al. 1990); programs that facilitate the development of these skills in
adolescents are likely to result in a lower prevalence of youth smoking (Conrad, Flay et al. 1992), presumably leading to lower smoking rates overall.

In 2009, the United States congress passed the Family Smoking Prevention and Tobacco Control Act, which gave the FDA the power to regulate tobacco products (CDC 2012a). This Act bans the sale of cigarettes with flavourings that appeal to youth (e.g., “candy-like” flavours) (CDC 2012a); these are used disproportionately by adolescent smokers (Klein, Giovino et al. 2008). In addition, the Act prohibits the sale of single cigarettes (CDC 2012a), which may be an effective deterrent of smoking in price-sensitive populations, particularly youth, as teen smokers are estimated to be up to three times more sensitive to cigarette price compared to adults (Chaloupka, Cummings et al. 2002). The Act also calls for graphic warning labels on tobacco products, as well as prohibits tobacco companies from marketing their products to children, which will likely lead to further decreases in the prevalence of tobacco use among youth (CDC 2012a).

1.2 Smoking cessation

1.2.1 Health benefits of cessation

Although the majority of current cigarette smokers express an interest in quitting (Hymowitz, Cummings et al. 1997), only 3-5% of self-quitters (i.e., no cessation aids) quit for at least 6-12 months (Hughes, Keely et al. 2004). Smoking cessation is associated with numerous health benefits, including an increased life expectancy compared to someone who keeps smoking, with the largest increase in life expectancy occurring in those who quit at younger ages (Jha, Ramasundarahettige et al. 2013). For smokers who quit between 25 and 44 years of age, the gain in life expectancy is nine to 10 years on average compared to continuing smokers (Jha, Ramasundarahettige et al. 2013). Even smokers who quit in their mid-40s to early 60s can expect to gain an average of four to six years of life compared to smokers who do not quit (Jha, Ramasundarahettige et al. 2013). Although smokers often gain weight upon quitting smoking (Klesges, Winders et al. 1997), the health risks associated with this increase in body weight do not offset the health benefits, particularly to the cardiovascular system, that result from smoking cessation (Clair, Rigotti et al. 2013).
1.2.2. Motivation to quit smoking and subsequent quit attempts

1.2.2.1. Adults

In a multinational sample of predominantly adult smokers (mean age ~40 years), over 70% of individuals expressed a desire to stop smoking (Thyrian, Panagiotakos et al. 2008). The desire to quit was disproportionately higher in countries with stricter tobacco control policies compared to countries with weaker policies (Thyrian, Panagiotakos et al. 2008); possessing a strong desire to quit smoking is associated with an increased likelihood of successful cessation (Hymowitz, Cummings et al. 1997; Osler and Prescott 1998). Often the primary motivating factor in smoking cessation is concern regarding the negative impact of smoking on health (McCaul, Hockemeyer et al. 2006). Experiencing failed quit attempts does not appear to affect smokers’ overall motivation to quit smoking, suggesting these individuals are likely to make future attempts at smoking cessation (Boardman, Catley et al. 2005).

1.2.2.2. Adolescents

Like adults, the majority of adolescent smokers express a desire to quit smoking and make at least one quit attempt (Ershler, Leventhal et al. 1989; Stanton, Lowe et al. 1996; Bancej, O'Loughlin et al. 2007). In a systematic review of smoking cessation prevalence studies, an estimated 58%, 68%, and 71% of current adolescent smokers made a cessation attempt in the previous six months, 12 months, and ever, respectively (Bancej, O'Loughlin et al. 2007). The high prevalence of quit attempts was not restricted to older or heavier-smoking adolescents, as a large proportion of younger adolescents and lighter-smoking adolescents also made smoking cessation attempts (Bancej, O'Loughlin et al. 2007). A number of factors underlie the motivation to quit smoking in adolescence, including concerns about current and future health, physical appearance, the cost of cigarettes, and performance in athletics (Riedel, Robinson et al. 2002). Several milestones related to smoking cessation in adolescence have been identified, including possessing a strong desire to quit and awareness of the difficulty of quitting (O'Loughlin, Gervais et al. 2009).
1.2.3. Predictors of smoking cessation

As previously mentioned, only 3-5% of self-quitters (i.e., not treatment-seeking) will maintain long-term abstinence following a quit attempt (Hughes, Keely et al. 2004). Most relapse among self-quitters occurs within the first eight days following a cessation attempt (Hughes, Keely et al. 2004). In addition to being motivated to quit smoking, smoking cessation is influenced by a number of genetic and environmental factors (Fig. 4); twin studies in adults suggest a substantial (~50%) proportion of the ability to quit smoking is heritable (Xian, Scherrer et al. 2003; Broms, Silventoinen et al. 2006). Variation in genes encoding enzymes and receptors involved in nicotine pharmacology (Gu, Hinks et al. 2000; Budulac, Vonk et al. 2012; Chen, Bloom et al. 2014), as well as in genes encoding components of the dopaminergic system (Han, Joe et al. 2008; Omidvar, Stolk et al. 2009), among others, has been shown to be associated with smoking cessation. A number of the environmental and/or demographic factors that influence smoking cessation are discussed in more detail below.

Figure 4 | A number of factors influence smoking cessation success. The ability to quit smoking is influenced by a number of genetic, demographic, and smoking-related factors, as well as treatment; examples of factors from each of these categories are highlighted here.
1.2.3.1. Level of nicotine dependence and cigarette consumption

In general, lower levels of cigarette consumption and nicotine dependence are associated with an increased likelihood of smoking cessation (Cohen, Lichtenstein et al. 1989; Ferguson, Patten et al. 2003). For example, adults smoking ≤20 cigarettes/day were ~2 times more likely to quit relative to their heavier smoking (i.e., >20 cigarettes/day) counterparts (Cohen, Lichtenstein et al. 1989), and a higher proportion of smokers with Fagerstrom Test for Nicotine Dependence scores ≤5 achieved 6-month tobacco abstinence compared to those with scores of 6 or higher (41 vs. 30%, respectively) (Ferguson, Patten et al. 2003).

1.2.3.2. Age of initiation

Age of initiation is also associated with smoking cessation success; adult smokers who began smoking at a younger age have a greater difficulty quitting (Breslau and Peterson 1996; Hymowitz, Cummings et al. 1997; Chen and Millar 1998). Among individuals who initiated smoking at age 13 or younger, only 18% quit smoking within ten years of initiation, compared to 42% of individuals that began smoking at 20 years of age or older (Chen and Millar 1998). In a separate study of adult smokers followed for five years, 21% of those who began at age 15 or younger had quit by the end of follow-up, compared to 28% of those who began smoking at age 20 or older (Hymowitz, Cummings et al. 1997).

1.2.3.3. Sex

Smoking cessation is typically lower in adult women compared to men (Kabat and Wynder 1987; Bjornson, Rand et al. 1995; Perkins 2001). Female adolescents are also less likely to discontinue smoking relative to male adolescents (O'Loughlin, Sylvestre et al. 2014). Compared to adult men, women report a lower confidence in quitting smoking, are less likely to realize the health benefits of quitting smoking, and are more concerned about gaining weight after quitting; these factors may contribute to the lower cessation rates among women (Sorensen and Pechacek 1987; Audrain, Gomez-Caminero et al. 1997). Among women, cigarette craving, withdrawal effects, and depression scores appear to be higher in the luteal phase of the menstrual cycle.
(Perkins, Levine et al. 2000; Carpenter, Upadhyaya et al. 2006). Smoking cessation in women also appears to be influenced, in part, by menstrual cycle phase, although these findings are not consistent across all studies. Women in the follicular phase (pre-ovulation) have higher cessation rates compared to women in the luteal phase (post-ovulation) in some (Allen, Bade et al. 2008; Mazure, Toll et al. 2011) but not all (Craig, Parrott et al. 1992) studies.

1.2.3.4. Ethnicity

In addition to sex differences in smoking cessation success, ethnic disparities exist. Despite reporting a higher desire to quit smoking (Royce, Hymowitz et al. 1993), African Americans have lower quit ratios, which is the proportion of former smokers among ever smokers, compared to Caucasians (Stahre, Okuyemi et al. 2010). Among adult ever-smokers (≥100 cigarettes lifetime consumption) making a quit attempt for at least one day, a lower proportion of African American smokers (32%) remained abstinent for 30 days compared to Hispanic/Latino (51%), Asian American/Pacific Islander (50%), and Caucasian (42%) smokers (Trinidad, Perez-Stable et al. 2011). Moreover, African American smokers were approximately half as likely to remain abstinent for six months, after adjusting for age, gender, income, educational attainment, and smoking within 30 minutes of waking (Trinidad, Perez-Stable et al. 2011).

In both adolescence and adulthood, a larger proportion of African American smokers use mentholated cigarettes compared to Caucasian smokers (~70% vs. ~22%, respectively) (Giovino, Sidney et al. 2004; Wackowski and Delnevo 2007). Differences in the use of mentholated cigarettes may explain some of the inter-ethnic variability in cessation success. Quit ratios are lower among African American smokers using mentholated cigarettes compared to those using non-mentholated cigarettes (34% vs. 49%, respectively) (Stahre, Okuyemi et al. 2010). Although menthol is associated with greater dependence symptoms in smokers (Collins and Moolchan 2006; Wackowski and Delnevo 2007), a lower proportion of African American (vs. Caucasian) youth that experiment with cigarettes become regular smokers, suggesting many quit in adolescence (Ellickson, Orlando et al. 2004). Thus, the reasons for the ethnic disparities in smoking cessation success remain to be clarified.
1.2.3.5. Socioeconomic and marital status

Socioeconomic status, approximated by educational attainment, income level, and/or social class, is also associated with smoking cessation success. These variables are each positively associated with smoking cessation success, such that greater educational attainment and wealth are associated with increased quit rates (Gilman, Abrams et al. 2003; Broms, Silventoinen et al. 2004; van Loon, Tijhuis et al. 2005). Being married versus single also positively predicts smoking cessation, especially among males (Broms, Silventoinen et al. 2004; van Loon, Tijhuis et al. 2005). However, having a spouse that smokes is negatively associated with smoking cessation success (Homish and Leonard 2005). Similarly, in adolescence, smoking by romantic partners positively predicts smoking continuation and escalation in smoking behaviour (Branstetter, Horn et al. 2009).

1.2.4. Pharmacological approaches to smoking cessation

Currently there are three types of FDA-approved medications to treat nicotine dependence: nicotine replacement therapy (Henningfield 1995), bupropion (Hurt, Sachs et al. 1997), and varenicline (Faessel, Obach et al. 2010). These treatments increase quit rates compared to placebo/control (Table 1), however a large proportion of smokers that receive treatment do not experience sustained abstinence, underscoring the need for the development of new cessation medications and treatment approaches.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Abstinent</th>
<th>Relative Effect (95% CI) vs. Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRT Patch</td>
<td>26+ weeks</td>
<td>RR=1.64 (1.52, 1.78)</td>
</tr>
<tr>
<td>Gum</td>
<td>15.9</td>
<td>RR=1.49 (1.40, 1.60)</td>
</tr>
<tr>
<td>Nasal spray</td>
<td>16.3</td>
<td>RR=2.02 (1.49, 2.73)</td>
</tr>
<tr>
<td>Inhaler</td>
<td>23.9</td>
<td>RR=1.90 (1.36, 2.67)</td>
</tr>
<tr>
<td>Tablets/Lozenges</td>
<td>17.1</td>
<td>RR=1.95 (1.61, 2.36)</td>
</tr>
<tr>
<td>Bupropion 26 weeks</td>
<td>26+ weeks</td>
<td>RR=1.59 (1.44, 1.76)</td>
</tr>
<tr>
<td>Bupropion 26 weeks</td>
<td>21.9</td>
<td>RR=1.69 (1.45, 1.97)</td>
</tr>
<tr>
<td>Bupropion 52 weeks</td>
<td>18.9</td>
<td></td>
</tr>
<tr>
<td>Varenicline 26+ weeks</td>
<td>25.6</td>
<td>OR=2.41 (1.91, 3.12)</td>
</tr>
</tbody>
</table>

Abbreviation: NRT, nicotine replacement therapy; RR, risk ratio; OR, odds ratio; CI, confidence interval

References: 1 (Stead, Perera et al. 2012); 2 (Hughes et al., 2014); 3 (Eisenberg, Filion et al. 2008)
1.2.4.1. Nicotine replacement therapy

The postulated mechanism of action of nicotine replacement therapy (NRT) in aiding smoking cessation is via the reduction of withdrawal and craving symptoms associated with cessation of cigarette smoking (Stead, Perera et al. 2012). NRT comes in various forms, including nicotine patch, nasal spray, vapour, lozenge, and gum (Henningfield 1995). In a Cochrane review of 150 smoking cessation studies involving NRT, 149 of which recruited adult smokers, NRT was demonstrated to increase quit rates by 50-70% compared to placebo; little difference in efficacy across various forms of NRT was observed (Stead, Perera et al. 2012) (Table 1). NRT also increases the likelihood of cessation in the absence of intensive behavioural counseling (Stead, Perera et al. 2012). Compared to cigarette smoking, the nicotine patch is a relatively slow nicotine delivery system, and average plasma nicotine levels on patch are similar to trough levels found in heavy smokers (Stead, Perera et al. 2012). An enhanced benefit of dual therapy with the nicotine patch and a comparatively faster nicotine delivery system (e.g., spray) is observed compared to patch alone (Stead, Perera et al. 2012). However, long-term quit rates on the nicotine patch remain low, at approximately 21% and 16% at 6-months and 12-months, respectively (Fiore, Smith et al. 1994; Jorenby, Leischow et al. 1999). Continuous abstinence rates at 12-months on the nicotine patch are even lower, at approximately 10% (Jorenby, Leischow et al. 1999).

The efficacy of NRT in promoting smoking cessation has also been tested in adolescent smokers (Moolchan, Robinson et al. 2005). Adolescents aged 13 to 17 years that smoked at least 10 cigarettes per day were randomized to placebo, nicotine patch (21 mg nicotine) or nicotine gum (2 or 4 mg nicotine) for 12 weeks, and all received cognitive behavioural therapy (Moolchan, Robinson et al. 2005). End-of-treatment abstinence rates were significantly higher in those receiving patch (18%) compared to placebo (2.5%). The abstinence rate in those receiving nicotine gum was 6.5% and was not significantly different from patch or placebo (Moolchan, Robinson et al. 2005). Active patch and gum treatments were well tolerated, and rates of adverse events were similar to those reported in clinical trials in adults.

However, NRT may be less effective in women than in men. In a meta-analysis of placebo-controlled smoking cessation trials, the odds ratio for long term (6-months) quitting on patch (vs.
placebo) was 1.6 in women, compared to 2.2 in men. There was a significant interaction between sex and treatment arm on smoking cessation (odds ratio = 1.4; P = 0.04), which suggested that women derive less benefit from the nicotine patch than men (Perkins and Scott 2008). The poorer quit rates among women on NRT may be due to their smoking more for reasons unrelated to nicotine (Perkins 1999; Perkins, Donny et al. 1999). During a quit attempt, male, but not female, smokers self-administer more nicotine nasal spray compared to placebo nasal spray (Perkins 1999); these data suggest that women may not smoke to avoid withdrawal, and/or derive less reinforcement from nicotine itself, potentially lowering their cessation success on NRT (Perkins, Donny et al. 1999). Women may also be more likely than men to express concern about gaining weight upon smoking cessation (Pirie, Murray et al. 1991), although male and female smokers on NRT (nicotine nasal spray) appear to gain a similar amount of body weight upon cessation (Sutherland, Stapleton et al. 1992). Moreover, continued use of nicotine nasal spray significantly attenuated abstinence-induced weight gain at 12 months (3 kg vs. 6 kg weight gain in nicotine nasal spray vs. placebo spray group, respectively); this attenuation occurred to a similar degree in male and female smokers (Sutherland, Stapleton et al. 1992). These data suggest that NRT may reduce some of the weight gain that occurs in both men and women who effectively quit smoking, which may be an important counseling point for smokers concerned about post-cessation weight gain.

1.2.4.2. Bupropion

Bupropion was the first drug developed to treat tobacco dependence that is not a nicotine replacement aid (Warner and Shoaib 2005). The ability of bupropion to promote smoking cessation is thought to stem from its antagonist activity at the nicotinic acetylcholine receptor (nAChR) including the α4β2 subtype that is responsible for the development of nicotine dependence (Slemmer, Martin et al. 2000). Bupropion can also bind to the dopamine transporter in humans, which is consistent with its additional postulated role in dopamine reuptake inhibition (Learned-Coughlin, Bergstrom et al. 2003).

Bupropion is also a widely used antidepressant, however its efficacy in smoking cessation is not restricted to patients with depression (Hurt, Sachs et al. 1997). The ability of antidepressants to
promote smoking cessation is thought to result from the alleviation of depressive symptoms that are associated with smoking abstinence (Hughes, Stead et al. 2014). Antidepressants may also substitute for the antidepressant effects of smoking and could modulate neural circuits associated with nicotine dependence (Hughes, Stead et al. 2014).

Sustained-release bupropion is associated with a dose-dependent increase in smoking abstinence at end-of-treatment (Hurt, Sachs et al. 1997). Quit rates were 19% in the placebo group, compared to 29%, 39%, and 44% in those receiving 100, 150, and 300 mg bupropion, respectively (Hurt, Sachs et al. 1997). As for the nicotine patch, quit rates on bupropion decline over time, and aggregate data from a Cochrane review indicate that the abstinence rate on bupropion at six and 12 months is 22% and 19%, respectively (Hughes, Stead et al. 2014) (Table 1). Bupropion is well tolerated, and, like NRT, reduces some of the weight gain that occurs with smoking cessation (Hurt, Sachs et al. 1997; Farley, Hajek et al. 2012). A combined treatment approach may further attenuate smoking cessation induced weight gain; treatment with both the nicotine patch and bupropion was associated with lower weight gain (1.1 kg) compared to either treatment alone (1.6-1.7 kg) or placebo (2.1 kg) (Jorenby, Leischow et al. 1999).

Although few clinical studies have been conducted in adolescents compared to adults, bupropion appears to be modestly effective at promoting smoking cessation in young smokers (Muramoto, Leischow et al. 2007). Abstinence rates at end-of-treatment were higher in adolescents receiving 300 mg bupropion compared to placebo (15% vs. 6%, respectively) (Muramoto, Leischow et al. 2007). In contrast, no benefit of bupropion over placebo was observed at a lower bupropion dose (150 mg), and the overall quit rates on bupropion and placebo appear to be lower than those reported for adult smokers (Hurt, Sachs et al. 1997; Muramoto, Leischow et al. 2007). A separate trial tested the efficacy of bupropion and contingency management for adolescent smoking cessation (Gray, Carpenter et al. 2011). At end-of-treatment, neither bupropion nor contingency management alone improved the odds of abstinence over their respective controls (Gray, Carpenter et al. 2011). In contrast, among those receiving bupropion, contingency management increased the odds of abstinence approximately 4-fold, suggesting an effect of contingency management on cessation with concurrent bupropion treatment (Gray, Carpenter et al. 2011). In a clinical trial examining the efficacy of co-treatment with bupropion and nicotine patch,
bupropion did not improve quit rates over patch alone in adolescents (Killen, Robinson et al. 2004). Overall, the available clinical trial data suggest at best a minor effect of bupropion in promoting adolescent smoking cessation.

1.2.4.3. Varenicline

Varenicline, the most recent pharmacological aid approved for the treatment of nicotine dependence, is also the most efficacious in promoting smoking cessation; at end-of-treatment, abstinence rates are higher on varenicline compared to bupropion (Jorenby, Hays et al. 2006) and the nicotine patch (Aubin, Bobak et al. 2008). From a meta-analysis of clinical studies involving smoking cessation pharmacotherapy, the overall quit rate in smokers receiving varenicline was ~26% at ≥6-months follow-up (Eisenberg, Filion et al. 2008) (Table 1). Varenicline acts as a partial agonist of the \( \alpha_4\beta_2 \) nAChR, and attenuates nicotine-mediated responses due to its higher affinity (vs. nicotine) for \( \alpha_4\beta_2 \) receptors (Coe, Brooks et al. 2005). Thus, varenicline likely works through two mechanisms to promote smoking cessation. In the absence of nicotine (i.e., during a quit attempt), varenicline provides some relief of withdrawal and craving symptoms via its partial agonist activity. Should the smoker lapse, however, varenicline, with its superior affinity for \( \alpha_4\beta_2 \) nAChRs, would reduce nicotine binding and nicotine-evoked effects (Faessel, Obach et al. 2010).

Varenicline displays a highly favourable pharmacokinetic profile; its pharmacokinetic properties are consistent regardless of smoking status, food intake, age, sex, and ethnicity (Faessel, Obach et al. 2010). The majority of a varenicline dose is excreted as unchanged varenicline in the urine (Obach, Reed-Hagen et al. 2006). Like NRT and bupropion, varenicline reduces post-cessation weight gain at end-of-treatment (Farley, Hajek et al. 2012). The effect of bupropion on limiting weight gain is larger than that of varenicline, while NRT and varenicline attenuate post-cessation weight gain to a similar degree (Farley, Hajek et al. 2012). However, none of these pharmacological aids appear to have a lasting effect on reducing weight gain at six or 12 months after quitting (Farley, Hajek et al. 2012). The efficacy and safety of varenicline for treating nicotine dependence in adolescents is currently being investigated in a placebo-controlled clinical trial, with expected completion in 2017 (NCT01509547).
1.2.4.4. Novel strategy in nicotine dependence therapy development

The classical drug development pipeline takes ~14 years, from pre-clinical testing through to gaining FDA approval, and costs up to $2 billion (Perkins and Lerman 2011). Despite the extensive screening and testing involved, <10% of new compounds reaching phase I clinical testing gain FDA approval, with a large proportion (>33%) of drugs failing in Phase III (Perkins and Lerman 2011; Perkins and Lerman 2014). In the process of developing new drugs to treat addiction, a medication screening approach has recently been proposed and involves early phase II efficacy screening of new compounds (Perkins and Lerman 2011). This approach, incorporated into existing drug development paradigms, could save millions of dollars and years of development in instances where drugs do not show early efficacy (Perkins and Lerman 2011).

Early efficacy screening, which utilizes a within-subjects cross-over design, is ideally performed in smokers with a high interest in quitting (Perkins and Lerman 2014). The number of days of abstinence on the medication(s) of interest compared to placebo is assessed during separate week-long quit attempts (Perkins and Lerman 2014). The validity of this approach was recently tested using existing smoking cessation medications, where varenicline, nicotine patch, and bupropion each significantly increased the number of days of abstinence compared to placebo within participants with high quit interest (Perkins and Lerman 2014). In contrast, modafinil, a drug that does not promote smoking cessation (i.e., a negative control), did not increase the number of days of abstinence (vs. placebo), demonstrating the specificity of this paradigm; it is equally important to be able to identify compounds that should not undergo further clinical investigation (Perkins and Lerman 2014). Successful early abstinence was recently shown to be associated with prolonged abstinence in separate clinical trials involving the nicotine patch, NRT, and bupropion (Ashare, Wileyto et al. 2013). Among smokers that received nicotine patch therapy, those that did not lapse at all during the first week following the target quit date were more likely to be abstinent at end-of-treatment and at six months relative to smokers who lapsed (odds ratio = 7.4 and 4.8, respectively) (Ashare, Wileyto et al. 2013). Each additional day of abstinence during the first week increased the likelihood of abstinence at end-of-treatment and six months by 1.6- and 1.4-fold, respectively, as reflected by higher quit rates in those with a
higher number of consecutive days of abstinence in the first week (Fig. 5) (Ashare, Wileyto et al. 2013). Similar effects were noted in NRT and bupropion clinical trials, where no lapse during the first seven days was associated with an increase in the likelihood of abstinence at end-of-treatment (odds ratios ~ 9) (Ashare, Wileyto et al. 2013). Thus, medication screening approaches that evaluate early abstinence may show promise as adjuncts to more formal clinical testing paradigms that evaluate longer term abstinence.

Figure 5 | A higher number of days of consecutive abstinence in the first week following a quit attempt is associated with higher quit rates at end-of-treatment and six months in smokers that received standard or extended nicotine patch therapy. Data from Ashare et al., 2013.

1.2.5. Non-pharmacological approaches to smoking cessation and electronic cigarettes

A number of non-pharmacological approaches have shown moderate efficacy in aiding smoking cessation, including behavioural therapy and Internet- and computer-based cessation programs. Behavioural therapy, especially administered in an open, flexible group format over a period of several weeks, provides some benefit for smoking cessation (Hiscock, Murray et al. 2013). Behavioural and cognitive-behavioural therapy approaches center on the principle of substance use as a learned behaviour that can be modified. The goal of behavioural therapy is to understand
situations in which a person would be at high risk for relapse, and to reduce the risk for lapse or relapse through the use of effective coping strategies (Hendershot, Witkiewitz et al. 2011). In a Cochrane review, behavioural counseling was shown to produce sustained abstinence in approximately 11% of smokers, compared to 8% in control (Lancaster and Stead 2005). However, in certain smoking populations, such as patients with cancer, behavioural therapy may provide no enhanced benefit in aiding smoking cessation over basic health education (Schnoll, Rothman et al. 2005).

In adolescents, school-based cognitive behavioural counseling also aids smoking cessation, with extended courses being particularly advantageous. In adolescent smokers receiving 9-weeks of nicotine patch therapy and 10-weeks of school-based cognitive behavioural therapy group sessions, those that received an additional nine group sessions had significantly higher abstinence rates at 6-months compared to those that did not receive extended therapy (21% vs. 7%, respectively) (Bailey, Hagen et al. 2013).

Studies involving Internet- and computer-based smoking cessation programs have yielded conflicting results, however they show overall efficacy in helping adult, but not adolescent, smokers quit (Myung, McDonnell et al. 2009). In a meta-analysis of randomized controlled trials involving Internet- and computer-based approaches to smoking cessation, the relative risk for smoking cessation in nine studies involving an Internet-based approach was 1.4, and was 1.5 in 13 studies using a computer-based intervention (Myung, McDonnell et al. 2009).

The use of electronic cigarettes (i.e., e-cigarettes) is also an emerging tool with potential use for smoking cessation. However, the use of e-cigarettes to aid smoking cessation is controversial, due to toxicity concerns, potential unintended effects of promoting and normalizing smoking in adolescents, and lack of adequate efficacy testing. Regarding toxicity, the level of toxicants and carcinogens in e-cigarette vapour was shown to be up to 450-fold lower than in regular cigarettes, suggesting that e-cigarettes are a comparatively safer nicotine-delivery system (Goniewicz, Knysak et al. 2014). In adolescents, the use of e-cigarettes was associated with a greater likelihood of concurrent cigarette smoking (Delnevo, Manderski et al. 2014; Dutra and Glantz 2014). However, the use of other tobacco products (e.g., cigars, smokeless tobacco,
hookah) was also associated with a greater likelihood of concurrent cigarette smoking (Delnevo, Manderski et al. 2014). The prevalence of past 30-day e-cigarette use (2.0%) was also lower compared to other tobacco products, including cigarettes (9.4%), cigars (8.4%), smokeless tobacco (4.4%), and hookah (3.5%) (Delnevo, Manderski et al. 2014). To date, there is a scarcity of observational studies that evaluate the effectiveness of e-cigarettes for smoking cessation, and even fewer controlled clinical trials (McRobbie, Bullen et al. 2014). In one randomized-controlled clinical trial in adult smokers, there was a trend toward a higher rate of point prevalence abstinence at one and six months in those treated with nicotine-containing e-cigarettes compared to the nicotine patch; in contrast, there was no difference in abstinence rates between those receiving nicotine-containing e-cigarettes and placebo e-cigarettes (Bullen, Howe et al. 2013).

1.3. Nicotine pharmacokinetics

1.3.1. Nicotine absorption and distribution

The major psychoactive compound in cigarette smoke, nicotine, is a natural alkaloid derived from the Nicotiana tabacum and Nicotiana rustica plants (Dawson and Solt 1959). Nicotine is a weakly basic drug with a pKa of ~8 (Hukkanen, Jacob et al. 2005). Once tobacco smoke reaches the alveoli of the lungs, about 80-90% of nicotine is rapidly absorbed (Hukkanen, Jacob et al. 2005); the amount of nicotine absorbed per cigarette averages 1 mg (Benowitz and Jacob 1984). Following the smoking of a single cigarette, plasma levels of nicotine rise to reach a maximum level of ~15 ng/ml within 5-10 minutes (Benowitz 1988). Buccal absorption of nicotine can also occur following the use of smokeless tobacco products including snus and chewing tobacco; the pH of these products is increased to facilitate absorption (Benowitz, Jacob et al. 1987). Nicotine-containing lozenges, the application of transdermal nicotine patches, and the use of nicotine-containing nasal spray facilitate nicotine absorption via additional routes including the gastrointestinal tract, skin, and nasal mucosa, respectively (Hukkanen, Jacob et al. 2005). Nicotine readily distributes to a number of tissues, including liver, lung, and kidney, but less so to adipose tissue (Urakawa, Nagata et al. 1994). Following cigarette smoking, nicotine reaches the brain in less than 10 seconds (Rose, Mukhin et al. 2010), contributing to rapid reinforcement from cigarette smoking.
1.3.2. Nicotine metabolism and clearance

Nicotine undergoes predominantly metabolic (i.e., non-renal) clearance (Hukkanen, Jacob et al. 2005). Due to the extensive metabolism of nicotine, less than 10% of a nicotine dose is excreted as unchanged nicotine in the urine (Hukkanen, Jacob et al. 2005). The majority (~80%) of nicotine is metabolized to cotinine, in a reaction principally (>90%) catalyzed by hepatic CYP2A6 in a two-step reaction involving aldehyde oxidase (Hukkanen, Jacob et al. 2005). Nicotine can also undergo phase II glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes to nicotine N-glucuronide (Hukkanen, Jacob et al. 2005). Additional minor pathways of nicotine metabolism involve its conversion to nornicotine and nicotine N-oxide, occurring largely via CYP2B6 and flavin-containing monooxygenase (FMO)-3, respectively (Hukkanen, Jacob et al. 2005).

Cotinine is also extensively metabolized, albeit at a slower rate, to a variety of compounds including 3’hydroxycotinine, cotinine N-glucuronide, norcotinine, and cotinine N-oxide (Hukkanen, Jacob et al. 2005). The first of these metabolites, 3’hydroxycotinine, is formed exclusively via CYP2A6 metabolism, and comprises up to 40% of a nicotine dose as measured in the urine of smokers (Hukkanen, Jacob et al. 2005). Major pathways of nicotine and cotinine metabolism are shown in Fig. 6.
1.3.3. Age-related differences in nicotine pharmacokinetics

To date, no age-related differences in CYP2A6 activity have been detected between youth and adults, suggesting the rate of nicotine metabolism is likely similar across these age groups. In a sample of adult smokers aged 18-70 years that received nicotine patch therapy, plasma concentrations of either nicotine or cotinine derived from the patch did not vary as a function of age (Gourlay, Benowitz et al. 1997). In a post-mortem study of 67 human livers whose donors ranged in age from 2-64 years, there was no association between donor age and either CYP2A6 protein levels or the in vitro rate of nicotine metabolism to cotinine (Al Koudsi, Hoffmann et al. 2010). In a separate study, there were no differences in CYP2A6 activity in vitro according to age group (<20 vs. 20-60 vs. >60 years) in liver microsomes from 150 donors (Parkinson, Mudra et al. 2004). Finally, age (range 2-89 years) was not correlated with hepatic CYP2A6 protein
levels in 28 autopsy samples (Baker, Satarug et al. 2001). Collectively the findings from human liver studies suggest there is no relationship between age, from 2 years through to adulthood, and CYP2A6 protein level or activity in vitro.

Age-related differences in nicotine pharmacokinetics do exist in neonates and the elderly, compared to adolescents and adults. In human neonates, the half-life of nicotine is approximately 3-4-fold longer than in adults (Dempsey, Jacob et al. 2000). In contrast, there is no difference in the half-life of cotinine between neonates and adults (Dempsey, Jacob et al. 2000; Dempsey, Sambol et al. 2013). The age-related difference in the half-life of nicotine, a high extraction drug, but not cotinine, a lower extraction drug, suggests potential differences in hepatic blood flow in neonates which would likely have a comparatively greater effect on the clearance of nicotine (vs. cotinine) (Dempsey, Jacob et al. 2000). Slower nicotine clearance is also observed in elderly individuals compared to younger adults (Molander, Hansson et al. 2001). The total, metabolic, and renal clearances of nicotine are reduced by 23%, 21%, and 49%, respectively, among elderly individuals (aged 65-75 years) compared to young adults (aged 22-34 years) (Molander, Hansson et al. 2001). Similarly, coumarin metabolism is reduced in the elderly (Sotaniemi, Lumme et al. 1996). These effects are likely mediated by physiological changes associated with aging such as decreased liver mass, liver blood flow, glomerular filtration rate, and renal tubular function (Rowe, Andres et al. 1976; Wynne, Cope et al. 1989; Klotz 2009), as CYP2A6 protein and activity levels (measured per unit protein) do not appear to change with advancing age in vitro (Baker, Satarug et al. 2001; Parkinson, Mudra et al. 2004; Al Koudsi, Hoffmann et al. 2010). Together the findings suggest that the rate of nicotine clearance is slower in neonates and the elderly compared to other ages, but that CYP2A6 activity and nicotine clearance is similar in adolescents and adults.
1.4. Genetic variability in the rate of nicotine metabolism

1.4.1. CYP2A6

1.4.1.1. Overview of CYP2A6 genetic variation

The CYP2A6 gene is highly polymorphic and consists of nine exons and eight introns (Mwenifumbo and Tyndale 2007). Transcription of the CYP2A6 gene yields an mRNA transcript specifying a CYP2A6 protein with 494 amino acids (Yamano, Nagata et al. 1989). As of March 2015, 45 numbered CYP2A6 alleles have been discovered (http://www.cypalleles.ki.se/cyp2a6.htm). Much of the CYP2A6 allelic variation can be attributable to single nucleotide polymorphisms (SNPs), as well as nucleotide insertions and deletions. CYP2A6 gene duplications, conversions, and hybrid alleles have also been described (Mwenifumbo and Tyndale 2007). The CYP2A7 pseudogene shares 95% nucleotide identity with CYP2A6 (Hoffman, Nelson et al. 2001). The high homology and close proximity of CYP2A6 and CYP2A7 is thought to have contributed to allelic variation in CYP2A6 (Oscarson, McLellan et al. 2002; Mwenifumbo, Al Koudsi et al. 2008). For example, the CYP2A6*31 allele results from possession of the CYP2A7 reference nucleotide sequence at that location (Mwenifumbo, Al Koudsi et al. 2008), and CYP2A6*12, a CYP2A6-CYP2A7 hybrid allele, has likely arisen from unequal crossover between CYP2A6 and CYP2A7 during meiosis (Oscarson, McLellan et al. 2002).

1.4.1.2. Frequency and impact of CYP2A6 alleles

The frequency of CYP2A6 variant allele expression varies according to ethnicity (Tanner, Chenoweth et al. 2015). For example, in Caucasian populations, the CYP2A6 variant alleles with relatively high frequencies of expression that lead to lower CYP2A6 activity are CYP2A6*2, CYP2A6*4, CYP2A6*9, and CYP2A6*12; the allele frequencies range from ~1% for CYP2A6*4 to ~8% for CYP2A6*9 (Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Audrain-McGovern, Al Koudsi et al. 2007; Tanner, Chenoweth et al. 2015). The frequencies of CYP2A6*4 and CYP2A6*9 are much higher in East Asians relative to Caucasians, at 11-24% and 16-20%, respectively. In contrast to its relatively high expression in Caucasians (~1-5%), CYP2A6*2 is thought to have lower expression in East Asian populations. Consistent with its
low expression in Caucasians, CYP2A6*12 is also expressed at low frequencies in East Asian populations (≤1%) (Mwenifumbo and Tyndale 2007).

Each of the CYP2A6*2, CYP2A6*4, CYP2A6*9, and CYP2A6*12 alleles has a detrimental impact on CYP2A6 activity, which varies in magnitude. CYP2A6*2 and CYP2A6*4 both lead to a complete loss of CYP2A6 function; CYP2A6*2 is a SNP in exon 3 of CYP2A6 that prevents heme from being incorporated into the enzyme (Yamano, Tatsuno et al. 1990), while CYP2A6*4 leads to a deletion of the CYP2A6 gene (Mwenifumbo, Zhou et al. 2010). Following an oral dose of nicotine (4 mg), individuals with two copies of CYP2A6*4 form less than 10% and 0% of the cotinine and 3’hydroxycotinine, respectively, that individuals with two wild type CYP2A6 alleles do (Mwenifumbo and Tyndale 2007). Individuals homozygous for the CYP2A6*4 gene deletion also form no 7’-hydroxycoumarin from an oral dose of coumarin (50 mg), another CYP2A6 substrate (Mwenifumbo and Tyndale 2007). People with one wild type CYP2A6 allele and one CYP2A6*2 or CYP2A6*4 loss-of-function allele display an intermediate level of CYP2A6 activity (Ho, Mwenifumbo et al. 2009; Binnington, Zhu et al. 2012).

The CYP2A6*9 and CYP2A6*12 alleles are associated with decreased CYP2A6 activity; CYP2A6*9 results from a SNP in the TATA box of CYP2A6, leading to a reduction in promoter activity and consequently reduced amounts of CYP2A6 mRNA and protein (Pitarque, von Richter et al. 2001; Kiyotani, Yamazaki et al. 2003), while the CYP2A6-CYP2A7 hybrid allele CYP2A6*12 results in a decrease in both CYP2A6 protein and activity levels (Oscarson, McLellan et al. 2002; Benowitz, Swan et al. 2006).

In African North Americans, several CYP2A6 alleles with relatively high frequencies of expression include CYP2A6*17, CYP2A6*20, CYP2A6*23-*28, and CYP2A6*35, with allele frequencies ranging from ~1 to 11%; the vast majority of these alleles (except CYP2A6*24 and CYP2A6*28) have been consistently shown to be associated with lower CYP2A6 activity in vivo (Ho, Mwenifumbo et al. 2008; Mwenifumbo, Al Koudsi et al. 2008; Al Koudsi, Ahluwalia et al. 2009). More recently, seven novel rare CYP2A6 alleles (CYP2A6*39-*45) were discovered in African Americans with phenotypically slow nicotine metabolism, with an overall frequency of ~3% (Piliguian, Zhu et al. 2014). Each of these cDNA-expressed variants was associated with
lower CYP2A6 activity \textit{in vitro}, and collectively these novel alleles were associated with lower nicotine metabolism \textit{in vivo} (Piliguian, Zhu et al. 2014).

1.4.2. Additional nicotine-metabolizing enzymes and supportive enzymes

1.4.2.1. CYP2B6

While CYP2A6 mediates \(~90\%\) of the inactivation of nicotine to cotinine, CYP2B6 is thought to contribute the remaining \(~10\%\) of this process (Al Koudsi and Tyndale 2010). In baculovirus expression systems, the affinity of CYP2B6 for nicotine is \(~10\)-fold lower compared to CYP2A6 (\(K_m = 105\) vs. \(11 \mu M\), respectively) (Yamazaki, Inoue et al. 1999). Although the role of CYP2B6 in peripheral nicotine metabolism is minimal, the inhibition of brain CYP2B activity in rats was associated with higher rates of acquisition of nicotine self-administration but no change in peripheral nicotine levels (Garcia, Coen et al. 2015). Thus, brain CYP2B, and possibly brain CYP2B6 in humans, may play an important role in the central metabolism of nicotine.

The \textit{CYP2B6*6} allele, a haplotype comprising two amino acid changes (Q172H and K262R), was also associated with lower CYP2B6 hepatic protein expression (Al Koudsi and Tyndale 2010) and slower CYP2B6-mediated metabolism of bupropion and efavirenz (Thorn, Lamba et al. 2010) (\textbf{Table 2}). Consistent with a potential role of CYP2B6 in nicotine metabolism in the brain, \textit{CYP2B6} genetic variation is associated with smoking cessation outcomes. In the placebo arm of a bupropion smoking cessation trial, 32\% of \textit{CYP2B6*1/*1} individuals were abstinent compared to only 15\% of individuals with one or two copies of \textit{CYP2B6*6}, suggesting higher smoking relapse in those with the \textit{CYP2B6*6} allele (Lee, Jepson et al. 2007a). These data suggest that slower CYP2B6 metabolism may predispose to smoking relapse, consistent with animal data showing higher nicotine reinstatement (following extinction) in rats with slower brain CYP2B6 activity (Garcia, Coen et al. 2015). Conversely, the effect of \textit{CYP2B6} genetic variation on smoking outcomes may be due to \textit{CYP2B6} variants being in linkage disequilibrium with \textit{CYP2A6} variants, which have been shown to modify smoking behaviour (Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Wassenaar, Dong et al. 2011).
1.4.2.2. Flavin-containing monooxygenase

Unlike P450 enzymes, FMO enzymes possess a flavin adenine dinucleotide binding domain and do not contain heme (Ziegler 1993; Krueger and Williams 2005). FMO enzymes catalyze the N-oxidation of a variety of substrates, the metabolites of which typically possess lower activity relative to their parent compounds (Stormer, Roots et al. 2000; Ziegler 2002; Krueger and Williams 2005).

To date, six isoforms of FMO enzymes have been discovered, five of which are functional (Hisamuddin and Yang 2007). The six FMO genes that encode the FMO enzymes are located on chromosome 1 (Hisamuddin and Yang 2007). Ontogenic and regional patterns of expression of FMO enzymes exist. FMO1 is the major isoform found in the fetal liver, however its hepatic expression is suppressed soon after birth, beginning at three days post-partum (Koukouritaki, Simpson et al. 2002). In contrast, hepatic FMO3 is not detectable in the fetus or neonate, but is expressed starting at around 1-2 years of age and undergoes a rapid increase in expression in the liver throughout adolescence (Koukouritaki, Simpson et al. 2002). In adulthood, FMO3 is the predominant FMO isoform expressed in the liver, while FMO1 is primarily expressed in the kidney, and possibly the brain (Zhang and Cashman 2006). Drastically reduced FMO3 activity, through loss-of-function FMO3 genetic variants, leads to a condition known as trimethylaminuria, or ‘fish odour syndrome’ (Hisamuddin and Yang 2007). The vast majority of trimethylamine (TMA), an odourous, volatile compound associated with the smell of rotting fish, is converted to TMA-N-oxide when FMO3 activity is intact (Hisamuddin and Yang 2007).

FMO3 also plays a minor role in nicotine inactivation, converting nicotine to nicotine N-oxide (Cashman, Park et al. 1992), which typically comprises ~4-7% of a total dose of nicotine recovered in urine after cigarette smoking (Benowitz, Hukkanen et al. 2009). Following oral nicotine administration to individuals with genetically deleted CYP2A6 (CYP2A6*4/*4 homozygotes), urinary nicotine N-oxide represents ~30% of a nicotine dose (Yamanaka, Nakajima et al. 2004), suggesting that FMO3-mediated nicotine metabolism may be more important when CYP2A6 activity is reduced. Large interindividual variation (~7-fold) in the rate of hepatic nicotine N-oxidation exists (Cashman, Park et al. 1992), with FMO3 genetic variability potentially contributing to these differences. Several FMO3 haplotypes have been
associated with altered ratios of cotinine/(cotinine + nicotine) and cigarette consumption in Caucasian heavy smokers (Bloom, Murphy et al. 2013), however the impact of FMO3 variation on total nicotine clearance is not known. The common non-synonymous FMO3 variant rs2266782 (E158K; G>A) is expressed widely across populations (19-42% in Koreans, Hispanics, Caucasians, and Africans) (Cashman, Zhang et al. 2001; Park, Kang et al. 2002), and is associated with lower in vitro FMO3 activity toward benzydamine (Stormer, Roots et al. 2000) and ranitidine (Park, Kang et al. 2002) (Table 2). Whether this reduced-function FMO3 variant influences nicotine clearance is currently not known. Of potential importance to centrally-mediated drug metabolism, FMO1 is expressed in the brain and may influence the nicotine concentration in this tissue (Zhang and Cashman 2006). Therefore, variation in FMO1 may also contribute to interindividual differences in smoking behaviours, as common polymorphisms in FMO1 are associated with nicotine dependence (Hinrichs, Murphy et al. 2011).
Table 2 | Selected variants in genes encoding additional nicotine metabolizing enzymes and supporting enzymes, and their impact on drug metabolism

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<th>Gene</th>
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<td></td>
<td>D67Y (UGT2B10*2)</td>
<td>4 (African) 11 (Caucasian)</td>
<td>Nicotine Cotinine Decreased (in vitro/vivo) Decreased (in vitro/vivo)</td>
<td>8-10</td>
</tr>
<tr>
<td>UGT2B10</td>
<td></td>
<td>21 (African) 30 (Caucasian)</td>
<td>3’Hydroxycotinine Decreased (in vivo)</td>
<td>10, 11</td>
</tr>
<tr>
<td>UGT2B17</td>
<td></td>
<td>19 (African) 26 (Caucasian)</td>
<td>Dextromethorphan (CYP2D6 substrate) Decreased (in vitro)</td>
<td>12-16</td>
</tr>
<tr>
<td>POR</td>
<td>A503V (POR*28)</td>
<td>19 (African) 26 (Caucasian)</td>
<td>Midazolam (CYP3A4 substrate) Decreased in vitro, Increased in vivo</td>
<td></td>
</tr>
</tbody>
</table>

References: 1(Benowitz et al., 2013); 2(Lee, Jepson et al. 2007a); 3(Thor, Lamba et al. 2010); 4(Zhu, Cox et al. 2012); 5(Cashman, Zhang et al. 2001); 6(Park, Kang et al. 2002); 7(Stormer, Roots et al. 2000); 8(Berg, Mason et al. 2010); 9(Chen, Blevins-Primeau et al. 2007); 10(Zhu, Zhou et al. 2013); 11(Wassenaar et al., 2015); 12(Agrawal, Choi et al. 2010); 13(Huang, Agrawal et al. 2008); 14(Oneda et al., 2009); 15(Sandee, Morrissey et al. 2010); 16(Yang et al., 2011)

1.4.2.3. UDP-glucuronosyltransferases

The UDP-glucuronosyltransferase (UGT) enzymes catalyze the transfer of glucuronic acid to a variety of endogenous and exogenous substrates (Kaivosaari, Toivonen et al. 2007). The inactive glucuronidated metabolites of nicotine, cotinine, and 3’hydroxycotinine account for a substantial proportion (~25%) of the urinary metabolites of nicotine (Zhu, Binnington et al. 2013). However, in individuals with two copies of the CYP2A6*4 gene deletion, the amount of nicotine excreted in the urine as nicotine N-glucuronide may be much higher compared to those with normal CYP2A6 activity (Yamanaka, Nakajima et al. 2004). Conversely, the urinary levels of cotinine N-glucuronide and 3’hydroxycotinine O-glucuronide are much lower in those with deleted CYP2A6, consistent with a lack of CYP2A6-mediated production of cotinine and 3’hydroxycotinine from nicotine and cotinine, respectively (Yamanaka, Nakajima et al. 2004).

The N-glucuronidation of nicotine and cotinine is catalyzed by the same enzyme, UGT2B10 (Kaivosaari, Toivonen et al. 2007), consistent with the high degree of correlation between the rates of nicotine- and cotinine-glucuronide formation (Chen, Blevins-Primeau et al. 2007).
Genetic variation in UGT2B10 is associated with altered glucuronidation of nicotine and cotinine. In human liver microsomes, livers with one copy of the UGT2B10 variant allele rs61750900 (Asp67Tyr; G>T) displayed 21% and 30% lower nicotine and cotinine glucuronidation activity, respectively, compared to livers that did not express the variant allele (Chen, Blevins-Primeau et al. 2007). A similar reduction (20%) in nicotine and cotinine glucuronidation was observed in vivo in smokers that possessed the rs61750900 variant allele (Berg, Mason et al. 2010), which is expressed at a frequency of ~4% and ~11% in African Americans and Caucasians, respectively (Chen, Giambrone et al. 2010; Zhu, Zhou et al. 2013) (Table 2). Compared to Caucasians, African Americans typically display lower levels of nicotine and cotinine glucuronidation (Berg, Mason et al. 2010), which may be due, in part, to a second UGT2B10 variant, rs116294140; this splice variant was shown to account for much of the reduced glucuronidation activity in African Americans (Murphy, Park et al. 2014). In contrast to nicotine and cotinine, 3’hydroxycotinine is O-glucuronidated largely via a second enzyme, UGT2B17 (Chen, Giambrone et al. 2012). UGT2B17 is also a polymorphic enzyme, and UGT2B17 variability is associated with altered 3’hydroxycotinine glucuronidation. The common UGT2B17 gene deletion (allele frequency ranges from 25-80%) is associated with lower 3’hydroxycotinine O-glucuronidation in vivo (Zhu, Zhou et al. 2013; Wassenaar, Conti et al. 2015) (Table 2). Although variability in UGT genes affects glucuronidation activities in vivo, the UGT variants studied to date do not appear to be associated with smoking behaviours or nicotine intake, consistent with their relatively small contribution to overall nicotine inactivation (Zhu, Zhou et al. 2013).

1.4.2.4. NADPH-cytochrome P450 oxidoreductase

It is thought that the activities of microsomal P450 enzymes, which include those involved in drug metabolism and steroid and fatty acid synthesis, require NADPH-cytochrome P450 oxidoreductase (POR), hereafter referred to as P450 oxidoreductase to match the nomenclature used in Chapter 2 (Gomes, Winter et al. 2009). POR contains both flavin adenine dinucleotide and flavin mononucleotide domains, which facilitate interactions between POR and P450 enzymes (Miller, Agrawal et al. 2011). Consistent with its essential role in numerous physiological reactions, disrupting the POR gene leads to embryonic lethality in mice (Shen,
O'Leary et al. 2002). In humans, POR deficiency is compatible with life, but leads to genital malformations in association with Antley-Bixler skeletal malformation syndrome, as well as congenital adrenal hyperplasia (Miller, Huang et al. 2009; Miller, Agrawal et al. 2011). These conditions are thought to result from disordered steroidogenesis, caused by deficiencies in the activity of several steroidogenic P450 enzymes including CYP17A1, CYP21A1, and CYP19A1 (Miller, Huang et al. 2009).

Variation in POR is also associated with a number of P450- and substrate-specific effects on drug metabolism; whether POR variability influences CYP2A6-mediated nicotine metabolism is not known. For example, the common non-synonymous POR variant rs1057868 (Ala503Val; C>T) is associated with decreased activity of some drug-metabolizing P450 enzymes (e.g., CYP3A4 and CYP2D6) in in vitro expression systems (Agrawal, Choi et al. 2010; Sandee, Morrissey et al. 2010). Specifically, the 503V allele (vs. wild type POR) reduced CYP2D6 activity to 62% with the substrate dextromethorphan (Sandee, Morrissey et al. 2010). In addition, 503V reduced the in vitro CYP3A4-mediated metabolism of midazolam by up to ~40% (Agrawal, Choi et al. 2010), but was associated with increased midazolam metabolism in vivo (Oneda, Crettol et al. 2009; Yang, Fu et al. 2011) (Table 2). In contrast, the A503V polymorphism did not appear to substantially affect CYP1A2 and CYP2C19 activity, as assessed in vitro (Agrawal, Huang et al. 2008). The 503V allele is present at a high frequency in African (19%), Caucasian (26%), Mexican (31%) and Chinese (37%) populations (Huang, Agrawal et al. 2008) (Table 2), and thus may meaningfully impact drug metabolism in a variety of populations.

1.5. Phenotypic measure of nicotine metabolism rate: the nicotine metabolite ratio

1.5.1. Association with CYP2A6 genotype and correlation with nicotine clearance

As described, CYP2A6 is responsible for >90% of the inactivation of nicotine to cotinine, and 100% of the conversion of cotinine to 3’hydroxycotinine (Nakajima, Yamamoto et al. 1996a; Nakajima, Yamamoto et al. 1996b; Messina, Tyndale et al. 1997). The ratio of these metabolites (3’hydroxycotinine/cotinine) is known as the nicotine metabolite ratio (NMR). The NMR is a phenotypic indicator of the rate of nicotine metabolism that is strongly concordant with CYP2A6
genotype (Dempsey, Tutka et al. 2004; Johnstone, Benowitz et al. 2006; Malaiyandi, Goodz et al. 2006; Malaiyandi, Lerman et al. 2006; Ho, Mwenifumbo et al. 2009). The NMR provides an advantage over \textit{CYP2A6} genotype when assessing the rate of nicotine metabolism in that it captures both genetic and environmental sources of variability in \textit{CYP2A6} activity (Swan, Lessov-Schlaggar et al. 2009). The NMR is highly correlated with total nicotine clearance following an oral nicotine dose (Dempsey, Tutka et al. 2004). Although \textit{CYP2A6} genotype is also associated with the rate of nicotine clearance (Benowitz, Swan et al. 2006), the NMR is more strongly related to nicotine clearance (Dempsey, Tutka et al. 2004), consistent with its ability to capture both genetic and environmental sources of variation in the rate of nicotine metabolism. Several potential disadvantages of using NMR as a surrogate of nicotine clearance include the fact that it is technically a measure of cotinine clearance, and that variation in the rate of 3’hydroxycotinine clearance could alter NMR measurements. However, as mentioned, NMR is strongly correlated with nicotine clearance (Dempsey, Tutka et al. 2004), and variation in the metabolism of 3’hydroxycotinine to 3’hydroxycotinine glucuronide does not appear to substantially alter NMR (Zhu, Zhou et al. 2013). However, unlike \textit{CYP2A6} genotype, NMR may be altered by transient influences on \textit{CYP2A6} activity, such as hormonal therapy (Benowitz, Lessov-Schlaggar et al. 2006) or pharmacological inducers/inhibitors (Zhang, Kilicarslan et al. 2001; Onica, Nichols et al. 2008), described below. Factors influencing hepatic blood flow, such as having a meal, may also influence NMR. However, an effect of hepatic blood flow on NMR, a measure of cotinine clearance, is likely to be minor compared to the effect of hepatic blood flow on nicotine clearance since cotinine is a lower extraction drug (Zhu, Renner et al. 2013). In general, the clearance of higher extraction drugs (e.g., nicotine) is affected more by differences in hepatic blood flow, whereas the clearance of lower extraction drugs (e.g., cotinine) is affected more by alterations in enzymatic activity (Zhu, Renner et al. 2013).

1.5.2. Stability of the nicotine metabolite ratio in smokers

The utility of NMR as a phenotypic marker of \textit{CYP2A6} activity is strengthened by cotinine’s long half-life (~16-19 hours) (Benowitz and Jacob 1994). The half-life of 3’hydroxycotinine is comparatively short (~6 hours) (Benowitz and Jacob 1994) and at steady state its clearance rate is dependent on its formation. The NMR is measurable in a variety of biological fluids, including
saliva, blood, plasma, and urine (St Helen, Novalen et al. 2012). NMR measurements derived from the blood of smokers smoking as usual show strong agreement with those derived from saliva and plasma, however greater variability is noted when comparing urinary NMR to NMR from the other source fluids (St Helen, Novalen et al. 2012). Cotinine and 3’hydroxycotinine also appear to be physically stable, as NMR measurements appear to be relatively robust to different storage temperatures (Lea, Dickson et al. 2006; St Helen, Novalen et al. 2012).

The NMR is relatively consistent in regular smokers over time and does not appear to vary throughout the day (Lea, Dickson et al. 2006). In ad libitum smokers smoking a mean of 26 cigarettes per day, urinary NMR was reproducible (i.e., did not significantly vary) over a period of eight weeks (Mooney, Li et al. 2008). During a smoking reduction phase, in which participants were asked to gradually reduce their cigarette consumption while receiving NRT (nicotine gum and patch), only slight variation in NMR was observed over time (Mooney, Li et al. 2008). In addition, the NMR measured in plasma was relatively stable in smokers gradually reducing their nicotine intake by smoking progressively reduced nicotine content cigarettes (Mooney, Li et al. 2008). Over a period of six months where the nicotine content was gradually reduced from 12 to 1 mg, the reliability coefficient for repeated measurements (weeks one, eight, 16, and 24) of NMR was >0.7, suggesting measures of NMR were relatively stable (Mooney, Li et al. 2008). In a separate study, within-person variation in NMR was observed over six study visits conducted during a 44-week period; because smokers’ 3’hydroxycotinine, but not cotinine, levels varied over time, 3’hydroxycotinine was likely not in steady state at all time-points, leading to fluctuations in NMR (St Helen, Novalen et al. 2012). Together the data suggest that NMR is reproducible within smokers over time even as they adjust their smoking behaviours, as long as 3’hydroxycotinine levels are in steady state. Thus, NMR measurements may not reliability replicate over time in occasional smokers, whose 3’hydroxycotinine levels may not be in steady state.

1.5.3. Known sources of variation in the nicotine metabolite ratio

1.5.3.1. Ethnicity
On a population level, African Americans and Japanese display lower NMR relative to Caucasians (Derby et al., 2008; Kandel et al., 2007; Moolchan, Franken, & Jaszyna-Gasior, 2006; Rubinstein, Shiffman, Rait, & Benowitz, 2013). This finding consistently replicates across a variety of ages, including adolescents (Moolchan, Franken, & Jaszyna-Gasior, 2006; Rubinstein, Shiffman, Rait, & Benowitz, 2013), young adults (aged 18-26 years) (Kandel, Hu et al. 2007), and middle-aged adults (median age ~60 years) (Derby, Cuthrell et al. 2008). Inter-ethnic variation in CYP2A6 allele frequencies likely explains some of this variation in NMR. The frequencies of CYP2A6 alleles that lead to reduced or null CYP2A6 activity are higher in African Americans and Japanese relative to Caucasians (Nakajima, Fukami et al. 2006; Mwenifumbo and Tyndale 2007), consistent with an overall lower NMR in African Americans and Japanese (Kandel, Hu et al. 2007; Derby, Cuthrell et al. 2008). Moreover, compared to Caucasians, livers from Japanese donors display lower CYP2A6 protein levels (Shimada, Yamazaki et al. 1996), potentially due to their higher frequency of CYP2A6*4 and CYP2A6*9 alleles which lead to reductions in CYP2A6 protein levels (Mwenifumbo and Tyndale 2007).

More recently, the rate of nicotine metabolism was characterized in Yupik, a population of Alaska Natives found in the Bristol Bay region of South Western Alaska (Binnington, Zhu et al. 2012). Compared to Caucasians and African Americans, Yupik possess an elevated rate of nicotine metabolism, as demonstrated by higher NMR (mean = 0.48, vs. 0.43 and 0.35 in Caucasians and African Americans, respectively). Yupik also possess a unique pattern of CYP2A6 allele frequencies relative to other populations, with higher expression of both CYP2A6*1B and CYP2A6*4 compared to Caucasians and African Americans (Binnington, Zhu et al. 2012). After restricting the analysis to those without tested CYP2A6 variants to reduce the influence of genotype variation, the elevated NMR in Yupik became even more pronounced (mean NMR = 0.61 in Yupik, vs. 0.44 and 0.43 in Caucasians, and African Americans, respectively) (Binnington, Zhu et al. 2012). The reason for the higher NMR observed in Yupik remains to be determined, and may be due to novel genetic variants in CYP2A6 or in genes regulating CYP2A6, and/or environmental sources of variation in NMR such as diet. Similar to the higher NMR in Alaska Natives, American Indians also possess higher NMR relative to Caucasians and African Americans, the reasons for which remain to be determined (Tanner, Henderson et al. 2015).
1.5.3.2. Sex

The NMR is higher in women relative to men (Benowitz, Lessov-Schlaggar et al. 2006; Benowitz, Hukkanen et al. 2009). Compared to men, pre-menopausal women have ~18% and 24% higher rates of nicotine and cotinine clearance, respectively (Benowitz, Lessov-Schlaggar et al. 2006). In contrast, there are no differences in NMR and nicotine and cotinine clearance in menopausal or post-menopausal women compared to men (Benowitz, Lessov-Schlaggar et al. 2006). In adolescents, NMR is similarly higher in girls than in boys (mean NMR = 0.32 and 0.25, respectively) (Berlin, Gasior et al. 2007). The higher NMR observed among women relative to men could be due to an inducing effect of estrogen on CYP2A6 (Higashi, Fukami et al. 2007). In human hepatocytes, estradiol treatment led to an induction of CYP2A6 mRNA levels by 1.2- to 1.5-fold. Further investigation yielded a putative estrogen response element on the CYP2A6 gene, with which ER-α was able to bind, suggesting it is a functional estrogen response element (Higashi, Fukami et al. 2007) that may be important for mediating the higher NMR observed in women relative to men.

1.5.3.3. Hormonal therapy

In addition to NMR being higher in women than in men, among women, NMR is higher in those taking estrogen-containing birth control pills (Benowitz, Lessov-Schlaggar et al. 2006). This is also likely due to an effect of CYP2A6 induction via estrogen (Higashi, Fukami et al. 2007). Among women taking estrogen-only contraceptives, mean NMR was 56% higher compared to women taking no hormones (Benowitz, Lessov-Schlaggar et al. 2006). Nicotine clearance rates were higher in women taking estrogen-only and combined contraceptives, but not in women taking progesterone-only pills (Benowitz, Lessov-Schlaggar et al. 2006). In adolescent girls, hormonal contraception is also associated with higher NMR (Berlin, Gasior et al. 2007).

1.5.3.4. Body mass index

Smokers typically display a lower body mass index (BMI) relative to non-smokers (Albanes, Jones et al. 1987; Akbartabartoori, Lean et al. 2005), and gain weight upon smoking cessation (Klesges, Winders et al. 1997). Smoking is thought to cause lower BMI through nicotine-
mediated appetite suppression, and weight gain occurs due to a removal of this suppression upon smoking cessation (Perkins, Epstein et al. 1991; Mineur, Abizaid et al. 2011). BMI is inversely related to NMR (Mooney, Li et al. 2008; Ho, Mwenifumbo et al. 2009; Binnington, Zhu et al. 2012), however whether this is due to an influence of BMI on NMR, or vice versa, and the mechanism remains to be determined.

1.5.3.5. Pharmacological and dietary inducers and inhibitors

CYP2A6 mRNA, protein, and/or activity is known to be increased by a few clinical drugs, including the barbiturate phenobarbital (Meunier, Bourrie et al. 2000), the anti-tuberculoid rifampin (Rae, Johnson et al. 2001), and the steroid dexamethasone (Onica, Nichols et al. 2008). Pyrazole and cadmium can also induce CYP2A6 (Kirby, Nichols et al. 2011). In addition, several compounds have been shown to inhibit CYP2A6 enzyme activity, including, but not limited to, the anti-fungal ketoconazole (Zhang, Ramamoorthy et al. 2002), the psoriasis treatment drug methoxsalen (Zhang, Kilicarslan et al. 2001), and the monoamine oxidase inhibitor tranylcypromine (Zhang, Kilicarslan et al. 2001). To our knowledge, the contribution of these compounds to variation in NMR has not been explicitly studied, however it is possible they may alter NMR through their effects on CYP2A6 expression levels and/or activity.

Dietary components can also affect CYP2A6 activity, potentially contributing to variation in NMR if consumed in sufficient amounts. For example, a compound found in broccoli has been shown to increase CYP2A6 activity in vivo in humans consuming 500 g of broccoli/day for six days (Hakooz and Hamdan 2007). However, it is not known whether more typical levels of broccoli consumption (i.e., 7.6 pounds of broccoli per person per year (Chun, Kim et al. 2005)) increase CYP2A6 activity. Isoflavones, present in soy products, have also been shown to affect CYP2A6 activity. Isoflavones decreased CYP2A6 activity toward nicotine in vitro in a cell expression system, and in vivo as demonstrated by lower cotinine/nicotine ratios following the consumption of an isoflavone supplement (60 mg/day for five days) (Nakajima, Itoh et al. 2006). Whether these findings are generalizable to smokers regularly consuming these compounds remains to be determined.
1.6. Association of the nicotine metabolite ratio with smoking cessation

1.6.1. Retrospective prediction of treatment outcomes by the nicotine metabolite ratio

In secondary analyses using data from smoking cessation clinical trials, the NMR predicted treatment outcomes on the nicotine patch (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009; Lerman, Jepson et al. 2010) as well as placebo (Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009). Conversely, NMR did not significantly predict quit rates on bupropion (Patterson, Schnoll et al. 2008) (Fig. 7). In two trials involving the nicotine patch, conducted in predominantly Caucasian treatment-seeking smokers, lower NMR (i.e., slower nicotine metabolism) was associated with increased quit rates relative to higher NMR. In the first study to show this association, pre-treatment NMR (measured when participants were smoking as usual) was associated with quit rates at both end-of-treatment and 6-months follow-up (Lerman, Tyndale et al. 2006). Nearly half (46%) of the smokers in the lowest NMR quartile (i.e., slowest nicotine metabolism group) were abstinent at end-of-treatment, relative to 28% of smokers in the highest NMR quartile (i.e., fastest nicotine metabolism group). This difference in quit rate (18%) between the slowest and fastest metabolizers persisted at 6-months follow-up, when 30% of participants in the lowest NMR quartile were abstinent, compared to only 11% in the highest NMR quartile (Lerman, Tyndale et al. 2006). In contrast, NMR was not associated with smoking cessation outcomes on nicotine nasal spray, although CYP2A6 genotype group was associated with the number of doses acquired (~6 more doses/day in CYP2A6 normal metabolizers relative to slow metabolizers) (Malaiyandi, Lerman et al. 2006). Thus, smokers receiving nicotine spray appear to titrate their level of nicotine intake according to their rate of metabolism, which would not be possible on the nicotine patch; this offers a potential explanation for the lack of differences in quitting by NMR on nicotine nasal spray.

In a larger study that sought to validate the initial findings with NMR and nicotine patch, pre-treatment NMR was significantly associated with abstinence at end-of-treatment (Schnoll, Patterson et al. 2009). Consistent with previous findings (Lerman, Tyndale et al. 2006), the abstinence rates were 42% and 28% in the lowest and highest NMR quartile, respectively (Schnoll, Patterson et al. 2009). NMR differences in smoking cessation success were also
observed in a clinical trial involving extended (six months) vs. standard (eight weeks) treatment with the nicotine patch (Lerman, Jepson et al. 2010). At six months, participants in the lowest NMR quartile had significantly higher quit rates on extended transdermal nicotine therapy relative to the standard regimen. In contrast, extended therapy provided no benefit over standard therapy in participants in the next three NMR quartiles (i.e., faster metabolizers) (Lerman, Jepson et al. 2010). Treatment with a higher dose of nicotine patch may increase quit rates in faster metabolizers; there was a trend toward a higher rate of 24-hour abstinence at end-of-treatment in faster metabolizers that received 42 mg nicotine patch compared to 21 mg nicotine patch (Schnoll, Wileyto et al. 2013).

The NMR also retrospectively predicted cessation success in a placebo-controlled clinical trial involving nicotine gum (Ho, Mwenifumbo et al. 2009). While nicotine gum treatment did not improve overall quit rates in treatment-seeking African American light smokers (Ahluwalia, Okuyemi et al. 2006), NMR differences in cessation rates were observed. Overall, participants in the slowest NMR quartile displayed a higher likelihood of abstinence (odds ratio = 1.9) than those in the other three NMR quartiles (Ho, Mwenifumbo et al. 2009). The association between NMR and abstinence was more pronounced in women. In women receiving placebo, quit rates were higher in the lowest NMR quartile (30% vs. 17% in the other three quartiles), which persisted at the end of 26 weeks of follow-up (quit rates were 30% vs. 18%, respectively) (Ho, Mwenifumbo et al. 2009). While lower NMR was not associated with increased quitting in the nicotine gum arm, a significantly higher proportion of women in the CYP2A6 slow genotype group were abstinent at end-of-treatment relative to those in the CYP2A6 normal and intermediate combined genotype group (32% vs. 16%, respectively) (Ho, Mwenifumbo et al. 2009).

The NMR also predicted cessation outcomes in a smoking cessation trial involving bupropion (Patterson, Schnoll et al. 2008). The association between NMR and cessation was restricted to the placebo arm, where 32% of participants in the lowest NMR quartile were abstinent at end-of-treatment (10 weeks), compared to only 10% of individuals in the highest NMR quartile. In contrast, there was little difference in quit rates on bupropion by NMR quartile (32% vs. 34% in lowest vs. highest NMR quartile, respectively) (Patterson, Schnoll et al. 2008). While those in
the lowest NMR quartile had equivalent quit rates on placebo and bupropion (32%), bupropion significantly enhanced end-of-treatment quit rates in participants in the highest NMR quartile (34% vs. 10% on placebo) (Patterson, Schnoll et al. 2008). Collectively, slow metabolizers quit better than normal metabolizers on both placebo pill (Patterson, Schnoll et al. 2008) and placebo gum (Ho, Mwenifumbo et al. 2009), consistent with their enhanced ability to quit smoking in general (Gu, Hinks et al. 2000).

Figure 7 | NMR predicted end-of-treatment quit rates on placebo and the nicotine patch in retrospective analyses of smoking cessation clinical trials. Placebo and bupropion data were derived from Patterson et al., 2008, where fast metabolizers comprised the fourth (highest) NMR quartile, and slow metabolizers comprised the first (lowest) NMR quartile. Nicotine patch data were derived from Schnoll et al., 2009, where fast metabolizers comprised the upper three (quartiles 2-4) NMR quartiles, and slow metabolizers comprised the first (lowest) NMR quartile.

1.6.2. Prospective prediction of treatment outcomes by the nicotine metabolite ratio

In a recently completed phase III smoking cessation clinical trial (NCT01314001), over 1200 adult smokers were randomized based on NMR to nicotine patch, varenicline, or placebo, which was assessed at baseline when participants were smoking as usual (Lerman, Schnoll et al. 2015).
This smoking cessation trial was novel in two ways: it was the first to assign treatment based on a genetically informed biomarker (i.e., NMR), and the first to investigate potential associations between NMR and treatment outcomes on varenicline. The trial was designed to test the primary hypothesis that there would be an interaction between active treatment arm and NMR on smoking abstinence (verified by exhaled carbon monoxide $\leq 8$ ppm) at end-of-treatment. An NMR cut-point of 0.31 was used to distinguish slow (NMR $<0.31$) from normal (NMR $\geq 0.31$) metabolizers. At end-of-treatment, the likelihood of abstinence was higher on varenicline compared to nicotine patch in normal metabolizers (OR $= 2.2$; $P = 0.001$), but not in slow metabolizers (OR $= 1.1$; $P = 0.56$), with a significant treatment-by-NMR interaction observed (ORR $= 1.9$; $P = 0.04$) (Lerman, Schnoll et al. 2015) (Fig. 8). In normal metabolizers, the enhanced efficacy of varenicline compared to nicotine patch persisted at six months. Overall side effect profiles indicated greater summary side effects on varenicline compared to placebo for slow metabolizers, but not normal metabolizers. In contrast, there was no difference in side effects on nicotine patch compared to placebo according to NMR group (Lerman, Schnoll et al. 2015). These findings could be implemented clinically and suggest that when assigning treatment based on NMR, varenicline is likely a better treatment for faster metabolizers, whereas the nicotine patch is more suitable for slower metabolizers.
Figure 8 | The likelihood of end-of-treatment abstinence is higher on varenicline (vs. nicotine patch) in normal, but not in slow, nicotine metabolizers, as tested prospectively in an NMR-stratified randomized trial. Data from Lerman et al., 2015.
1.7. STATEMENT OF RESEARCH HYPOTHESES

In Chapter 1 “CYP2A6 slow nicotine metabolism is associated with increased quitting by adolescent smokers”, we hypothesized that in adolescence, CYP2A6 slow metabolizers would have an increased likelihood of quitting smoking compared to CYP2A6 normal metabolizers, as has been observed in adult smokers.

In Chapter 2, “Variation in P450 oxidoreductase (POR) A503V and flavin-containing monooxygenase (FMO)-3 E158K is associated with minor alterations in nicotine metabolism, but does not alter cigarette consumption”, we hypothesized that POR 503V would be associated with higher nicotine and cotinine metabolism, and that FMO3 158K would be associated with lower nicotine metabolism particularly in CYP2A6 slow metabolizers. We further hypothesized that the influence of FMO3 E158K and POR A503V variation on nicotine metabolism would be sufficient to affect the level of cigarette consumption and total nicotine equivalents.

In Chapter 3, “Known and novel sources of variability in the nicotine metabolite ratio in a large sample of treatment-seeking smokers”, we hypothesized that Caucasian ethnicity, female sex, estrogen-containing birth control pill and hormone replacement therapy use, lower BMI, higher cigarette consumption, and higher alcohol use would be associated with higher NMR in univariate analyses. We further speculated that these factors would significantly contribute to overall variability in NMR.

In Chapter 4, “The nicotine metabolite ratio is associated with early smoking abstinence even after controlling for factors that influence the nicotine metabolite ratio”, we hypothesized that NMR would be a significant predictor of one-week smoking abstinence, with lower NMR being associated with a higher likelihood of one-week abstinence compared to higher NMR. We further hypothesized that demographic and lifestyle factors that are associated with variation in NMR would not alter the ability of NMR to predict one-week abstinence.
CHAPTER 1: CYP2A6 SLOW NICOTINE METABOLISM IS ASSOCIATED WITH INCREASED QUITTING BY ADOLESCENT SMOKERS

Meghan J. Chenoweth, Jennifer O’Loughlin, Marie-Pierre Sylvestre, and Rachel F. Tyndale

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JL and RFT designed the overall epidemiological study; a description of the overall study is found in Appendix A. Following a literature search, MJC found gaps in the existing literature and developed a research question. Using the data dictionary, MJC designed an analytic plan to answer the research question within the overall epidemiological study. MJC performed genotyping for CYP2A6 variant alleles. MJC, JL, MS, and RFT all contributed to the analytic strategy. MS performed statistical analyses. MJC, JL, MS, and RFT all interpreted the results. MJC and RFT wrote the manuscript.
2.1. Abstract

Variation in the *CYP2A6* gene, which decreases the rate of nicotine metabolic inactivation, is associated with higher adult smoking cessation rates during clinical trials. We hypothesized that slow metabolism is associated with increased quitting during adolescence. White adolescent smokers (N=308, aged 12-17, 36.3% male) from a cohort study were genotyped for *CYP2A6*, resulting in 7.8% slow, 14.0% intermediate and 78.2% normal metabolizers. Overall, 144 smokers quit smoking, as indicated by being abstinent for at least 12 months. In logistic regression analyses, the odds ratio for quitting was 2.25 (95% confidence interval 1.05, 4.80; *P*=0.037) for slow metabolizers relative to normal metabolizers. A linear trend toward increased quitting with decreasing *CYP2A6* activity was also observed (odds ratio = 1.44, 95% confidence interval 1.02, 2.01; *P*=0.034). Thus, *CYP2A6* slow metabolism is associated with increased adolescent smoking cessation, indicating that even early in the smoking history, genetic variation is influencing smoking cessation.
2.2. Introduction

Adolescence is a critical period for smoking acquisition (O'Loughlin, Karp et al. 2009). Although a large proportion of adolescents smoke during their teen years, only a fraction progress to regular, dependent smoking as adults, suggesting that many quit smoking before adulthood.

Interindividual variation in the ability to quit smoking in adolescence may be related, in part, to pharmacogenetics. Genetic variation in the CYP2A6 gene is associated with the ability to quit smoking among adults (Ray, Tyndale et al. 2009). CYP2A6 plays a major role in the inactivation of nicotine to cotinine, and the conversion of cotinine to 3'-hydroxycotinine (Benowitz, Swan et al. 2006). Slow nicotine metabolism, measured by the CYP2A6 genotype or the nicotine metabolite ratio (3'-hydroxycotinine to cotinine ratio), is associated with higher spontaneous quitting rates in adults as well as increased quitting on placebo and the nicotine patch (Ray, Tyndale et al. 2009).

In adolescence, slow nicotine metabolism is associated with increased risk and rate of acquisition of nicotine dependence relative to normal metabolizers (NM) (O'Loughlin, Paradis et al. 2004; Al Koudsi, O'Loughlin et al. 2010), possibly because of increased initial nicotine reinforcement leading to an enhanced risk of acquiring nicotine dependence. However, once dependent, slow nicotine metabolizers display relatively limited cigarette consumption (O'Loughlin, Paradis et al. 2004; Audrain-McGovern, Al Koudsi et al. 2007) and reduced escalation in dependence (Audrain-McGovern, Al Koudsi et al. 2007; Al Koudsi, O'Loughlin et al. 2010) potentially increasing the ability of adolescent slow metabolizers (SM) to quit smoking.

We undertook a prospective cohort investigation of the association between CYP2A6 genotype and smoking cessation, defined here as smoking abstinence for at least 12 months, in a cohort of White adolescent smokers. We hypothesized that being a CYP2A6 slow nicotine metabolizer is associated with an increased likelihood of smoking cessation.
2.3. Materials and Methods

Participants were selected from the ongoing Nicotine Dependence in Teens (NDIT) study (O'Loughlin, Karp et al. 2009) on the basis of ethnicity, smoking status, and provision of a blood or saliva sample for DNA extraction, and CYP2A6 genotyping. A total of 610 White adolescents were genotyped for CYP2A6; 308 White adolescents who were either smokers at baseline or initiated smoking during follow-up were retained for analysis. Data on sociodemographic variables and smoking behaviors were collected in self-report questionnaires administered every 3-4 months between 1999 and 2004. All participants were asked about their cigarette smoking behaviours using two indicators: (a) ‘check the one box that describes you best: I have never smoked a cigarette, even just a puff; I have smoked cigarettes, even just a puff, but not at all in the past 12 months; I smoked cigarettes once or a couple of times in the past 12 months; I smoke cigarettes once or a couple of times each month; I smoke cigarettes once or a couple of times each week; I smoke cigarettes everyday’, and (b) a recall of cigarette use in each of the 3 months preceding completion of the survey. Here, the number of days smoked was multiplied by the average number of cigarettes smoked per day on smoking days. Smokers were defined as participants who had ever puffed on a cigarette, and quitting smoking (abstinence for ≥12 months) was defined as subsequently having at least four consecutive surveys with a value of 0 for the total number of cigarettes smoked. Among those who quit smoking, measures of nicotine dependence were computed in the survey immediately preceding the onset of quitting smoking, including craving scores, derived from 14 items assessing craving frequency, physical and mental addiction, and the ability to refrain from smoking; nicotine withdrawal scores, assessing irritability, restlessness, anxiety, urge to smoke, and difficulty sleeping and concentrating while abstinent; and self-medication scores, where participants either endorsed or refuted statements that smoking improves their functioning, concentration, affect, energy, and stress.

All participants provided assent and a parent or guardian provided written informed consent at baseline. The study was approved by the Montreal Department of Public Health Ethics Review Committee, the McGill University Faculty of Medicine Institutional Review Board, and the University of Toronto Research Ethics Board.
DNA was extracted from saliva or blood samples using Oragene kits (DNA Genotek Inc., Kanata, Ontario, Canada) and QIAamp blood kits (Qiagen Inc., Valencia, California, USA), respectively. Genotyping for CYP2A6 variant alleles was performed using a previously validated two-step gene and allele specific polymerase chain reaction (Lerman, Jepson et al. 2010), or an allele-specific TaqMan single nucleotide polymorphism genotyping assay (Applied Biosystems) and real-time PCR. Individuals were genotyped for CYP2A6*2, CYP2A6*4, CYP2A6*9, and CYP2A6*12 alleles, which were selected on the basis of their known role in decreasing nicotine metabolism (Benowitz, Swan et al. 2006). Individuals were categorized as normal, intermediate, or slow CYP2A6 genotype metabolizers based on the metabolic impact of CYP2A6 genotype (Benowitz, Swan et al. 2006). NM had no variant alleles (100% CYP2A6 activity), whereas intermediate metabolizers (IM) had one copy of a decreased-function variant allele (CYP2A6*9 or CYP2A6*12; ~80% CYP2A6 activity), and SM had two copies of a decreased-function allele or one or two copies of a loss-of-function variant allele (CYP2A6*2 or CYP2A6*4; ≤50% CYP2A6 enzymatic activity).

Bootstrap-based multiple imputation was used to manage missing survey data, according to the protocol by Honaker and King (Honaker and King 2010), which utilizes the longitudinal nature of the data to improve the imputation process. Ten imputed sets of data were generated, and multiple logistic regression within a generalized estimating equation (GEE) framework was performed to examine the association between CYP2A6 metabolic group (independent variable) and cessation (dependent variable) while accounting for the correlation between measures within individuals. No covariates were included. The additive model assumed a linear relationship between the CYP2A6 metabolic group, consistent with genotype group impact on metabolism (Benowitz, Swan et al. 2006), and the odds of quitting. The statistical programs used to complete the analysis were R (version 2.14.1, available online: http://www.r-project.org/), the Amelia II package for multiple imputation (version 1.5-5, available online: http://gking.harvard.edu/amelia/), and the Zelig package for GEE models (version 3.5.4, available online: http://gking.harvard.edu/zelig).
2.4. Results

The demographic characteristics of the entire population as well as smoking variables among quitters are reported in Table 3. A total of 144 smokers quit smoking for at least 1 year. A greater proportion of SM quit smoking (Fig. 9), consistent with an increased likelihood of quitting by SM relative to NM (odds ratio (OR) 2.3, 95% confidence interval (CI) 1.1, 4.8; \(P=0.037\)) (Fig. 9). There was no significant difference in the probability of quitting by IM compared with NM (OR 1.3, 95% CI 0.7, 2.2; \(P=0.436\)) (Fig. 9), however a significant linear trend towards increased quitting with decreasing \(CYP2A6\) metabolism (SM>IM>NM) was observed (OR for linear trend across \(CYP2A6\) activity groups = 1.44, 95% CI 1.0, 2.0; \(P=0.034\)).

In an exploratory analysis restricted to regular smokers (n=188, defined as monthly smokers), we observed a similar direction and size of effect, where SM (n=12) were more likely to quit than NM (n=154; OR 1.7, 95% CI 0.58, 4.74), although the smaller numbers reduced power.
Table 3 | Selected characteristics of the study participants according to the *CYP2A6* metabolic group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal (%)</th>
<th>Intermediate (%)</th>
<th>Slow (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample (%)</td>
<td>78.2</td>
<td>14.0</td>
<td>7.8</td>
</tr>
<tr>
<td>Male (95% CI) (95% CI) (%)</td>
<td>33.8 (28.0, 40.0)</td>
<td>51.0 (36.3, 65.4)</td>
<td>35.1 (18.4, 55.7)</td>
</tr>
<tr>
<td>Mean age at baseline, (95% CI)(years)</td>
<td>14.4 (14.2, 14.6)</td>
<td>14.2 (13.9, 15.4)</td>
<td>14.0 (13.4, 14.6)</td>
</tr>
<tr>
<td>Francophone (95% CI) b</td>
<td>22.0 (16.9, 29.2)</td>
<td>13.0 (4.5, 32.0)</td>
<td>17.9 (2.7, 62.7)</td>
</tr>
<tr>
<td>Single parent family (95% CI) b</td>
<td>12.3 (8.4, 17.7)</td>
<td>7.0 (2.1, 20.9)</td>
<td>17.7 (6.8, 39.0)</td>
</tr>
<tr>
<td>Parents smoke (95% CI) b</td>
<td>41.7 (35.6, 48.3)</td>
<td>43.3 (28.8, 59.1)</td>
<td>26.7 (11.9, 49.9)</td>
</tr>
<tr>
<td>Friends smoke (95% CI) b</td>
<td>88.6 (83.7, 92.2)</td>
<td>92.8 (75.1, 98.2)</td>
<td>85.4 (62.5, 95.4)</td>
</tr>
<tr>
<td>Mean monthly cigarettes a (95% CI) b</td>
<td>17.6 (-4.7, 39.8)</td>
<td>18.5 (-14.2, 51.3)</td>
<td>22.0 (-62.0, 106.0)</td>
</tr>
<tr>
<td>Mean craving score a (95% CI) b</td>
<td>5.1 (3.8, 6.5)</td>
<td>3.3 (1.4, 5.2)</td>
<td>3.0 (-0.1, 6.1)</td>
</tr>
<tr>
<td>Mean withdrawal score a (95% CI) b</td>
<td>0.8 (0.5, 1.2)</td>
<td>0.7 (0.2, 1.3)</td>
<td>0.4 (-0.3, 1.0)</td>
</tr>
<tr>
<td>Mean self-medication score a (95% CI) b</td>
<td>1.7 (1.1, 2.3)</td>
<td>1.2 (0.03, 2.5)</td>
<td>0.9 (-0.6, 2.5)</td>
</tr>
</tbody>
</table>

CI, confidence interval
aComputed in quitters (n = 144) in a survey immediately preceding the onset of cessation
b95% confidence that the population mean will lie in this interval; overlap in 95% CI between *CYP2A6* metabolic groups suggests no differences
Figure 9 | Association between the CYP2A6 metabolic group and quitting smoking for at least 12 months in adolescence.

Three hundred and eight adolescent White smokers who were genotyped for CYP2A6 variant alleles were grouped according to the projected CYP2A6 metabolic activity: CYP2A6 normal metabolizers, CYP2A6 intermediate metabolizers, and CYP2A6 slow metabolizers. A) The proportion in each CYP2A6 metabolic group that quit smoking is shown as a percentage of the total for that CYP2A6 metabolic group. B) The likelihood of quitting smoking for at least 12 months by CYP2A6 intermediate metabolizers and CYP2A6 slow metabolizers relative to CYP2A6 normal metabolizers is shown as odds ratios with 95% confidence intervals (CIs). No covariates were used in the model. *P = 0.037.
2.5. Discussion

This is the first prospective cohort investigation of the association between CYP2A6 genotype and quitting smoking in adolescence, and showed that CYP2A6 slow nicotine metabolism is associated with an increased likelihood of quitting smoking. This is similar to the effect of CYP2A6 slow metabolism on increasing quit rates in adults (Ray, Tyndale et al. 2009), providing further evidence that SM have better quit rates in general.

The current findings add novel information regarding the association between CYP2A6 and the tobacco use continuum in adolescence: although SM convert to dependence faster (O'Loughlin, Paradis et al. 2004), they smoke fewer cigarettes (O'Loughlin, Paradis et al. 2004; Audrain-McGovern, Al Koudsi et al. 2007), escalate in dependence slower (Audrain-McGovern, Al Koudsi et al. 2007; Al Koudsi, O'Loughlin et al. 2010), and, we now show, are more likely to quit. This enhanced quitting ability may be mediated by reduced brain response to smoking-related cues (Tang, Hello et al. 2012). Among young adult smokers, SM (vs. NM with similar smoking behaviors) displayed attenuated responses to visual smoking cues in reward and cue processing areas of the brain, as assessed by fMRI (Tang, Hello et al. 2012), which may reduce cravings and potentially improve quitting.

Among adults, SM have higher quit rates on the nicotine patch relative to NM, whereas bupropion may be more useful for faster metabolizers than slower metabolizers (Ray, Tyndale et al. 2009). If these findings extend to younger populations, there may be utility in tailoring smoking cessation treatments based on nicotine metabolism rate in adolescent smokers. For example, the nicotine patch, which is safe and effective in adolescent smokers (Moolchan, Robinson et al. 2005), may be a more effective treatment for adolescent SM.

Strengths of this analysis include the prospective design and repeated sampling, which may minimize recall bias among participants. In addition, multiple imputation was used to manage missing data. Limitations include potential selection bias due to convenience sampling, restriction of the study population to Whites (to minimize population stratification), as well as
genotyping for prevalent CYP2A6 alleles in Whites known to reduce CYP2A6 activity. The presence of undetected CYP2A6 alleles conferring slow nicotine metabolism may misclassify some individuals as NM or IM; however this would likely reduce, rather than enhance, the likelihood of observing increased quitting among SM.

Overall, the findings herein reveal that in adolescence, genetically slow nicotine metabolizers are more likely to quit smoking than normal nicotine metabolizers. A greater understanding of the factors underpinning cessation in adolescence may improve quit rates early in the course of smoking onset, reducing subsequent long-term illnesses and mortality that result from cigarette smoking.
2.6. Significance to Thesis

The finding that \textit{CYP2A6} slow metabolism is associated with an increased likelihood of smoking abstinence in adolescence contributes to the literature in two ways. First, it shows that the relationship between slow nicotine metabolism and increased smoking cessation that has been observed in adults also extends to adolescent smokers. As slow nicotine metabolism influences cessation in a similar manner in adults and adolescents (i.e., it increases quit rates in both age groups), it is possible that smoking cessation strategies tested in adults that seek to optimize quit rates using information on a smoker’s nicotine metabolism rate may also work in adolescence. Thus, the recent clinical trial finding in adult smokers that showed varenicline may optimize quit rates in normal metabolizers, while the nicotine patch may be a more suitable treatment for slow metabolizers \cite{Lerman2015}, may also apply to adolescents who are trying to quit smoking.

In addition, this chapter highlights the role of genetic factors in adolescent smoking behaviours, in particular cessation. In studies of adolescent smoking, psychological and social factors are often investigated in relation to various smoking behaviours. We extend this work by showing that variation in the rate of \textit{CYP2A6} nicotine inactivation can influence an adolescent’s ability to quit smoking. Variation in the \textit{CYP2A6} gene, together with other genetic/biological factors as well as psychological, social, demographic, and environmental causes, contribute to variation in smoking, a complex behavioural phenotype. Genetic studies, including this chapter, provide support for future work aimed at investigating gene-gene and gene-environment interactions in adolescent smoking.
CHAPTER 2: VARIATION IN P450 OXIDOREDUCTASE (POR) A503V AND FLAVIN-CONTAINING MONOOXYGENASE (FMO)-3 E158K IS ASSOCIATED WITH MINOR ALTERATIONS IN NICOTINE METABOLISM, BUT DOES NOT ALTER CIGARETTE CONSUMPTION


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LSC, JSA, NLB, and RFT designed the overall clinical trial, while RFT designed the overall pharmacokinetic study. A description of both studies is found in Appendix B. After identifying gaps in the literature, MJC proposed research questions and planned a sub-study to answer specific research questions. MJC performed FMO3 and POR genotyping. AZXZ performed CYP2A6 genotyping. MJC and RFT contributed to the analytic strategy. MJC created a study database and performed all statistical analyses. MJC, AZXZ, LSC, JSA, NLB, and RFT all interpreted the results. MJC and RFT wrote the manuscript.
3.1. Abstract

The rates of nicotine metabolism differ widely, even after controlling for genetic variation in the major nicotine-metabolizing enzyme, CYP2A6. Genetic variants in an additional nicotine-metabolizing enzyme, flavin-containing monooxygenase (FMO)-3, and an obligate microsomal CYP-supportive enzyme, cytochrome P450 reductase (POR), were investigated. We examined the impact of FMO3 E158K and POR A503V before and after stratifying by CYP2A6 metabolism group. In 130 nonsmokers of African descent who received 4 mg oral nicotine, FMO3 158K trended toward slower nicotine metabolism in reduced CYP2A6 metabolizers (P=0.07) only, whereas POR 503V was associated with faster CYP2A6 activity (nicotine metabolite ratio) in normal (P=0.03), but not reduced, CYP2A6 metabolizers. Neither FMO3 158K nor POR 503V significantly altered the nicotine metabolic ratio (N=659), cigarette consumption (N=667), or urine total nicotine equivalents (N=418) in smokers of African descent. Thus, FMO3 E158K and POR A503V are minor sources of nicotine metabolism variation, insufficient to appreciably alter smoking.
3.2. Introduction

Smoking remains a leading preventable cause of morbidity and mortality (St Helen, Dempsey et al. 2013), with African North Americans showing a disproportionately elevated risk of disease despite smoking fewer cigarettes compared with Whites (St Helen, Dempsey et al. 2013). Nicotine is primarily inactivated by CYP2A6 to cotinine (COT), which undergoes further CYP2A6-mediated metabolism to 3'-hydroxycotinine (3HC), and 3HC/COT, a phenotypic measure of CYP2A6 activity, is highly correlated with nicotine clearance (Schoedel, Hoffmann et al. 2004). CYP2A6 variants such as CYP2A6*2, *4, *9, *12, *17, *20, *23-*28, and *35 (each with ≤7% frequency in African North Americans (Schoedel, Hoffmann et al. 2004; Mwenifumbo, Al Koudsi et al. 2008; Al Koudsi, Ahluwalia et al. 2009)) that reduce CYP2A6 activity lead to lower nicotine clearance and 3HC/COT (Mwenifumbo, Al Koudsi et al. 2008; Al Koudsi, Ahluwalia et al. 2009) and influence smoking (Schoedel, Hoffmann et al. 2004). Less is known on the contributions of other enzymes toward nicotine clearance, smoking, and tobacco-related disease.

Flavin-containing monoxygenase (FMO)-3 catalyzes hepatic nicotine N'-oxidation, a pathway responsible for ~4-7% of nicotine urinary recovery, but up to 30% in individuals with deleted CYP2A6 (Bloom, Murphy et al. 2013), suggestive of a greater reliance on FMO3-mediated nicotine metabolism when CYP2A6 activity is reduced. Variation in FMO3, which may impact nicotine metabolism, is associated with altered ratios of COT/(COT + nicotine) and cigarette consumption in Whites (Bloom, Murphy et al. 2013). We extend this investigation by examining the impact of FMO3 E158K (rs2266782 G>A), a variant with reduced FMO3 activity (e.g. for benzydamine and ranitidine (reviewed in Hisamuddin and Yang 2007), on systemic nicotine metabolism and smoking in populations of African descent. Here, the FMO3 pathway may be more important for nicotine clearance, relative to Whites, because of both higher frequencies of FMO3 158K (~48% vs. 39% in Whites (reviewed in Hisamuddin and Yang 2007)) and a greater

We also examined the impact of genetic variation in cytochrome P450 oxidoreductase (POR), a coenzyme required for microsomal CYP-mediated drug metabolism (Hu, Zhuo et al. 2012), on CYP2A6 activity and smoking. POR donates electrons to CYPs, and several variants in POR are associated with altered CYP activity, including reduced CYP3A4 and CYP2D6 activity (reviewed in Hu, Zhuo et al. 2012). POR A503V (rs1057868 C>T) (~19% frequency in African Americans; ~25-30% in Whites (Hu, Zhuo et al. 2012)), is associated with CYP-specific and substrate-specific effects on drug metabolism in vitro and higher CYP3A-mediated midazolam metabolism in vivo (reviewed in Hu, Zhuo et al. 2012). We hypothesized that POR 503V will increase nicotine and COT metabolism.

3.3. Materials and Methods

Study 1: Nicotine pharmacokinetics, following 4 mg oral nicotine, was studied in African Canadians (Mwenifumbo, Sellers et al. 2007). The area under the plasma nicotine–concentration time curve (AUC) was estimated over 360 min, and 3HC/COT was determined at 270 min (Mwenifumbo, Sellers et al. 2007). Participants were excluded if they had unreliable kinetic or CYP2A6 genetic data. The final sample utilized kinetic data from 130 nonsmokers and cigarette consumption data from 133 smokers. Study 2: Total nicotine equivalents were determined as the molar sum of nicotine and nine major metabolites in urine, corrected for creatinine, in 534 African American light smokers (≤10 cigarettes/day) (Cox, Nollen et al. 2012). DNA was extracted from blood samples for genotyping CYP2A6 (Mwenifumbo, Sellers et al. 2007), FMO3, and POR. The demographic characteristics of the participants have been published previously (Mwenifumbo, Sellers et al. 2007; Cox, Nollen et al. 2012).

also genotyped in study 1 (Mwenifumbo, Al Koudsi et al. 2008). Individuals with 1 or more variant alleles were grouped as ‘reduced CYP2A6 metabolizers’; individuals without were grouped as ‘normal CYP2A6 metabolizers’. \textit{FMO3} E158K and \textit{POR} A503V were genotyped using allele-specific TaqMan SNP genotyping assays (Applied Biosystems, Foster City, California, USA) with ~99% call confidence. Studies were approved by the University of Toronto Ethics Board, Institutional Review Board, University of Kansas human subjects committee, and the University of California San Francisco human subjects committee.

Hardy–Weinberg equilibrium was tested using chi-square tests. Shapiro–Wilk tests were used to determine data normality. Mann–Whitney U-tests (two-tailed) were used to compare outcomes between two genotype groups. Analyses were carried out using SPSS (version 21; IBM, Armonk, New York, USA).

**3.4. Results**

\textit{FMO3} 158K frequencies were 41.9 and 45.9% in study 1 and study 2, respectively. \textit{POR} 503V frequencies were 16.5 and 17.8% in study 1 and study 2, respectively. \textit{FMO3} E158K and \textit{POR} A503V frequencies, consistent with previous reports (Hisamuddin and Yang 2007; Hu, Zhuo et al. 2012), were in Hardy–Weinberg equilibrium.

We first examined associations between \textit{FMO3} E158K (G > A) variation and nicotine pharmacokinetics in study 1 nonsmokers who received oral nicotine using plasma nicotine AUC as the outcome measure. No association was observed between \textit{FMO3} E158K and nicotine AUC in the total group or in normal \textit{CYP2A6} metabolizers (Fig. 10). However, in reduced \textit{CYP2A6} metabolizers, there was a trend toward higher nicotine AUC in the \textit{FMO3} GA/AA versus GG group (mean, 2.0 vs. 1.4 µg/ml x min, respectively; \(P = 0.07\)) (Fig. 10).
Figure 10 | Association of FMO3 E158K and POR A503V variation with nicotine metabolism in African Canadian nonsmokers who received 4 mg oral nicotine. One hundred and thirty adult African Canadian nonsmokers received 4 mg oral nicotine and were genotyped for CYP2A6, FMO3 E158K, and POR A503V. Nicotine AUC according to FMO3 E158K (G > A) genotype group (GG vs. GA + AA) is shown in the total group (a), in individuals with normal CYP2A6 genotype (b), and in individuals with reduced CYP2A6 genotype (c). The 3HC/COT ratio according to the POR A503V (C > T) genotype group (CC vs. CT + TT) is shown in the total group (d), in individuals with normal CYP2A6 genotype (e), and in individuals with reduced CYP2A6 genotype (f). All P values indicated were derived from Mann–Whitney U-tests comparing FMO3 GG and FMO3 GA + AA groups, or comparing POR CC and POR CT + TT groups. The number of participants fluctuates slightly between FMO3 and POR genotype analyses, reflecting the number of participants for whom the allele was successfully genotyped and included in the analyses. AUC, area under the curve; COT, cotinine; FMO, flavin-containing monooxygenase; 3HC, 3’-hydroxycotinine; NIC, nicotine; POR, P450 oxidoreductase.
In contrast to FMO3, POR likely influences nicotine metabolism indirectly by supporting CYP2A6 activity. Therefore, we used 3HC/COT, a marker of CYP2A6 activity, to assess associations between POR A503V and nicotine metabolism. No association was observed between POR A503V and 3HC/COT in the total group or in reduced CYP2A6 metabolizers (Fig. 10). However, in normal CYP2A6 metabolizers, 3HC/COT was higher in the POR CT/TT relative to the CC group (mean, 0.25 vs. 0.18, respectively; \( P = 0.03 \)) (Fig. 10), suggesting faster CYP2A6 activity supported by the POR T allele. This was insufficient to significantly alter nicotine AUC in the POR CT/TT relative to the CC group (mean, 0.9 vs. 1.3 \( \mu \text{g/ml x min} \), respectively; \( P = 0.21 \)). When examining baseline plasma 3HC/COT from 659 smokers, no association was observed with POR A503V in the total group (\( P = 0.63 \)), or in reduced (\( P = 0.89 \)) or normal (\( P = 0.66 \)) CYP2A6 metabolizers. Similar relationships were observed for analyses of CT individuals alone, as were observed for the CT/TT group. Moreover, in 44 White livers with wild-type CYP2A6, there was no association between POR A503V and Vmax [mean, 30.2 vs. 30.7 nmol/mg protein/h in POR CC (\( N = 22 \)) vs. CT/TT (\( N = 22 \)), respectively; \( P = 0.45 \)] or Km (mean, 55.6 vs. 58.7 \( \mu \text{mol/l} \) in POR CC (\( N = 22 \)) vs. CT/TT (\( N = 22 \)), respectively; \( P = 0.81 \)) in nicotine C-oxidation assays (described in (Al Koudsi, Hoffmann et al. 2010)). We also found no significant relationship between POR rs17148944 (G > A), associated with reduced POR mRNA expression (Gomes, Winter et al. 2009), and nicotine AUC [mean, 1.5 vs. 1.2 \( \mu \text{g/ml x min} \) in POR GG (\( N = 118 \)) vs. GA (\( N = 12 \)), respectively; \( P = 0.58 \)] or 3HC/COT [mean, 0.16 vs. 0.21 in GG (\( N = 118 \)) vs. GA (\( N = 12 \)), respectively; \( P = 0.15 \)] in African Canadian nonsmokers.

Regression analyses (number of minor allele copies entered as the predictor) indicated that FMO3 E158K accounted for less than 1% and ~4% of the variation in nicotine AUC in normal and reduced CYP2A6 metabolizers, respectively, whereas POR A503V accounted for ~6 and ~1% of the variation in 3HC/COT in normal and reduced CYP2A6 metabolizers, respectively.

In contrast to the modest impact of FMO3 E158K and POR A503V on nicotine metabolism, CYP2A6 was associated strongly with differences in nicotine AUC and 3HC/COT. Relative to normal CYP2A6 metabolizers, reduced CYP2A6 metabolizers showed 50% higher nicotine AUC.
(mean, 1.8 vs. 1.2 µg/ml x min, respectively; \(P < 0.001\)) and 40% lower 3HC/COT (mean, 0.12 vs. 0.20, respectively; \(P < 0.001\)).

On examining relationships between \(FMO3\) E158K (G > A) and \(POR\) A503V (C > T) with cigarettes smoked per day (CPD) and urine nicotine equivalents (reflecting daily nicotine intake), a trend toward lower CPD was noted in the \(FMO3\) GA/AA group relative to the \(FMO3\) GG group (mean, 7.5 vs. 10.1, respectively; \(P = 0.05\)) among African Canadian reduced \(CYP2A6\) metabolizers, but this was not replicated in the African American smokers (Table 4). No other associations were observed between \(FMO3\) E158K and \(POR\) A503V with CPD, and neither SNP was associated with differences in total nicotine equivalents (Table 4).
<table>
<thead>
<tr>
<th>Genotype Group</th>
<th>Total group</th>
<th>Normal CYP2A6</th>
<th>Reduced CYP2A6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean cigarettes per day (± SD; N) in Study 1 African Canadian Smokers (N=133°)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>FMO3</em> E158K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA + AA</td>
<td>9.4 (5.7; 85)</td>
<td>10.5 (6.5; 53)</td>
<td>7.5 (3.3; 32)</td>
</tr>
<tr>
<td>GG</td>
<td>9.3 (5.0; 46)</td>
<td>8.9 (5.0; 29)</td>
<td>10.1 (5.2; 17)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.81</td>
<td>.30</td>
<td>.05</td>
</tr>
<tr>
<td><em>POR</em> A503V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT + TT</td>
<td>9.0 (5.4; 47)</td>
<td>9.3 (5.8; 31)</td>
<td>8.5 (4.6; 16)</td>
</tr>
<tr>
<td>CC</td>
<td>9.5 (5.4; 86)</td>
<td>10.2 (6.1; 53)</td>
<td>8.4 (4.0; 33)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.41</td>
<td>.29</td>
<td>.97</td>
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<tr>
<td><strong>Mean cigarettes per day (± SD; N) in Study 2 African American Smokers (N=534°)</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>FMO3</em> E158K</td>
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</tr>
<tr>
<td>GA + AA</td>
<td>7.9 (2.5; 369)</td>
<td>8.1 (2.5; 193)</td>
<td>7.8 (2.5; 176)</td>
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<tr>
<td>GG</td>
<td>7.9 (2.6; 161)</td>
<td>8.1 (2.9; 73)</td>
<td>7.8 (2.4; 88)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.84</td>
<td>.93</td>
<td>.85</td>
</tr>
<tr>
<td><em>POR</em> A503V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT + TT</td>
<td>7.8 (2.5; 160)</td>
<td>8.1 (2.6; 78)</td>
<td>7.5 (2.4; 82)</td>
</tr>
<tr>
<td>CC</td>
<td>8.0 (2.5; 373)</td>
<td>8.1 (2.6; 191)</td>
<td>7.9 (2.5; 182)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.31</td>
<td>.87</td>
<td>.20</td>
</tr>
<tr>
<td><strong>Mean total nicotine equivalents&lt;sup&gt;d&lt;/sup&gt; (± SD; N) in Study 2 African American Smokers (N=418°)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>FMO3</em> E158K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA + AA</td>
<td>59.1 (60.9; 292)</td>
<td>60.5 (40.7; 153)</td>
<td>57.6 (77.4; 139)</td>
</tr>
<tr>
<td>GG</td>
<td>56.5 (45.7; 122)</td>
<td>58.4 (58.1; 56)</td>
<td>54.9 (32.0; 66)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.88</td>
<td>.45</td>
<td>.26</td>
</tr>
<tr>
<td><em>POR</em> A503V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT + TT</td>
<td>54.3 (36.7; 128)</td>
<td>54.4 (37.5; 64)</td>
<td>54.1 (36.2; 64)</td>
</tr>
<tr>
<td>CC</td>
<td>60.2 (63.5; 289)</td>
<td>62.5 (48.8; 148)</td>
<td>57.9 (76.1; 141)</td>
</tr>
<tr>
<td>P value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>.50</td>
<td>.22</td>
<td>.73</td>
</tr>
</tbody>
</table>

FMO, flavin-containing monooxygenase; POR, P450 oxidoreductase.

<sup>a</sup>The number of participants fluctuates slightly between *FMO3* and *POR* genotype analyses, reflecting the number of participants for whom the allele was successfully genotyped and included in the analyses.

<sup>b</sup>Derived from the Mann-Whitney U test (*FMO3* GG group vs. *FMO3* GA + AA groups).

<sup>c</sup>Derived from the Mann-Whitney U test (*POR* CC group vs. *POR* CT + TT groups).

<sup>d</sup>Measured in nmol/mg creatinine.
3.5. Discussion

Our findings suggest that *FMO3* E158K and *POR* A503V modestly influence systemic nicotine metabolism within *CYP2A6* subgroups, but do not appreciably alter CPD or daily nicotine intake. The trend toward lower CPD with *FMO3* 158K in African Canadian reduced *CYP2A6* metabolizers was not replicated in African American smokers. Although these latter smokers consumed 10 or less CPD (a clinical trial inclusion criteria), their total nicotine equivalents values are similar to those reported in White heavier smokers (St Helen, Dempsey et al. 2013), suggesting similar levels of nicotine intake because of greater smoking intensity.

In European American heavy smokers (~20–25 CPD), variation in the *FMO3* haplotype was reported to be associated with CPD, although the effect (~3 CPD difference) was restricted to individuals predicted to have faster CYP2A6 activity (Bloom, Murphy et al. 2013). Discrepancies between these findings and ours may reflect differences in study design related to *CYP2A6/FMO3* grouping strategies, haplotype versus allele assessments, and/or heterogeneity in study populations attributable to ethnicity and heaviness of smoking.

Six human *FMO* genes exist, encoding five functional FMO enzymes (Hisamuddin and Yang 2007). FMO1 is the major fetal hepatic FMO isoform, but in adults, it is expressed in extrahepatic tissues, likely including the brain (Hinrichs, Murphy et al. 2011). FMO1 also catalyzes nicotine metabolism (Hinrichs, Murphy et al. 2011), and variation in *FMO1* is associated with nicotine dependence, perhaps through altered brain metabolism of nicotine (Hinrichs, Murphy et al. 2011); further study of associations between this enzyme family and smoking behaviors may be warranted.

In conclusion, *FMO3* E158K and *POR* A503V do not considerably impact nicotine metabolism in African North Americans, consistent with the lack of effect on daily smoking or nicotine intake. Although African Americans smoke fewer cigarettes than Whites, they appear to achieve similar levels of exposure to nicotine and experience a disproportionately higher prevalence of tobacco-related diseases (St Helen, Dempsey et al. 2013). Future investigations examining the genetic and environmental determinants of tobacco consumption and tobacco-related diseases among populations of African descent are warranted.
3.6. Significance to Thesis

This chapter adds to the existing literature regarding sources of variation in nicotine metabolism and the resultant impact on smoking behaviour. While it is biologically plausible that genetic variation in a relatively minor nicotine-metabolizing enzyme (i.e., FMO3) and in a coenzyme required for CYP2A6 activity (i.e., POR) could influence nicotine metabolism, we found little association between the investigated FMO3 and POR variant alleles and altered nicotine clearance (i.e., nicotine AUC) or CYP2A6 activity (i.e., the NMR). It is not surprising, then, that these variant alleles did not substantially affect smoking behaviour (i.e., cigarettes/day) or nicotine intake (i.e., total nicotine equivalents). Overall, this chapter provides evidence that these gene variants do not substantially contribute to nicotine metabolism variation/NMR and smoking behaviours. The process of ruling out sources of variation in nicotine metabolism/NMR may be as important as identifying significant sources of NMR variation; this chapter suggests that variability in FMO3 and POR are unlikely to confound associations between the rate of nicotine metabolism and smoking behaviours and/or tobacco dose.

This chapter is also important from a clinical perspective, as assays to assess NMR are currently being developed for use in treatment settings to assign pharmacotherapy in order to optimize quit rates. While genetic variation in general would not change a smoker’s NMR throughout the course of treatment, it is likely worthwhile to understand sources of variation in any biomarker that is being developed to optimize clinical outcomes.
4 CHAPTER 3: KNOWN AND NOVEL SOURCES OF VARIABILITY IN THE NICOTINE METABOLITE RATIO IN A LARGE SAMPLE OF TREATMENT-SEEKING SMOKERS

Meghan J. Chenoweth, Maria Novalen, Larry W. Hawk Jr, Robert A. Schnoll, Tony P. George, Paul M. Cinciripini, Caryn Lerman, and Rachel F. Tyndale

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CL and RFT designed and implemented the overall clinical trial. A description of the overall study is found in Appendix C. After identifying gaps in the literature and developing specific research questions, MJC used a data dictionary to design a secondary analysis to answer the research questions. MN performed LC-MS/MS analysis to determine participants’ NMR. All authors contributed to the analytic strategy. MJC created a study database and performed all statistical analyses. MJC, LWH Jr., RAS, TPG, PMC, CL and RFT interpreted the results. MJC and RFT wrote the manuscript.
4.1. Abstract

**Background:** The ratio of 3’hydroxycotinine to cotinine, or nicotine metabolite ratio (NMR), is strongly associated with CYP2A6 genotype, CYP2A6-mediated nicotine and cotinine metabolism, and nicotine clearance. Higher NMR (faster nicotine clearance) is associated retrospectively with heavier smoking and lower cessation rates.

**Methods:** NMR as a predictive biomarker of cessation outcomes is being investigated (NCT01314001). In addition to strong CYP2A6 genetic influences on NMR, demographic and hormonal factors alter NMR. Here, we analyzed, for the first time together, these sources of variation on NMR in smokers screened for this clinical trial (N = 1,672).

**Results:** Participants (mean age = 45.9) were 65.1% Caucasian, 34.9% African American, and 54.8% male. Mean NMR (SD) was higher in Caucasians versus African Americans [0.41 (0.20) vs. 0.33 (0.21); \(P < 0.001\)], and in females versus males [0.41 (0.22) vs. 0.37 (0.20); \(P < 0.001\)]. Among females, birth control pill use (N = 17) and hormone replacement therapy (N = 14) were associated with 19.5% (\(P = 0.09\)) and 29.3% (\(P = 0.06\)) higher mean NMR, respectively, albeit nonsignificantly. BMI was negatively associated with NMR (Rho = -0.14; \(P < 0.001\)), whereas alcohol use (Rho = 0.11; \(P < 0.001\)) and cigarette consumption (Rho = 0.12; \(P < 0.001\)) were positively associated with NMR. NMR was 16% lower in mentholated cigarette users (\(P < 0.001\)). When analyzed together in a linear regression model, these predictors (each \(\leq 2\%\)) accounted for <8% of total NMR variation.

**Conclusions:** Although these factors significantly affected NMR, they contributed little (together <8%; each \(\leq 2\%\)) to total NMR variation.

**Impact:** Thus, when using NMR, for example, to prospectively guide smoking cessation therapy, these sources of variation are unlikely to cause NMR misclassification.
4.2. Introduction

Tobacco use remains a leading cause of morbidity and mortality worldwide, and life expectancy is shortened by more than 10 years in smokers (Jha, Ramasundarahettige et al. 2013). If current trends in smoking prevalence continue, tobacco use is projected to kill one billion people worldwide during the 21st century (World Health Organization, 2008), underscoring the need for improved smoking prevention and cessation strategies. One approach to improving smoking cessation rates and reducing the global burden of disease from tobacco may involve the personalization of smoking cessation pharmacotherapies using validated biomarkers that predict treatment success (Bough, Lerman et al. 2013). A diagnostic and predictive biomarker of smoking cessation outcomes with potential clinical utility is the nicotine metabolite ratio (NMR; (Bough, Lerman et al. 2013)).

Nicotine is the major psychoactive compound in cigarette smoke responsible for the reinforcing properties associated with cigarette smoking and the development of tobacco addiction (Benowitz 2010). The majority (~80%) of nicotine is metabolically inactivated to cotinine, in a reaction predominantly catalyzed by CYP2A6 (Nakajima, Yamamoto et al. 1996a). Cotinine undergoes further metabolism to 3’hydroxycotinine, in a reaction exclusively mediated by CYP2A6 (Nakajima, Yamamoto et al. 1996b; Dempsey, Tutka et al. 2004). The ratio of 3’hydroxycotinine/cotinine, known as the NMR, is a biomarker of CYP2A6 genotype activity, as well as nicotine metabolism rate, and it correlates strongly with total nicotine clearance (Dempsey, Tutka et al. 2004; Binnington, Zhu et al. 2012). The NMR has been shown retrospectively to be associated with smoking cessation success in multiple clinical trials involving heavy and light smokers, of Caucasian and African American descent, respectively (Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009; Schnoll, Patterson et al. 2009). Individuals with lower NMR, indicative of lower CYP2A6 activity and slower nicotine clearance, displayed higher quit rates on transdermal nicotine (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009) and nicotine gum (Ho, Mwenifumbo et al. 2009), relative to individuals with higher NMR. In contrast, there were no differences in quit rates on bupropion (a non-CYP2A6 substrate) between NMR groups; however, among those receiving counseling and placebo, those with lower NMR had higher quit rates (Patterson, Schnoll et al. 2008).
In addition to cessation, NMR and CYP2A6 genotype are associated with smoking acquisition, the level of cigarette consumption, as well as nicotine dependence (O’Loughlin, Paradis et al. 2004; Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Audrain-McGovern, Al Koudsi et al. 2007; Wassenaar, Dong et al. 2011; Sofuoglu, Herman et al. 2012; Schnoll, George et al. 2014). Those with slower nicotine metabolism rates, determined via NMR or CYP2A6 genotype, display lower self-reported cigarettes smoked per day (Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Wassenaar, Dong et al. 2011), lower total nicotine intake (Benowitz, Pomerleau et al. 2003; Johnstone, Benowitz et al. 2006; Falcone, Jepson et al. 2011; Zhu, Binnington et al. 2013), lower nicotine dependence (Sofuoglu, Herman et al. 2012; Schnoll, George et al. 2014), and lower total puff volumes resulting in lower carcinogen exposure (Strasser, Benowitz et al. 2011). The relationship between lower NMR and reduced cigarette consumption/nicotine dependence scores may be more pronounced in men than in women (Schnoll, George et al. 2014) and in younger cohorts and smokers not seeking treatment (Schoedel, Hoffmann et al. 2004; Lerman, Tyndale et al. 2006). CYP2A6 genotype is also associated with lung cancer risk; those with reduced activity CYP2A6 genotypes (i.e., slower metabolizers) have a lower risk of developing lung cancer (Ariyoshi, Miyamoto et al. 2002; Fujieda, Yamazaki et al. 2004; Tamaki, Arai et al. 2011; Wassenaar, Dong et al. 2011). The reduced lung cancer risk among slower metabolizers likely stems from both lower levels of smoking and lower metabolic activation of tobacco-specific nitrosamines (Zhu, Binnington et al. 2013).

One advantage to using NMR rather than CYP2A6 genotype as a biomarker of nicotine metabolism rate is that it includes both genetic and environmental sources of variation in nicotine metabolism and clearance. Here, we investigate the influence of nongenetic sources (specifically non-CYP2A6 genetic variation) of variation on NMR. If these sources of variation have a relatively small impact on NMR, and NMR is shown to prospectively predict cessation outcomes, this would further support the utility of NMR as a prospective biomarker to guide treatment assignment. In addition to CYP2A6 genotype (Swan, Lessov-Schlaggar et al. 2009), a number of factors contribute to interindividual variability in NMR, including ethnicity (Benowitz, Pomerleau et al. 2003; Kandel, Hu et al. 2007; Derby, Cuthrell et al. 2008), sex
(Benowitz, Lessov-Schlaggar et al. 2006; Mwenifumbo, Sellers et al. 2007), birth control pill use (Benowitz, Lessov-Schlaggar et al. 2006), body mass index (BMI; (Mooney, Li et al. 2008)), and potentially mentholated cigarette use (MacDougall, Fandrick et al. 2003; Benowitz, Herrera et al. 2004). NMR is higher among Caucasians relative to African Americans and Asians (Benowitz, Pomerleau et al. 2003; Kandel, Hu et al. 2007; Derby, Cuthrell et al. 2008; Binnington, Zhu et al. 2012), reflecting the lower frequency of slower-activity CYP2A6 genetic variants in Caucasians relative to African and Asian populations (Schoedel, Hoffmann et al. 2004; Haberl, Anwald et al. 2005; Nakajima, Fukami et al. 2006; Mwenifumbo and Tyndale 2007). NMR is also higher among premenopausal women relative to men, and even higher among women taking estrogen-containing birth control pills (Benowitz, Lessov-Schlaggar et al. 2006; Mwenifumbo, Sellers et al. 2007). In contrast, there are no differences in NMR between men and menopausal or postmenopausal women (Benowitz, Lessov-Schlaggar et al. 2006).

Although several smaller studies have investigated individual influences on NMR, a comprehensive analysis to characterize these relationships simultaneously in one large population has not been performed. Moreover, to date the relationship between NMR and alcohol use has not been investigated, despite the common co-use of smoking and alcohol and the impact of alcohol on smoking cessation success (Shiffman, Paty et al. 1996; Bobo and Husten 2000). In contrast with the well-characterized CYP2A6 genotype contribution to variation in NMR, this article describes environmental influences that are less understood. We divided our analysis into three parts. We first examined previously known influences on NMR (i.e., ethnicity, gender, exogenous estrogen-based hormonal therapies, and BMI). We next characterized relationships between NMR, alcohol use, mentholated cigarette use, and the level of cigarette consumption. Our final objective was to quantify the overall influence of these predictors on NMR, to determine if they, alone or together, represent a substantial source of variation in this biomarker.

4.3. Materials and Methods

Study subjects and data collection
Treatment-seeking adults (ages 18–65) smoking ≥10 cigarettes per day for the past 6 months responded to advertisements for a smoking cessation clinical trial (NCT01314001). Exclusion
criteria included the use of chewing tobacco, snuff or snus; recent treatment for substance abuse; current cocaine or opiate abuse; the consumption of >25 standard alcoholic drinks/week; current depression, mania, schizophrenia, or post-traumatic stress disorder; recent use of antipsychotics, antidepressants, prescription stimulants, metformin, cimetidine, cardiac medications, or other anticoagulants; and the daily use of prescription opiates/inhalers. Those interested in participating after meeting eligibility criteria provided a blood sample for NMR determination, collected when participants were smoking as usual. The detailed study protocol, including NMR determination, is described in a previous analysis of NMR and three self-report measures of nicotine dependence in a subset of the trial participants (N = 833 of 1,807 screened by NMR; (Schnoll, George et al. 2014)). Briefly, cotinine and 3’hydroxycotinine were assessed from whole blood by liquid chromatography–tandem mass spectrometry (LC/MS-MS) using a previously validated method (Jacob, Yu et al. 2011; St Helen, Novalen et al. 2012). NMR data were available on a total of 1,807 eligible participants screened at the four clinical sites: the University of Pennsylvania (N = 487), the Centre for Addiction and Mental Health (CAMH) at the University of Toronto (N = 430), the MD Anderson Cancer Center (Houston, TX; N = 443) and the University at Buffalo, SUNY (Buffalo, NY; N = 447). Survey data on demographic variables (including age, gender, and ethnicity) and smoking history were collected, as well as height and weight measurements to compute BMI. Data were also collected from female participants on the use of oral contraceptives and hormone replacement therapies. Data on mentholated cigarette use were collected from the subset of participants in the intent-to-treat (ITT) group (N = 1,155), assessed when they received their study medication and completed the first counseling session. Informed consent was obtained from each participant. The study was approved by Institutional Review Boards at each site.

Statistical analysis

All statistical analyses were completed using SPSS Version 22 (IBM Corporation). The Shapiro–Wilk test was used to determine whether continuous variables were normally distributed. Mann–Whitney U Tests (two-tailed) and chi-square tests were used to compare continuous and categorical outcome measures, respectively, between two groups. The strength of correlation between continuous variables was assessed using Spearman rank correlation coefficient.
A univariate analysis of variance model was used to determine whether ethnicity and gender interact to influence NMR (2 x 2 factorial design). Hierarchical linear regression models were used to determine whether cigarette consumption confounds the association (i) between BMI and NMR, or (ii) between alcohol use and NMR. In these models, breath CO (carbon monoxide; a biomarker of cigarette consumption) was entered in block 1, and the predictor (BMI or alcohol use) was entered in block 2. Separate hierarchical linear regression models were also used to test whether BMI and alcohol use interact with ethnicity and/or gender to influence NMR. The single predictors (BMI and alcohol use) together with ethnicity or gender were entered in block 1, and the interaction term (e.g., BMI x gender) was entered in the second block. We used a univariate analysis of variance model to determine whether mentholated cigarette use and ethnicity interact to influence NMR (2 x 2 factorial design). A univariate analysis of variance model was also used to determine whether NMR stratum (faster vs. slower metabolism) interacts with either ethnicity and/or gender to influence cigarettes per day (CPD) (2 x 2 x 2 factorial design).

A linear regression model was also used to assess the variation in NMR accounted for by each of the predictors, and their combined overall contribution to NMR variability. The predictors [ethnicity, gender, birth control pill use, hormone replacement therapy (HRT) use, BMI, alcohol consumption (number of standard drinks/wk), and cigarettes/d) were entered simultaneously into the model. The overall contribution of the predictors to NMR variation was assessed by examining the model $R^2$ value. The unique and individual contribution of each predictor to overall NMR variation was assessed by squaring the part correlation coefficients and multiplying by 100%. A separate and similar model was also run in the ITT subgroup (N = 1,155), with and without the use of mentholated cigarettes as a predictor. A Pearson chi-square test was used to determine whether the prevalence of mentholated cigarette use in African Americans and Caucasians was different.

4.4. Results

Participant demographics

Of the 1,807 eligible subjects with NMR, 55.7% were male with a mean age of 45.4 years and mean BMI of 29.4. The majority of the subjects were Caucasian (60.3%) and African American (32.3%), with a small number of subjects reporting Asian (3.4%), American Indian/Alaska native
(0.3%), Hawaiian/Polynesian (0.1%), or "more than one" or "other" race (3.7%). We restricted all further analyses herein to Caucasians and African Americans with NMR (N = 1,672), as the small numbers of subjects from the four additional racial groupings (N = 135 total) precluded meaningful statistical analysis. Participant characteristics of the final analytic sample are shown in Table 5.
Table 5 | Demographic characteristics of Caucasian and African American participants with NMR (N = 1672)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Female, N=756</td>
<td>45.2</td>
</tr>
<tr>
<td>Mean age (SD), N=1672</td>
<td>45.9 (11.0)</td>
</tr>
<tr>
<td>% Caucasian, N=1089</td>
<td>65.1</td>
</tr>
<tr>
<td>% African American, N=583</td>
<td>34.9</td>
</tr>
<tr>
<td>Mean BMI (SD), N=1672</td>
<td>29.5 (6.5)</td>
</tr>
<tr>
<td>Mean number of drinks/wk (SD), N=1672</td>
<td>3.3 (5.2)</td>
</tr>
<tr>
<td>Mean cigarettes/d (SD), N=1672</td>
<td>18.7 (7.5)</td>
</tr>
<tr>
<td>Mean breath CO, ppm (SD), N=1672</td>
<td>23.3 (10.2)</td>
</tr>
<tr>
<td>NMR in total group, N=1672</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>0.38 (0.21)</td>
</tr>
<tr>
<td>Median</td>
<td>0.35</td>
</tr>
<tr>
<td>Range</td>
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<tr>
<td>Skewness</td>
<td>1.51</td>
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<tr>
<td>Kurtosis</td>
<td>4.87</td>
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<tr>
<td>NMR in ITT group, N=1155^</td>
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</tr>
<tr>
<td>Mean (SD)</td>
<td>0.35 (0.20)</td>
</tr>
<tr>
<td>Median</td>
<td>0.30</td>
</tr>
<tr>
<td>Range</td>
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<td>1.39</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>2.63</td>
</tr>
</tbody>
</table>

Abbreviation: ppm, parts per million.

^Participants with lower NMR were oversampled in the clinical trial, reflecting the lower NMR values observed in the ITT group versus in the total group.
Factors (ethnicity, gender, exogenous estrogen, and BMI) known to influence NMR

Mean NMR was higher in Caucasians than in African Americans [0.41 (0.20) vs. 0.33 (0.21), \( P < 0.001 \)] and in females compared with males [0.41 (0.22) vs. 0.37 (0.20), \( P < 0.001 \); Fig. 11].

The interaction term (ethnicity x gender) was not significant \( [F(1, 1,668) = 1.06, P = 0.30] \). Next, we examined the influence of estrogen-containing birth control pill use and estrogen-containing HRT use on NMR in women. Relative to nonusers (N = 739), females that reported current birth control pill use (N = 17) had 19.5% higher mean (SD) NMR [0.49 (0.24) vs. 0.41 (0.22), respectively, \( P = 0.09 \); Fig. 12]. Mean (SD) NMR was 29.3% higher among HRT users (N = 14) relative to nonusers [N = 742; 0.53 (0.29) vs. 0.41 (0.22), respectively, \( P = 0.06 \); Fig. 12]. We noted similar relationships in Caucasian females (Fig. 12); in the African Americans, there were only 4 females in total using either birth control pills or HRT, precluding our ability to assess this impact.

In the total group, BMI was negatively correlated with NMR (Rho = -0.14, \( P < 0.001 \); Fig. 13), which remained significant after controlling for breath CO, a biomarker of cigarette consumption (linear regression model \( R^2 \) change after controlling for CO = 0.018, \( P < 0.001 \)). In a separate linear regression model, after controlling for main effects (BMI and ethnicity), the interaction term (BMI x ethnicity) was not significant (\( P = 0.91 \); model \( R^2 \) change = 0.0), suggesting that the relationship between BMI and NMR is similar in Caucasians and African Americans. Likewise, after controlling for main effects, BMI and gender did not interact to influence NMR (\( P = 0.68 \); model \( R^2 \) change = 0.0), suggesting a similar relationship between BMI and NMR in both males and females.
Figure 11 | Variation in the NMR according to ethnicity and gender in Caucasian and African American adult smokers. NMR is shown as a function of ethnicity (A) and gender (B) in the total group (Mann–Whitney U tests).
Figure 12 | Association between exogenous estrogen-containing therapy and NMR among females. NMR is shown as a function of estrogen-containing birth control (BC) pill use in all females and Caucasian females (A) and as a function of estrogen-containing HRT use in all females and Caucasian females (B; Mann–Whitney U tests).
Figure 13 | Associations for NMR with BMI, alcohol consumption, cigarette use, and menthol. Correlations between BMI and NMR (A) and alcohol use (# standard drinks/week) and NMR (B) are shown in the total group. The association between mentholated cigarette use and NMR in the ITT group (in which it was available) is shown in (C), whereas the association between NMR, as a median split, and CPD is depicted in the total group in (D; Mann–Whitney U tests).
Associations of alcohol use, mentholated cigarette use, and cigarette consumption with NMR

Alcohol use (range = 0–25 standard drinks/wk) was positively associated with NMR in the total group (Rho = 0.11, P < 0.001; Fig. 13), even after controlling for levels of smoking using breath CO (linear regression model $R^2$ change after controlling for CO = 0.008, $P < 0.001$).

In a linear regression model, after controlling for main effects (alcohol use and ethnicity), the interaction term (alcohol use x ethnicity) was not significant ($P = 0.73$; model $R^2$ change = 0.0), suggesting a similar relationship in Caucasians and African Americans. Similarly, after controlling for alcohol use and gender, the interaction term (alcohol use x gender) was not significant ($P = 0.65$; model $R^2$ change = 0.0), suggesting a similar relationship in males and females.

We next investigated the potential influence of mentholated cigarette use on NMR. Menthol inhibits CYP2A6 activity in vitro (MacDougall, Fandrick et al., 2003) and nicotine clearance in vivo (Benowitz, Herrera et al., 2004), and, therefore, may result in lower NMR. In the ITT subgroup (N = 1,155), in which menthol use data were available, the prevalence of mentholated-cigarette use was 22.7% and 85.6% among Caucasian and African American smokers, respectively ($P < 0.001$). Those smoking mentholated cigarettes (N = 550) displayed significantly lower mean (SD) NMR compared with those smoking nonmentholated cigarettes [(N = 601; 0.32 (0.20) vs. 0.37 (0.20), respectively; $P < 0.001$; Fig. 13]. We used a 2 x 2 factorial design to evaluate whether the association between mentholated-cigarette use and NMR was similar across ethnicities. There was no significant main effect of mentholated-cigarette use on NMR [$F(1, 1,147) = 1.62, P = 0.20$], whereas a significant effect of ethnicity [$F(1, 1,147) = 10.82, P = 0.001$] was observed. The interaction term (mentholated-cigarette use x ethnicity) was not significant [$F(1, 1,147) = 3.51, P = 0.06$].

We next evaluated the relationship between NMR and CPD. In contrast with the influence of selected variables on NMR described above, variation in CYP2A6 activity, measured by CYP2A6 genotype or NMR, has been previously shown to influence cigarette consumption (i.e., faster metabolizers smoke more heavily (O'Loughlin, Paradis et al. 2004; Audrain-McGovern, Al
Koudsi et al. 2007; Wassenaar, Dong et al. 2011; Schnoll, George et al. 2014)). Thus, we were interested in investigating a potential effect of NMR (slow vs. fast nicotine metabolism) on CPD. We included NMR [median split; ≤0.350 (N = 838) vs. >0.350 (N = 834)] as the predictor variable and CPD (continuous measure) as the outcome variable. Mean (SD) CPD was 17.9 (6.8) and 19.5 (8.1) in those with lower versus higher NMR, respectively (P < 0.001; Fig. 13). We ran a 2 x 2 x 2 factorial model to evaluate potential interactions between ethnicity, gender, and NMR on CPD. There were significant main effects of NMR [F(1, 1,664) = 5.91, P = 0.02], ethnicity [F(1, 1,664) = 89.03, P < 0.001], and gender [F(1, 1,664) = 15.51, P < 0.001] on CPD. The only significant interaction term in the model was ethnicity x gender [F(1, 1,664) = 6.16, P = 0.01].

The NMR cutoff point of 0.31 was used in the clinical trial (NCT01314001) to differentiate slower from faster metabolizers, to randomize participants to treatment based on NMR, and to compare treatment outcomes from this clinical trial. Thus, we also used an NMR cutoff point of 0.31 to stratify participants into slower (≤0.31; N = 679) and faster (>0.31; N = 993) NMR groups and to evaluate potential interactions between ethnicity, gender, and NMR (2 x 2 x 2 factorial model) on CPD. There were significant main effects of NMR [F(1, 1,664) = 5.87, P = 0.02], ethnicity [F(1, 1,664) = 84.93, P < 0.001], and gender [F(1, 1,664) = 11.34, P = 0.001] on CPD. The only significant interaction term in the model was ethnicity x gender [F(1, 1,664) = 6.16, P = 0.01].

To further illustrate these various relationships with NMR, we compared alcohol consumption, BMI, as well as CPD and CO across NMR groups using the cutoff point of 0.31. Consistent with the analyses above, slower metabolizers (lower NMR) displayed lower alcohol and cigarette consumption, but higher BMI, relative to faster metabolizers in the total group (Table 6, also shown for the ITT subgroup).
Table 6 | Comparison of BMI, alcohol consumption, cigarette consumption, and breath CO according to NMR metabolism group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Slower metabolizers (NMR ≤0.310)</th>
<th>Faster metabolizers (NMR &gt;0.310)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group (N = 1672)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BMI&lt;sup&gt;a&lt;/sup&gt; (SD; N)</td>
<td>30.3 (7.0; 679)</td>
<td>29.0 (6.2; 993)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean # drinks/wk (SD; N)</td>
<td>2.9 (4.9; 679)</td>
<td>3.7 (5.3; 993)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean cigarettes/d (SD; N)</td>
<td>17.6 (6.6; 679)</td>
<td>19.5 (8.0; 993)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Breath CO, ppm (SD; N)</td>
<td>22.5 (9.9; 679)</td>
<td>23.9 (10.4; 993)</td>
<td>0.008</td>
</tr>
<tr>
<td>Intent-to-Treat group (N = 1155)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BMI&lt;sup&gt;a&lt;/sup&gt; (SD; N)</td>
<td>30.4 (7.1; 611)</td>
<td>29.3 (6.2; 544)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean # drinks/wk (SD; N)</td>
<td>2.9 (5.0; 611)</td>
<td>3.7 (5.3; 544)</td>
<td>0.002</td>
</tr>
<tr>
<td>Mean cigarettes/d (SD; N)</td>
<td>17.6 (6.6; 611)</td>
<td>19.0 (7.4; 544)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Breath CO, ppm (SD; N)</td>
<td>22.9 (10.0; 611)</td>
<td>23.6 (10.3; 544)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Abbreviation: ppm, parts per million.
<sup>a</sup>Measured in kg/m<sup>2</sup>
<sup>b</sup>Derived from Mann-Whitney U tests (two-tailed).
Regression analysis identifying significant predictors of NMR

In the overall model, ethnicity, gender, HRT use, BMI, CPD, and number of alcohol drinks per week were significant predictors of NMR, whereas birth control pill use trended toward significance (Table 7, men were coded as "0" for birth control pill use and HRT). The overall model $R^2$ value was 0.076, indicating these variables accounted for 7.6% of the variation in NMR; each variable uniquely contributed ≤2% of the variation in NMR. We then ran the same model in females only (see footnote to Table 7). The overall $R^2$ value for the model, which included all predictors except gender, was 0.066. The impact of birth control pill use and HRT on NMR was of a similar magnitude in the female-only group, as for the total group.

We also ran a separate model examining the impact of these predictors on NMR among those in the ITT subgroup (N = 1,155; Table 8); a similar percentage of contribution (6.5%) to NMR variability was observed. Mentholated cigarette use (available in this subgroup) did not significantly contribute to NMR variation, and its inclusion in the model did not substantially alter the regression coefficients of the other variables (Table 8, footnote contains data on women only). Notably, HRT use was associated with a relatively large unstandardized (B) coefficient in both the total (B = 0.11) and ITT (B = 0.16) groups, suggesting a potential impact on NMR in those receiving HRT.
### Table 7 | Linear regression analysis of the predictors of the NMR in the total sample (N = 1672)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>NMR</th>
<th>$R^2 = 0.076^a; P &lt;0.001$</th>
<th>$B$</th>
<th>Standard error</th>
<th>$\beta$</th>
<th>$P$</th>
<th>% of Variation$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity$^c$</td>
<td>0.071</td>
<td>0.011</td>
<td>0.162</td>
<td>&lt;0.001</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender$^{d,e}$</td>
<td>0.057</td>
<td>0.010</td>
<td>0.136</td>
<td>&lt;0.001</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth control pill use</td>
<td>0.036</td>
<td>0.050</td>
<td>0.017</td>
<td>0.47</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT use</td>
<td>0.114</td>
<td>0.054</td>
<td>0.050</td>
<td>0.036</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.003</td>
<td>0.001</td>
<td>-0.108</td>
<td>&lt;0.001</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol use (# drinks/wk)</td>
<td>0.003</td>
<td>0.001</td>
<td>0.070</td>
<td>0.004</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cigarettes/d</td>
<td>0.002</td>
<td>0.001</td>
<td>0.062</td>
<td>0.012</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Together the predictors account for 7.6% of the variation in NMR.
$^b$Calculated by squaring the part correlation coefficient (not shown), and multiplying by 100.
$^c$African Americans and Caucasians were coded as “0” and “1”, respectively, in the model.
$^d$Males and females were coded as “0” and “1”, respectively, in the model.
$^e$When we restricted the model to females only (N = 756), to further examine the effect of birth control pill and HRT use, the predictors (gender is excluded) together explained 6.6% of the variation in NMR. The standardized beta values for birth control pill and HRT use were 0.025 (P = 0.48) and 0.069 (P = 0.052), respectively, in the female-only group. They uniquely contributed 0.06% and 0.48% of the variation in NMR, respectively, in females.
Table 8 | Linear regression analysis of the predictors of the NMR in the ITT group (N = 1155)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>B</th>
<th>Standard error</th>
<th>β</th>
<th>P</th>
<th>% of Variationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicityc</td>
<td>0.048 (0.053)</td>
<td>0.015 (0.012)</td>
<td>0.118 (0.130)</td>
<td>0.002 (&lt;0.001)</td>
<td>0.81 (1.5)</td>
</tr>
<tr>
<td>Genderd,e</td>
<td>0.058 (0.058)</td>
<td>0.012 (0.012)</td>
<td>0.144 (0.144)</td>
<td>&lt;0.001 (&lt;0.001)</td>
<td>1.9 (1.9)</td>
</tr>
<tr>
<td>Birth control pill use</td>
<td>0.031 (0.030)</td>
<td>0.057 (0.057)</td>
<td>0.016 (0.015)</td>
<td>0.58 (0.60)</td>
<td>0.03 (0.02)</td>
</tr>
<tr>
<td>HRT use</td>
<td>0.158 (0.158)</td>
<td>0.062 (0.062)</td>
<td>0.074 (0.073)</td>
<td>0.010 (0.011)</td>
<td>0.53 (0.53)</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.003 (-0.003)</td>
<td>0.001 (0.001)</td>
<td>-0.087 (-0.088)</td>
<td>0.004 (0.003)</td>
<td>0.69 (0.71)</td>
</tr>
<tr>
<td>Alcohol use (# drinks/wk)</td>
<td>0.002 (0.002)</td>
<td>0.001 (0.001)</td>
<td>0.056 (0.055)</td>
<td>0.062 (0.062)</td>
<td>0.29 (0.28)</td>
</tr>
<tr>
<td>Cigarettes</td>
<td>0.002 (0.002)</td>
<td>0.001 (0.001)</td>
<td>0.077 (0.077)</td>
<td>0.010 (0.011)</td>
<td>0.55 (0.53)</td>
</tr>
<tr>
<td>Menthol</td>
<td>0.007</td>
<td>0.015</td>
<td>0.018</td>
<td>0.627</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE: Numbers in parentheses indicate values when the mentholated cigarette use variable is removed from the model to facilitate comparison with the total group in Table 2 in which this variable is missing.

*Together the predictors account for 6.5% of the variation in NMR with and without mentholated cigarette use in the model.
*Calculated by squaring the part correlation coefficient (not shown), and multiplying by 100.
*African Americans and Caucasians were coded as “0” and “1”, respectively, in the model.
*Males and females were coded as “0” and “1”, respectively, in the model.
*When we restricted the model to females only (N = 516), to further examine the effect of birth control pill and HRT use, the predictors (gender and menthol are excluded) together explained 5.3% of the variation in NMR. The standardized beta values for birth control pill and HRT use were 0.021 (P = 0.63) and 0.10 (P = 0.021), respectively, in the female-only group. They uniquely contributed 0.04% and 1% of the variation in NMR, respectively, in females.
4.5. Discussion

The NMR is currently being investigated prospectively as a predictive biomarker of response to smoking cessation treatments in an ongoing NMR-stratified clinical trial (NCT01314001). If NMR displays favorable efficacy and economic feasibility in predicting treatment response (Bough, Lerman et al. 2013), the NMR could be used to tailor smoking cessation pharmacotherapy in treatment-seeking smokers. Although CYP2A6 genotype, and potentially other genetic factors, cause substantial interindividual variability in NMR (Swan, Lessov-Schlaggar et al. 2009), within-person variability is relatively minor as the NMR is stable and reproducible over time in cigarette smokers (Mooney, Li et al. 2008; St Helen, Novalen et al. 2012).

We first examined known influences (i.e., ethnicity, gender, exogenous estrogen-based hormonal therapies, and BMI) on NMR. The higher NMR observed among Caucasians relative to African Americans is likely largely due to the lower frequency of reduced CYP2A6 activity variants among populations of Caucasian descent (Haberl, Anwald et al. 2005; Nakajima, Fukami et al. 2006; Mwenifumbo and Tyndale 2007). Among individuals without CYP2A6 variants (i.e., CYP2A6*1/*1 wild-type individuals), there is no difference in NMR between Caucasians and African Americans (Binnington, Zhu et al. 2012), suggesting that the variability in the NMR observed between Caucasians and African Americans in this study is likely attributable to variation in CYP2A6. Interethnic variability in NMR may also arise when there are large differences between ethnicities in exposure to nongenetic factors that affect CYP2A6 expression and/or activity. For instance, the prevalence of mentholated cigarette use was much higher among African American relative to Caucasian smokers in our study, consistent with previous findings (Blot, Cohen et al. 2011). Menthol has been shown to inhibit CYP2A6 activity in vitro (MacDougall, Fandrick et al. 2003) and nicotine clearance in vivo (Benowitz, Herrera et al. 2004), and may be associated with lower average NMR in African Americans compared with populations with lower prevalence of menthol cigarette use. This effect on NMR, although not a significant predictor of variation in overall NMR (Table 3), may represent a source of variability in NMR under certain circumstances.
We observed higher NMR among women relative to men, and even higher NMR in women taking estrogen-containing birth control pills or HRT. The higher NMR is likely attributable to enhancement of CYP2A6 transcriptional activity through estrogen binding of the estrogen response element located within the CYP2A6 gene (Higashi, Fukami et al. 2007). We observed no interaction between gender and ethnicity on NMR, suggesting that the influence of estrogen on NMR is similar between ethnicities.

BMI was negatively associated with NMR, consistent with previous reports (Mooney, Li et al. 2008; Ho, Mwenifumbo et al. 2009; Swan, Lessov-Schlaggar et al. 2009; Binnington, Zhu et al. 2012). Negative associations were also observed for plasma cotinine (Rho = -0.10; P < 0.001) and 3’hydroxycotinine (Rho = -0.18; P < 0.001) with BMI, in line with prior findings (Perez-Stable, Benowitz et al. 1995; Ho, Faseru et al. 2009). We postulate three potential explanations. First, when we controlled for the level of smoking (breath CO), as smoking is associated with lower body weight (Albanes, Jones et al. 1987; Akbartabartoori, Lean et al. 2005), the negative association between BMI and NMR remained significant, suggesting that the lower BMI observed among those with higher NMR may not result from heavier smoking in those with faster nicotine metabolism. In African Americans (Cox, Nollen et al. 2012), we observe no association between the CYP2A6 genotype group and BMI, despite observing significant correlations between NMR and BMI in both the total population and in reduced CYP2A6 metabolizers (A.Z.X. Zhu; unpublished data), further suggesting that this relationship is due to an effect of higher BMI (or obesity) on NMR rather than an effect of CYP2A6 activity or NMR on risk for obesity. Second, higher BMI may differentially affect the distribution pharmacokinetics of cotinine and 3’hydroxycotinine, resulting in an overall net reduction in NMR. However, it seems unlikely that this occurs through unique effects on the volume of distribution of cotinine versus 3’hydroxycotinine, as the pKa values of these compounds are similar (~4.4 vs. ~4.3, respectively (Li, Li et al. 1992)). Likewise, it seems unlikely that higher BMI would differentially affect the urinary excretion of these compounds, because negative associations for BMI with urinary cotinine and 3’hydroxycotinine are similar to those for plasma cotinine and 3’hydroxycotinine (A.Z.X. Zhu; unpublished data from another study (Zhu, Binnington et al. 2013)). Together these findings suggest that the relationship between higher BMI and lower
NMR is not mediated by differential effects of obesity on the volume of distribution or excretion of cotinine and 3’hydroxycotinine. Third, higher BMI and adiposity may uniquely affect the enzymes involved in the metabolism of cotinine and 3’hydroxycotinine, for example, UDP-glucuronosyltransferases; however, this remains to be explicitly tested in a pharmacokinetic study.

NMR was positively associated with cigarette consumption. This is likely an effect of NMR on the level of smoking, in which faster metabolism is associated with heavier smoking and greater total nicotine intake (Benowitz, Pomerleau et al. 2003; Zhu, Binnington et al. 2013). Consistent with this, the inhibition of CYP2A6 activity using oral methoxsalen treatment lead to reductions in both smoking and CYP2A6-mediated metabolic activation of the procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Sellers, Ramamoorthy et al. 2003). We also observed a positive relationship between alcohol consumption and NMR, which could suggest that CYP2A6 activity increases with alcohol use, or alternatively may represent an indirect effect of NMR on smoking, as nicotine is commonly used with alcohol (Bobo and Husten 2000; Grant, Hasin et al. 2004). When we controlled for smoking level, the association between higher alcohol use and higher NMR remained significant \( P < 0.001 \), suggesting that the higher NMR in those consuming larger amounts of alcohol is not due exclusively to higher smoking among this group. In rodents, liver damage (Gilmore, Hartmann et al. 2003; Kirby, Nichols et al. 2011) and 3-week ethanol treatment (Lu, Zhuge et al. 2011) induced CYP2A5, which is the murine ortholog of CYP2A6. In contrast, there was no impact on hepatic CYP2A6 levels or activity following 5 weeks of alcohol self-administration (~24 mmol/L blood ethanol levels; equivalent to ~4 standard drinks/d) in African green monkeys (Ferguson, Miksys et al. 2012). The reason for the higher NMR in those with higher alcohol consumption remains to be determined. However, given that the number of drinks per week uniquely explained <1% of the total variation in NMR, typical variation in alcohol use is not likely to substantially alter the utility of NMR, in particular as a prospective biomarker to guide treatment.

In both Caucasians and African Americans, lower NMR is associated retrospectively with greater smoking cessation (Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009; Schnoll, Patterson et al. 2009). This study used NMRs collected during
participant screening for the first prospectively stratified study of NMR as a predictive biomarker of cessation. This trial is currently underway (NCT01314001). We studied multiple sources of variation in NMR, which together accounted for <8% of NMR variation; the greatest unique contribution made by any one factor to NMR variation was <2%, suggesting these factors contribute little to overall variation in NMR on a population level. When NMR is used prospectively to guide therapy, relatively permanent or long-term sources of variation (e.g., ethnicity, gender, and BMI) are unlikely to cause treatment misclassifications, whereas potentially more transient influences on NMR (e.g., HRT and birth control pill use) may need to be considered if they are likely to change during the course of treatment. Together, we extend our understanding of the type, and degree, of influence of demographic factors on NMR. Each factor examined, alone and together, contributed little variation to NMR, supporting the NMR as a stable, reliable, and independent biomarker with potential clinical utility to guide smoking cessation pharmacotherapy.
4.6. Significance to Thesis

This chapter expands on existing literature that has identified demographic and/or environmental sources of variation in the NMR. While many smaller studies have identified sources of variation in NMR, this is the largest study to date that has investigated relationships between demographic/environmental factors and NMR in a treatment-seeking sample of adult smokers. Moreover, the association between these sources of variation and NMR was examined in both univariate and multivariate models; this is the first study to investigate these sources of variability simultaneously in the same model, in order to characterize their unique, as well as their overall, contribution to NMR variability. Similar to the finding from chapter 2 that FMO3 and POR variation contributed little to nicotine metabolism/NMR variation, this chapter showed that the associations between demographic/environmental factors and NMR were relatively minor. The factors that are relatively permanent (e.g., ethnicity and gender) and not going to change throughout an 8 to 12-week course of smoking cessation treatment would not affect NMR-based optimization of cessation outcomes. However, it is still important to consider these types of factors in studies of NMR and smoking, as previous work has shown that smoking behaviours (e.g., cigarettes/day, cessation outcomes) vary as a function of ethnicity and gender. One potentially transient influence on NMR, the use of hormone replacement therapy, had a comparatively large effect size in regression models evaluating sources of NMR variation. As this factor could change throughout the course of smoking cessation treatment, it may be worthwhile in the future to advise treatment-seeking smokers to delay making changes to existing hormonal treatment regimens if they are assigned pharmacotherapy based on their NMR.
5 CHAPTER 4: THE NICOTINE METABOLITE RATIO IS ASSOCIATED WITH EARLY SMOKING ABSTINENCE EVEN AFTER CONTROLLING FOR FACTORS THAT INFLUENCE THE NICOTINE METABOLITE RATIO

Meghan J. Chenoweth, Robert A. Schnoll, Maria Novalen, Larry W. Hawk Jr., Tony P. George, Paul M. Cinciripini, Caryn Lerman, and Rachel F. Tyndale

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CL and RFT designed and implemented the overall clinical trial. A description of the overall study is found in Appendix C. MJC read existing literature, identified gaps within the literature, consulted the study data dictionary, and designed a secondary analysis to answer the research questions. MN performed LC-MS/MS analysis to determine participants’ NMR. All authors contributed to the analytic strategy. MJC created a study database and performed all statistical analyses. MJC, LWH Jr., RAS, TPG, PMC, CL and RFT interpreted the results. MJC and RFT wrote the manuscript.
5.1. Abstract

**Introduction:** The decrease in smoking rates in North America has plateaued, underscoring the need for new approaches to treat nicotine dependence. Inter-individual differences in smoking behaviour result, in part, from variation in the rate of CYP2A6-mediated nicotine metabolism. A phenotypic measure of CYP2A6 activity is the nicotine metabolite ratio (NMR), the ratio of 3’hydroxycotinine/cotinine. The NMR is associated with smoking cessation. However, the NMR is also associated with genetic (e.g., *CYP2A6* genotype) and other (e.g., sex and ethnicity) factors. Here we aimed to determine if previously identified non-*CYP2A6* sources of variation in the NMR mitigated the association between the NMR and short-term abstinence.

**Methods:** The NMR was determined from blood samples collected at intake from daily smokers aged 18-65. Biochemically-verified point prevalence abstinence (exhaled carbon monoxide level ≤8 ppm) was measured at one week following the target quit date in participants from a smoking cessation clinical trial (NCT01314001). Analyses were restricted to N=462 blacks and N=693 whites in the intent-to-treat sample.

**Results:** Lower NMR (<0.31) was associated with a higher likelihood of one-week abstinence (OR=1.43; 95% CI=1.12, 1.84). NMR was associated with abstinence even after controlling for treatment arm (nicotine patch or varenicline) and factors previously associated with NMR variation including sex, ethnicity, estrogen-containing hormonal therapy, BMI, alcohol and cigarette consumption.

**Conclusions:** NMR was associated with one-week smoking abstinence; NMR may be a useful addition to medication screening approaches evaluating treatments for nicotine dependence.
5.2. Introduction

Smoking prevalence has stabilized at ~20% in North America (Agaku, King et al. 2014; Reid, Hammond et al. 2014), suggesting that the current efforts to reduce smoking initiation and/or increase cessation have plateaued. The Food and Drug Administration (FDA) has approved three medications (i.e., nicotine replacement therapy, bupropion, and varenicline) to treat nicotine dependence; each with modest success (reviewed in (Ashare, Wileyto et al. 2013)). Bringing a new drug to market typically takes up to 15 years and costs approximately $2 billion (Perkins and Lerman 2011). Despite these extensive costs, over one-third of drugs fail in Phase III trials (Perkins and Lerman 2011). New approaches that incorporate short-term efficacy screening of medications in early phase II may assist in selecting only those compounds with adequate efficacy for further development (Perkins, Stitzer et al. 2006). While short-term lab-based studies have been used to assess the ability of new compounds to reduce withdrawal, craving and/or drug reinforcement, they do not always accurately predict cessation outcomes in phase III (Perkins and Lerman 2011).

A new screening strategy in smoking cessation drug development has recently been validated using existing smoking cessation drugs (Perkins and Lerman 2014), which assesses short-term abstinence on the medication(s) of interest relative to an existing drug or placebo (Perkins, Stitzer et al. 2006; Perkins and Lerman 2011). Successful short-term abstinence increases the likelihood of prolonged cessation (Ashare, Wileyto et al. 2013), suggesting short-term screening approaches may serve as useful predictive models of both medication efficacy and long-term abstinence.

Nicotine, the principal psychoactive compound in cigarette smoke, is inactivated primarily by CYP2A6 to cotinine (Nakajima, Yamamoto et al. 1996a). CYP2A6 further converts cotinine to 3’hydroxycotinine (Nakajima, Yamamoto et al. 1996b), and the ratio of 3’hydroxycotinine/cotinine is known as the nicotine metabolite ratio (NMR) (Dempsey, Tutka et al. 2004). NMR, which is stable over time in smokers (St Helen, Novalen et al. 2012), is strongly associated with CYP2A6 genotype and nicotine clearance (Dempsey, Tutka et al. 2004), and is a genetically-informed biomarker of CYP2A6 activity incorporating environmental influences on
nicotine clearance (Bough, Lerman et al. 2013). NMR is associated with a variety of smoking behaviours, including cessation (Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Schnoll, Patterson et al. 2009) and early symptoms of withdrawal and craving (Lerman, Tyndale et al. 2006). Lower NMR, indicative of slower nicotine clearance, is associated with lower cigarette consumption (Chenoweth, Novalen et al. 2014), dependence scores (Schnoll, George et al. 2014), nicotine-mediated reward (Sofuoglu, Herman et al. 2012), as well as higher quit rates on placebo (Patterson, Schnoll et al. 2008) and nicotine patch (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). In an NMR-stratified smoking cessation clinical trial involving nicotine patch and varenicline, varenicline displayed a higher efficacy relative to patch in those with higher NMR, but quit rates were similar across treatments in those with lower NMR (Lerman, Schnoll et al. 2015). Treatment with varenicline (vs. placebo) was also associated with greater overall side-effect severity among those with lower NMR (Lerman, Schnoll et al. 2015). In these clinical trial participants, we previously quantified the effect of sex, ethnicity, estrogen-based hormonal therapy, BMI, alcohol use, and mentholated cigarette use on baseline NMR (Chenoweth, Novalen et al. 2014). Our current objectives were to determine if NMR is associated with one-week abstinence following the target quit date, and if controlling for factors associated with baseline NMR (Chenoweth, Novalen et al. 2014) mitigate the association between NMR and one-week abstinence. The incorporation of pharmacogenetic information into early screening approaches, especially factors such as NMR that influence cessation outcomes, may identify new medications for nicotine dependence that work better in some subgroups rather than others.

5.3. Materials and Methods

Study Participants
Adults (aged 18-65 years) smoking ≥10 cigarettes/day for the previous six months were recruited for participation in a smoking cessation clinical trial (NCT01314001). Participants were prospectively randomized to placebo, nicotine patch, or varenicline according to their NMR, assessed at baseline in those interested in participating in the clinical trial after meeting eligibility criteria. Detailed study procedures including trial inclusion/exclusion criteria, as well as participant characteristics, are provided elsewhere (Chenoweth, Novalen et al. 2014; Schnoll,
George et al. 2014; Lerman, Schnoll et al. 2015). A flow chart depicting recruitment to each arm of the study and exclusions at each stage is published (Lerman, Schnoll et al. 2015). The baseline NMR was assessed from blood while participants were smoking as usual, by liquid chromatography-tandem mass spectrometry (Chenoweth, Novalen et al. 2014). A clinical NMR cut-point was used to distinguish faster (≥0.31) from slower (<0.31) metabolizers based on previous clinical trial data as described (Lerman, Schnoll et al. 2015); participants were randomized by treatment site and NMR in a 1:1:1 ratio to treatment arm (Lerman, Schnoll et al. 2015). Point prevalence abstinence at one week following the target quit date was biochemically verified using exhaled carbon monoxide (CO). Those with CO ≤8 ppm were considered abstinent, while those with CO >8 ppm were classified as smoking.

Statistical Analyses
Mann-Whitney U (two-tailed) and \( \chi^2 \) tests were used to compare continuous and dichotomous characteristics, respectively, between abstainers and non-abstainers at week one. We used multiple logistic regression analysis to examine the relationship between NMR and one-week abstinence, after controlling for potential confounding effects of covariates. Covariates included treatment arm and factors previously associated with NMR variation in this study population (Chenoweth, Novalen et al. 2014), as well as treatment site. Dummy-coding was used to control for treatment arm, where the impact of varenicline and nicotine patch on one-week abstinence was assessed compared to placebo. Statistical analyses were performed using SPSS Version 22 (IBM Corporation).

5.4. Results
A total of 1246 comprised the intent-to-treat (ITT) group for the clinical trial (NCT01314001). We restricted all further analyses to white (N=693) and black (N=462) participants in the ITT group, as the small number of individuals from additional ethnic groups precluded statistical analyses (Chenoweth, Novalen et al. 2014). Of these, 997 (86.3%) completed the week one assessment. The remaining 158 participants that did not complete the week one assessment were assumed to be smoking and were included in the ‘non-abstinent’ group, as is standard for an ITT analysis (Ashare, Wileyto et al. 2013). NMR, treatment, and other characteristics according to
abstinence status at one week are shown in Table 9. Similar results were obtained when these participants were excluded from the analysis (Tables 10 and 11).
Table 9 | NMR, treatment, and factors associated with NMR, according to abstinence status at one week in ITT group (N=1155)

<table>
<thead>
<tr>
<th></th>
<th>Abstinent&lt;sup&gt;b&lt;/sup&gt; (N=588)</th>
<th>Not Abstinent&lt;sup&gt;c&lt;/sup&gt; (N=567)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Slow metabolizers, NMR&lt;0.31</td>
<td>56.1</td>
<td>49.2</td>
<td>0.019</td>
</tr>
<tr>
<td>(ref. normal metabolizers, NMR≥0.31)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% On patch or varenicline (ref. placebo)</td>
<td>75.7</td>
<td>58.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factors associated with NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% White (ref. black)</td>
<td>63.6</td>
<td>56.3</td>
<td>0.01</td>
</tr>
<tr>
<td>% Female (ref. male)</td>
<td>47.3</td>
<td>42.0</td>
<td>0.07</td>
</tr>
<tr>
<td>% Using estrogen therapy&lt;sup&gt;d&lt;/sup&gt; (ref. no estrogen therapy)</td>
<td>4.3</td>
<td>4.2</td>
<td>0.95</td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>29.9 (6.8)</td>
<td>29.8 (6.6)</td>
<td>0.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean # drinks/week (SD)</td>
<td>3.6 (5.4)</td>
<td>2.8 (4.9)</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean cigarettes per day (SD)</td>
<td>17.5 (6.4)</td>
<td>19.0 (7.6)</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Using mentholated cigarettes (ref. non-menthol cigarettes)</td>
<td>45.1</td>
<td>50.6</td>
<td>0.059</td>
</tr>
<tr>
<td>Additional demographic factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% College or higher</td>
<td>71.1</td>
<td>65.8</td>
<td>0.052</td>
</tr>
<tr>
<td>% Income &gt; $50,000</td>
<td>41.5</td>
<td>30.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% Single</td>
<td>56.8</td>
<td>61.2</td>
<td>0.13</td>
</tr>
</tbody>
</table>

BMI = body mass index; CO = carbon monoxide
Note: P values are derived from chi-square tests unless otherwise indicated
<sup>a</sup>P values derived from Mann-Whitney U tests
<sup>b</sup>Includes those who completed the week one assessment and were abstinent (i.e., CO≤8 ppm)
<sup>c</sup>Includes those who were in the ITT group and completed the week one assessment and were not abstinent (i.e., CO >8 ppm), or did not complete the week one assessment (and were therefore assumed to be smoking)
<sup>d</sup>Includes the use of estrogen-containing birth control pills and hormone replacement therapy; assessed in women only
Table 10 | Association between NMR and one-week abstinence (CO≤8 ppm) after controlling for treatment and factors associated with NMR variation in those who completed the one-week assessment (N=997)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine patch (ref. placebo)</td>
<td>1.82</td>
<td>1.32, 2.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Varenicline (ref. placebo)</td>
<td>2.67</td>
<td>1.93, 3.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factors associated with NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female sex (ref. males)</td>
<td>1.20</td>
<td>0.91, 1.58</td>
<td>0.21</td>
</tr>
<tr>
<td>Caucasian ethnicity (ref. African Americans)</td>
<td>1.83</td>
<td>1.29, 2.60</td>
<td>.001</td>
</tr>
<tr>
<td>Estrogen therapy(^a) (ref. no estrogen therapy)</td>
<td>1.02</td>
<td>0.38, 2.74</td>
<td>0.97</td>
</tr>
<tr>
<td>BMI (continuous)</td>
<td>1.01</td>
<td>0.99, 1.03</td>
<td>0.32</td>
</tr>
<tr>
<td>Standard alcoholic drinks per week (continuous)</td>
<td>1.03</td>
<td>1.01, 1.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Cigarettes per day (continuous)</td>
<td>0.97</td>
<td>0.95, 0.99</td>
<td>0.002</td>
</tr>
<tr>
<td>Menthol cigarettes (ref. non-menthol cigarettes)</td>
<td>0.83</td>
<td>0.59, 1.16</td>
<td>0.28</td>
</tr>
<tr>
<td>NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow metabolism, NMR≤0.31 (ref. NMR≥0.31)</td>
<td>1.49</td>
<td>1.14, 1.96</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^a\)Includes the use of estrogen-containing birth control pills and hormone replacement therapy; women not taking estrogen therapy, as well as men, were coded as “0”, whereas women taking estrogen therapy were coded as “1”
Table 11 | NMR, treatment, and factors associated with NMR, in those who did not complete the one-week assessment (N=158)

<table>
<thead>
<tr>
<th>NMR</th>
<th>Participants who did not complete the one week assessment (N=158)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Slow metabolizers, NMR&lt;0.31 (ref. normal metabolizers, NMR≥0.31)</td>
<td>50.6</td>
</tr>
<tr>
<td>Treatment arm</td>
<td></td>
</tr>
<tr>
<td>% On patch or varenicline (ref. placebo)</td>
<td>59.5</td>
</tr>
<tr>
<td>Factors associated with NMR</td>
<td></td>
</tr>
<tr>
<td>% Caucasian (ref. African American)</td>
<td>69.6</td>
</tr>
<tr>
<td>% Female (ref. male)</td>
<td>34.8</td>
</tr>
<tr>
<td>% Using estrogen therapy* (ref. no estrogen therapy)</td>
<td>5.5</td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>29.4 (6.9)</td>
</tr>
<tr>
<td>Mean # drinks/week (SD)</td>
<td>3.4 (5.0)</td>
</tr>
<tr>
<td>Mean cigarettes per day (SD)</td>
<td>20.0 (8.6)</td>
</tr>
<tr>
<td>% Using mentholated cigarettes (ref. non-menthol cigarettes)</td>
<td>39.9</td>
</tr>
<tr>
<td>Additional demographic factors</td>
<td></td>
</tr>
<tr>
<td>% College or higher</td>
<td>63.3</td>
</tr>
<tr>
<td>% Income &gt; $50,000</td>
<td>39.2</td>
</tr>
<tr>
<td>% Single</td>
<td>59.5</td>
</tr>
</tbody>
</table>

*Includes the use of estrogen-containing birth control pills and hormone replacement therapy; assessed in women only
We first examined the association between NMR group and one-week abstinence; compared to normal metabolizers, slow metabolizers were significantly more likely to achieve abstinence (OR=1.32, 95% CI = 1.05, 1.67; P=0.019). Nicotine patch and varenicline, relative to placebo, significantly influenced one-week abstinence with odds ratios (95% CI) of 1.80 (1.35, 2.41) and 2.60 (1.94, 3.48), respectively (P<0.001 vs. placebo). The association between slow metabolism and increased abstinence likelihood did not change (OR=1.32; P=0.022) when we controlled for treatment.

In a final logistic regression model, we included treatment, NMR, and all variables previously associated with NMR in this population, as independent variables (Chenoweth, Novalen et al. 2014). After controlling for these factors, NMR was significantly associated with one-week abstinence (OR for abstinence in slow vs. normal metabolizers = 1.43; Table 12). In addition, white (vs. black) ethnicity, higher baseline alcohol consumption, and lower baseline cigarette consumption were all significantly associated with abstinence (Table 12), even while controlling for treatment and NMR. Including treatment site as a covariate in the logistic regression models did not alter any of these relationships (data not shown). In 2x2 models, there was no interaction between NMR and ethnicity (OR=0.84, 95% CI 0.52, 1.38; P=0.50), or between NMR and sex (OR=0.65, 95% CI 0.40, 1.03; P=0.067), on one-week abstinence, suggesting similar associations between NMR and abstinence in whites and blacks, and in men and women.
**Table 12 | Association between NMR and one-week abstinence (CO ≤ 8 ppm) after controlling for treatment and factors associated with NMR variation in the ITT group (N=1155)**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment arm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine patch (ref. placebo)</td>
<td>1.80</td>
<td>1.34, 2.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Varenicline (ref. placebo)</td>
<td>2.58</td>
<td>1.92, 3.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Factors associated with NMR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female sex (ref. males)</td>
<td>1.28</td>
<td>1.00, 1.65</td>
<td>0.054</td>
</tr>
<tr>
<td>White ethnicity (ref. blacks)</td>
<td>1.56</td>
<td>1.13, 2.16</td>
<td>0.007</td>
</tr>
<tr>
<td>Estrogen therapy (ref. no estrogen therapy)</td>
<td>1.00</td>
<td>0.41, 2.43</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI (continuous)</td>
<td>1.01</td>
<td>1.00, 1.03</td>
<td>0.16</td>
</tr>
<tr>
<td>Standard alcoholic drinks per week (continuous)</td>
<td>1.03</td>
<td>1.01, 1.06</td>
<td>0.009</td>
</tr>
<tr>
<td>Cigarettes per day (continuous)</td>
<td>0.97</td>
<td>0.95, 0.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Menthol cigarettes (ref. non-menthol cigarettes)</td>
<td>0.89</td>
<td>0.66, 1.21</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>NMR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow metabolism, NMR&lt;0.31 (ref. NMR≥0.31)</td>
<td>1.43</td>
<td>1.12, 1.84</td>
<td>0.004</td>
</tr>
</tbody>
</table>

BMI = body mass index; CI = confidence interval; CO = carbon monoxide; OR = odds ratio

*aIncludes the use of estrogen-containing birth control pills and hormone replacement therapy; women not taking estrogen therapy, as well as men, were coded as “0”, whereas women taking estrogen therapy were coded as “1”*
Table 13 | Association between NMR and one-week abstinence (CO≤8 ppm) after controlling for treatment and factors associated with NMR variation, all coded as categorical variables, in the ITT group (N=1155)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine patch (ref. placebo)</td>
<td>1.83</td>
<td>1.36, 2.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Varenicline (ref. placebo)</td>
<td>2.65</td>
<td>1.97, 3.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factors associated with NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female sex (ref. males)</td>
<td>1.31</td>
<td>1.02, 1.68</td>
<td>0.034</td>
</tr>
<tr>
<td>Caucasian ethnicity (ref. African Americans)</td>
<td>1.57</td>
<td>1.13, 2.16</td>
<td>0.006</td>
</tr>
<tr>
<td>Estrogen therapy (ref. no estrogen therapy)</td>
<td>0.94</td>
<td>0.39, 2.30</td>
<td>0.89</td>
</tr>
<tr>
<td>Higher BMI (ref. lower BMI)</td>
<td>1.05</td>
<td>0.82, 1.35</td>
<td>0.68</td>
</tr>
<tr>
<td>Alcohol consumption (ref. no alcohol consumption)</td>
<td>1.29</td>
<td>1.01, 1.66</td>
<td>0.04</td>
</tr>
<tr>
<td>Higher cigarettes/day (ref. lower cigarettes/day)</td>
<td>0.60</td>
<td>0.47, 0.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Menthol cigarettes (ref. non-menthol cigarettes)</td>
<td>0.91</td>
<td>0.67, 1.24</td>
<td>0.54</td>
</tr>
<tr>
<td>NMR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow metabolism, NMR&lt;0.31 (ref. NMR≥0.31)</td>
<td>1.43</td>
<td>1.12, 1.83</td>
<td>0.005</td>
</tr>
</tbody>
</table>

aIncludes the use of estrogen-containing birth control pills and hormone replacement therapy; women not taking estrogen therapy, as well as men, were coded as “0”, whereas women taking estrogen therapy were coded as “1”

bMedian split on BMI was performed: higher (>28.7; N=581) vs. lower BMI (≤28.7; N=574)

cMedian split on alcohol consumption was performed: N=576 participants reported no alcohol consumption (i.e., zero standard drinks/week) and N=579 participants reported consuming ≥1 drink/week

dMedian split on cigarettes/day was performed: higher (>19 cigarettes; N=581) vs. lower cigarettes/day (≤19 cigarettes; N=574)
5.5. Discussion

One-week abstinence was strongly associated with end-of-treatment abstinence (OR=9.6, 95% CI 6.7, 13.8; P<0.001) consistent with it being a good predictor of long-term abstinence (Ashare, Wileyto et al. 2013). At one week, lower NMR was associated with an increased likelihood of abstinence, as had been observed at later time-points (i.e., end-of-treatment and six months) (Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009; Schnoll, Patterson et al. 2009), even after controlling for factors that influence NMR (Chenoweth, Novalen et al. 2014). The relationship between NMR and early abstinence also remained significant when a median split on BMI, alcohol consumption, and cigarette consumption was performed (Table 13). Thus, it may be worthwhile to incorporate NMR, a genetically-informed biomarker of cessation (Bough, Lerman et al. 2013), into short-term efficacy screening approaches during the development of novel smoking cessation medications. These studies together provide support for the development of a test kit for assessing NMR for future use in clinical settings.

In addition to NMR and treatment arm, our overall model suggests a direct influence of ethnicity, alcohol consumption, and cigarette consumption on one-week abstinence. Sex had a modest influence on abstinence, which varied depending on whether BMI, alcohol consumption, and cigarette consumption were included as continuous measures (OR 1.28, p=0.054, Table 12) or as categorical variables (OR 1.31, p=0.034, Table 13). Compared to men, women may display an enhanced ability to maintain short-term abstinence, when nicotine withdrawal symptoms are strongest, as women appear to smoke more for reasons unrelated to nicotine (Perkins 1999). However, women generally have lower cessation rates in studies evaluating longer-term abstinence (Hymowitz, Cummings et al. 1997; Osler, Prescott et al. 1999). Taken together, each of these factors would likely need to be considered when tailoring smoking cessation strategies, but as separate entities from the NMR. Consistent with prior findings on smoking cessation, we observed a greater likelihood of abstinence among whites relative to blacks. Relative to whites, black smokers display lower quit ratios (i.e., a lower proportion of former smokers among ever smokers (Stahre, Okuyemi et al. 2010)). In our clinical trial population, >85% of black smokers used mentholated cigarettes, compared to <25% of white smokers (Chenoweth, Novalen et al.
The use of mentholated cigarettes was not associated with one-week abstinence in our study or at end-of-treatment (Lerman, Schnoll et al. 2015). Mentholated cigarette use has not been consistently associated with a reduced likelihood of cessation or with an increased risk for lung cancer (Blot, Cohen et al. 2011), however this remains to be investigated further.

Alcohol consumption (number of standard alcoholic drinks/week) was positively associated with one-week abstinence, with the caveat that self-reported alcohol consumption was measured at intake and may not accurately reflect the level of alcohol consumption one week following the target quit date. Follow-up of alternative drug usage during one-week abstinence studies might help clarify if there are compensatory increases in consumption levels over the course of a smoking cessation attempt. A previous cohort study in community-based adult smokers showed a negative influence of daily alcohol consumption on self-reported smoking cessation five years later (Hymowitz, Cummings et al. 1997), suggesting that this level of alcohol consumption impedes attempts to quit smoking. The association with the level of alcohol was weak, with abstainers consuming one more standard drink per week relative to non-abstainers (~4 versus ~3 drinks/week). The low level of consumption likely reflects clinical trial exclusion criteria (consumption of >25 standard drinks/week).

Consistent with previous findings (Hymowitz, Cummings et al. 1997), lower baseline cigarette consumption was associated with a higher likelihood of abstinence in our study. In these clinical trial participants, we previously showed that lower NMR was associated with lower cigarette consumption (Chenoweth, Novalen et al. 2014). In our overall model (Table 12), both baseline cigarette consumption and NMR were associated with abstinence, suggesting at least part of the effects were independent of one another.

Overall, we showed that NMR, which is associated with treatment outcome at end-of-treatment and 6-month follow-up (Lerman, Schnoll et al. 2015), was also associated with one-week abstinence, and factors which account for variability in NMR did not remove the association between NMR and one-week abstinence. This early period of abstinence represents a period of heightened vulnerability during the process of smoking cessation, and NMR may be useful in informing early efficacy screening approaches for compounds undergoing development.
Screening approaches may include NMR-based randomization to treatment, as we have done in this clinical trial (Lerman, Schnoll et al. 2015), or simply including NMR in analytic models as a covariate known to alter smoking cessation and treatment response.
5.6. Significance to Thesis

This chapter adds a novel finding to the smoking cessation literature by showing a significant association between NMR and early smoking abstinence. To our knowledge, the impact of variation in the rate of nicotine metabolism on one-week smoking abstinence has not been investigated. This work can be considered an extension of chapter 3, where demographic and/or environmental sources of variation in NMR were investigated. In this chapter, these sources of NMR variation were controlled for when the relationship between NMR and one-week smoking abstinence was investigated. Similar to the relationship between NMR and longer term smoking cessation outcomes (e.g., end-of-treatment and six-month abstinence), lower NMR (i.e., slower nicotine metabolism) was associated with a higher likelihood of one-week abstinence. This finding is potentially helpful for novel approaches to drug development that incorporate early efficacy screening of new compounds to treat tobacco dependence; at the very least, NMR should be included in analytic models as a covariate associated with early (i.e., one-week), as well as later, cessation outcomes.
6 GENERAL DISCUSSION

6.1. Summary of research findings

The work within this thesis investigated relationships between the rate of nicotine metabolism and smoking abstinence, in both adolescent and adult smokers, as well as sources of variation in the rate of nicotine metabolism. In chapter 1, we showed an association between slow CYP2A6 metabolism and a higher likelihood of smoking abstinence in a cohort of adolescent smokers who were not seeking treatment to quit smoking. We then investigated in chapter 2 potential associations between variation in FMO3 and nicotine clearance, and between POR and CYP2A6 activity (NMR), and found that this genetic variation contributed little to variation in nicotine metabolism, smoking behaviours, and total nicotine dose. In chapter 3, we examined known and novel sources of variation in NMR in a sample of treatment-seeking adult smokers, and found that a variety of demographic and environmental factors contributed little to the overall variability in the NMR. We extended this work in chapter 4, where we examined the association between NMR and early smoking abstinence in this same sample of treatment-seeking adult smokers; we observed a higher likelihood of one-week smoking abstinence among those with lower NMR, even after controlling for these demographic and environmental factors that were shown to be associated with NMR in chapter 3.

6.2. Why are slow nicotine metabolizers more likely to quit smoking?

Smoking cessation rates are typically higher in slower relative to faster nicotine metabolizers. The increased likelihood of quitting among slow metabolizers has been shown to occur spontaneously, in the absence of treatment, in both adults (Gu, Hinks et al. 2000) and adolescents (see chapter 1 of this thesis). Higher quit rates among slow metabolizers were also observed in clinical trials in treatment-seeking adults when participants were on placebo (Patterson, Schnoll et al. 2008) and the nicotine patch (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009; Lerman, Jepson et al. 2010). The mechanism(s) underlying the increased likelihood of cessation among slow nicotine metabolizers is not well understood, but could relate to differences in
nicotine pharmacokinetics and/or resulting pharmacodynamics differences between normal and slow metabolizers.

6.2.1. Nicotine pharmacokinetics

The classical addiction theory (Fig. 14) postulates that once drug dependence is acquired, removal of the drug from the system will produce a state of withdrawal that leads to future drug use (Piper 2015). In general, it is thought that a faster rate of drug delivery to the brain, as well as a faster rate of drug removal from the brain, increases abuse liability (O'Brien C 2005; Samaha and Robinson 2005) and withdrawal and craving (Koob and Volkow 2010).

Figure 14 | Classical addiction theory. The use of addictive drugs leads to drug dependence in some individuals. During a period of abstinence, dependent individuals will experience withdrawal symptoms, which can cause them to relapse and reinstate drug use. Modified from Piper, 2015.

6.2.1.1. Nicotine metabolism rate, withdrawal, and craving

As previous work has shown that withdrawal and craving can predict relapse (West, Hajek et al. 1989; Swan, Ward et al. 1996), variation in pharmacokinetics that influences the rate of drug removal may influence withdrawal and/or craving, in turn affecting relapse. Compared to a slower rate of clearance, faster drug clearance may lead to a more pronounced withdrawal syndrome, potentially increasing the likelihood of relapse. It is possible that variation in nicotine pharmacokinetics, particularly variation in the rate of nicotine metabolism (and therefore the rate of nicotine clearance), contributes to differences in abstinence rates between normal and slow nicotine metabolizers through altering the severity of withdrawal symptoms. In adult Japanese smokers, withdrawal symptoms were more severe during a quit attempt in normal versus slow
nicotine metabolizers (Kubota, Nakajima-Taniguchi et al. 2006). In adolescent light smokers (≤6 cigarettes/day) not seeking treatment, faster metabolizers reported greater withdrawal symptoms compared to slower metabolizers after 24 hours of abstinence (Rubinstein, Benowitz et al. 2008). Together the findings suggest that faster nicotine metabolizers may experience greater withdrawal symptoms, which could contribute to their higher rate of early relapse (vs. slow metabolizers); in chapter 4 of this thesis, we observed higher relapse rates at one week in normal metabolizers. Normal nicotine metabolizers may also experience greater craving; among adult smokers receiving nicotine patch therapy who were abstinent at one week, faster nicotine metabolism (i.e., higher pre-treatment NMR) was associated with greater craving intensity at one week (Lerman, Tyndale et al. 2006). This suggests that even while abstinent and on nicotine patch, normal metabolizers could experience greater craving than slow metabolizers, which may also contribute to their increased risk for relapse.

6.2.1.2. Nicotine metabolism rate and short-term versus prolonged cessation

The notion that faster nicotine removal may increase withdrawal and/or craving, leading to the greater risk for early relapse observed among normal metabolizers (see chapter 4), may partially explain why NRT is not as effective at treating tobacco dependence in normal metabolizers compared to slow metabolizers (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). In smokers who were abstinent at one week while on nicotine patch therapy, normal metabolizers acquired lower patch-derived plasma levels of nicotine compared to slow metabolizers (mean ~15 ng/ml versus ~21 ng/ml, respectively, at one week) (Schnoll, Patterson et al. 2009), which may have contributed to their lower quitting on patch (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). However, nicotine patch-derived plasma nicotine levels did not predict cessation at end-of-treatment (Lerman, Tyndale et al. 2006), suggesting lower patch-derived plasma nicotine does not mediate the reduced quitting rate observed at end-of-treatment among normal (vs. slow) metabolizers (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009).

A lower likelihood of cessation has also been observed in normal metabolizers relative to slow metabolizers among self-quitters (Gu, Hinks et al. 2000) and in smokers receiving placebo (Patterson, Schnoll et al. 2008). In these scenarios, nicotine (derived from smoking) would no
longer be present in the body after a short period of time; in general, the half-life of nicotine averages ~2 hours (Benowitz, Lessov-Schlaggar et al. 2006), thus it would take only ~14 hours (seven half-lives) for >99% of a nicotine dose to be cleared from the body (Benowitz, Hukkanen et al. 2009). In chapter 1 of this thesis, we showed an enhanced likelihood of quitting for at least one year in adolescent slow metabolizers compared to normal metabolizers, a time frame in which nicotine would no longer be present. Thus, while differences in withdrawal as a function of nicotine metabolism rate may contribute to short-term abstinence outcomes, it is likely that additional factors beyond immediate differences in nicotine pharmacokinetics could contribute directly to CYP2A6 and NMR differences in long-term quit rates, as observed in both adult and adolescent smokers. This idea is supported by prior research suggesting that there does not appear to be a protracted withdrawal syndrome; many nicotine withdrawal symptoms (e.g., anxiety, difficulty concentrating, irritability, restlessness) returned to baseline (i.e., pre-quit levels) within ~1 month of abstinence in a sample of self-quitters, although hunger and weight gain persisted beyond the one-month mark (Hughes 1992). In contrast to withdrawal symptoms, craving elicited by smoking abstinence may persist for a month or longer in some smokers after a quit attempt; in a sample of treatment-seeking smokers that received nicotine gum or placebo gum, ~75% of smokers that achieved abstinence continued to report cravings six months after quitting (Hughes, Gust et al. 1991). In former smokers who had quit 1-10 years prior, ~60% reported still having a desire to smoke (Hughes 2010). Thus, cigarette craving could influence relapse risk in former smokers who have achieved prolonged abstinence. Whether variation in the rate of nicotine metabolism influences long-term craving in abstinent smokers, in turn influencing later relapse, is currently not known. In the clinical trial participants from chapters 3 and 4 that achieved abstinence at six months (n=173), the level of craving symptoms (assessed by the Questionnaire on Smoking Urges- Brief (QSU-B)) was ~2 points higher in normal metabolizers (versus slow metabolizers), however this difference was not significant (mean craving score = 13.7 versus 11.5, respectively; P=0.13).

In addition to variation in nicotine pharmacokinetics, it is possible that differences in brain nAChR availability contribute to variation in smoking cessation outcomes (Brody, Mukhin et al. 2014); however, it remains to be determined whether receptor availability differs according to the rate of nicotine metabolism. In smokers, the availability of β2-containing nAChRs, which are
thought to be required for the development of nicotine dependence (Maskos, Molles et al. 2005; Benowitz 2010), was higher compared to that in non-smokers (Cosgrove, Batis et al. 2009). In a clinical study, a higher degree of upregulation of available β2-containing nAChRs was associated with a greater risk of relapse at 10 weeks in treatment-seeking smokers (Brody, Mukhin et al. 2014). If CYP2A6 genetic or NMR differences influence the level of nAChR availability, presumably via differential nicotine exposure and modeling of neural networks, this could affect the risk for relapse in abstinent smokers, with normal nicotine metabolizers (versus slow metabolizers) possibly having a higher degree of β2-containing nAChR upregulation. Differences in the strength of neural circuits within brain reward regions as a function of the rate of nicotine metabolism were recently demonstrated (Sufang Yi, et al. unpublished findings), and are discussed in further detail below; it is possible that nicotine metabolism-mediated neural changes also occur upstream of brain reward pathways, at the level of the nAChR, and influence relapse risk. However, within 6-12 weeks following smoking abstinence, the availability of β2-containing nAChRs on average returns to the level of that in a non-smoker (Cosgrove, Batis et al. 2009), although there could be subgroup differences between normal and slow nicotine metabolizers. If differences in nicotine metabolism rate influence cessation outcomes via changes in β2-containing nAChR availability, this may not fully account for differences in quit rates at later time-points, such as one year (see chapter 1). In addition, due to the protracted nature of craving in many former smokers (Herd and Borland 2009; Hughes 2010), it is unlikely that this long-lasting craving, experienced by ~60% of former smokers between 1-10 years after they quit smoking (Hughes 2010), is modulated by differences in β2-containing nAChRs.

Together the findings suggest that in addition to altered nicotine pharmacokinetics and potential differences in nAChR availability, there may be other explanations for the differences in abstinence outcomes according to the rate of nicotine metabolism. It is possible that differential effects of nicotine signaling downstream of the nAChR and altered neural circuitry within the brain also contribute to differences in quit rates between normal and slow nicotine metabolizers.
6.2.2. Nicotine pharmacodynamics and changes in neural circuitry

6.2.2.1. Effect of nicotine on dopamine release within the brain

Nicotine and other addictive drugs are hypothesized to cause prolonged functional alteration of the neural circuits that mediate their reinforcing effects, likely occurring via dopamine signaling initiated in the ventral tegmental area (VTA) and altered synaptic plasticity (Hyman and Malenka 2001; Kelley and Berridge 2002). Nicotine binds nAChRs located in the VTA (Pons, Fattore et al. 2008). The VTA is a component of the mesostriatal dopamine system and houses the cell bodies of the ventral mesolimbic pathway, while its terminals are found in the nucleus accumbens (Laviolette and van der Kooy 2004; Gotti, Clementi et al. 2009). There are also dopaminergic projections that extend from the VTA to the prefrontal cortex, comprising the ventral mesocortical pathway (Laviolette and van der Kooy 2004) (Fig. 15). Results from preclinical animal models and human imaging studies suggest that nicotine causes dopamine release in the nucleus accumbens, which in turn is thought to mediate the reinforcing properties associated with cigarette smoking (Corrigall, Franklin et al. 1992; Barrett, Boileau et al. 2004; Brody, Olmstead et al. 2004). Lesioning of the nucleus accumbens of rats using 6-hydroxydopamine led to reductions in nicotine self-administration behaviour in animals that previously self-administered nicotine at high levels, consistent with marked dopamine depletion in the nucleus accumbens (Corrigall, Franklin et al. 1992). The signals received by the nucleus accumbens from the VTA are likely integrated with excitatory input from glutamate-producing neurons in the amygdala, prefrontal cortex, and tegmental pedunculopontine nucleus (Laviolette and van der Kooy 2004; Kandel and Kandel 2014). Glutamate signals can lead to long-term potentiation of nicotine’s effects, described in more detail below. The VTA also houses gamma-aminobutyric acid (GABA) neurons, which may modulate dopamine signaling and the activity of the tegmental pedunculopontine nucleus (Laviolette and van der Kooy 2004) (Fig. 15). Lesioning of the tegmental pedunculopontine nucleus in rats blocked the rewarding effects of nicotine and appeared to enhance nicotine aversion in a conditioned place preference paradigm (Laviolette, Alexson et al. 2002).
Figure 15 | Mesolimbic and mesocortical dopamine pathways activated by nicotine.
Abbreviations: VTA, ventral tegmental area; TPP, tegmental pedunculopontine nucleus; GABA, gamma-aminobutyric acid. Adapted from Laviolette and van der Kooy, 2004.

6.2.2.2. Nicotine, nAChR desensitization, and long-term potentiation

Following cigarette smoking, nicotine binds and causes rapid desensitization of nAChRs, particularly the high affinity $\alpha_4\beta_2$ receptors which are chiefly responsible for the development of nicotine dependence (Dani, Ji et al. 2001; Paradiso and Steinbach 2003; Brody, Mandelkern et al. 2006). In rats, at nicotine levels comparable to those achieved in smokers, profound desensitization of a variety of nAChR types occurred in brain slices following bath application of nicotine (Pidoplichko, DeBiasi et al. 1997); a separate study showed that nAChRs containing $\alpha_4$ (vs. $\alpha_3$) and $\beta_2$ (vs. $\beta_4$) were particularly sensitive to the desensitizing effects of nicotine (Fenster, Rains et al. 1997). It is likely that the speed of arrival of nicotine in the brain, as well as the onset
of nAChR receptor occupancy by nicotine, would be similar in normal and slow nicotine metabolizers. In addition, the rapid rate of desensitization (within minutes) of nAChRs would presumably not be substantially affected by the rate of nicotine metabolism; however, a slow nicotine metabolizer may have more profound desensitization compared to a normal metabolizer due to their prolonged CNS exposure to each unit of nicotine consumed.

Despite the rapid desensitization of nAChRs following nicotine exposure, dopamine release has been observed in the nucleus accumbens >1 hour after a single nicotine injection in rats (Imperato, Mulas et al. 1986; Schilstrom, Nomikos et al. 1998; Di Chiara 2000). This is thought to be due to nicotine signaling at pre-synaptic α7-containing nAChRs on glutamatergic terminals, which enhances glutamate release in the VTA and N-methyl-D-aspartate (NMDA) receptor activation on dopaminergic neurons, leading to long-term potentiation (Mansvelder and McGehee 2000; Matsuyama, Matsumoto et al. 2000; Welsby, Rowan et al. 2006). Nicotine-induced glutamate secretion in the VTA also leads to increased dopamine levels in the nucleus accumbens, with potentiated glutamatergic signals possibly leading to sustained dopamine release in the nucleus accumbens (Schilstrom, Svensson et al. 1998; Fu, Matta et al. 2000; Mansvelder and McGehee 2000). Together these glutamatergic effects are thought to lead to a prolonged excitation of the reward system; these effects may be less pronounced in a slow nicotine metabolizer due to their presumably higher level of nAChR desensitization (via prolonged nicotine exposure) compared to normal nicotine metabolizers.

Nicotine-mediated dopamine release in the nucleus accumbens can be enhanced by both sustained and burst firing of non-NMDA and NMDA receptors, respectively on VTA neurons; burst firing is more efficient than sustained firing at inducing dopamine release in the nucleus accumbens (Suaudchagny, Chergui et al. 1992; Mansvelder and McGehee 2000). Normal metabolizers, who have faster nicotine metabolism and smoke more cigarettes per day, may have greater peaks and troughs in brain nicotine levels compared to slow metabolizers, and thus may experience more burst firing. Therefore, there may be stronger associations formed between nicotine and smoking-related cues in the brains of normal (vs. slow) nicotine metabolizers; this may in turn contribute to why smoking cue-evoked responses (vs. control cues) in brain reward regions (e.g., insula and amygdala) of normal nicotine metabolizers were stronger than those in
slow nicotine metabolizers (Tang, Hello et al. 2012). Findings from animal models have demonstrated that cues and experiences associated with drug use can eventually become reinforcing themselves and motivate animals to self-administer nicotine (Shaham, Adamson et al. 1997; Caggiula, Donny et al. 2001; Caggiula, Donny et al. 2002); thus, it is possible that the higher smoking cue-related reactivity observed in normal metabolizers (Tang, Hello et al. 2012) may partially explain their higher rates of relapse compared to slow nicotine metabolizers.

Consistent with the postulated role of α7 nAChRs in nicotine-mediated long-term potentiation, α7 nAChRs play a key role in neuronal plasticity throughout the CNS, with particular importance for processes involved in learning and memory (McKay, Placzek et al. 2007). Nicotine withdrawal leads to cognitive deficits, particularly in working memory, experienced by abstinent smokers; varenicline, a full agonist at α7 nAChRs, was shown to restore working memory in abstinent smokers (Loughead, Ray et al. 2010); it is possible that normal nicotine metabolizers, who have been shown to experience more severe withdrawal symptoms compared to slow nicotine metabolizers (Kubota, Nakajima-Taniguchi et al. 2006; Rubinstein, Benowitz et al. 2008), experience greater working memory deficits compared to slow metabolizers. Lower working-memory related brain activity was prospectively associated with smoking relapse (Loughead, Wileyto et al. 2014); as varenicline has ~20-fold higher affinity for α7 nAChRs compared to nicotine (Coe, Brooks et al. 2005), varenicline may more effectively mitigate abstinence-induced defects in working memory, contributing to its greater efficacy compared to nicotine patch in normal metabolizers (Lerman, Schnoll et al. 2015). Within this clinical trial, number needed to treat analyses that compared varenicline to placebo and nicotine to placebo indicated that among normal metabolizers, far fewer smokers would need to be treated with varenicline than patch to have one smoker quit (5 vs. 26, respectively) (Lerman, Schnoll et al. 2015).

6.2.2.3. Nicotine exposure, metabolism rate, and the modeling of neural networks

Recently, the strength of functional connectivity in brain reward regions was shown to be different in normal compared to slow nicotine metabolizers (Sufang Yi et al. unpublished findings). These differences were found in smokers but not in non-smokers, suggesting nicotine
exposure is required for the remodeling of these networks. Compared to slow nicotine metabolizers, normal nicotine metabolizers were shown to have higher functional connectivity strength in mesocorticolimbic reward circuits; this suggests greater neuroplastic changes occurred in normal compared to slow metabolizers in response to nicotine. These strengthened neural networks in normal metabolizers may partially explain their greater reactivity to smoking cues (vs. control cues) (Tang, Hello et al. 2012) and also possibly contribute to their higher rates of relapse.

6.3. Differences in smoking behaviours between adult and adolescent smokers

The vast majority of research investigating how variation in the rate of nicotine metabolism influences smoking behaviours has been conducted in adult smokers, with comparatively fewer studies examining adolescent smokers. Adolescent and adult smokers have demonstrated differences in a number of smoking behaviours including smoking duration, smoking quantity, and regularity of smoking; a large proportion of adolescent smokers were shown to be light, intermittent or occasional (i.e., non-daily) smokers (O'Loughlin, DiFranza et al. 2003). In addition, adolescent daily smokers have been shown to smoke fewer cigarettes than adult daily smokers (Reid, Hammond et al. 2014). Despite the lower level and more sporadic pattern of smoking among many adolescent smokers, adolescent smokers are often dependent on nicotine (Rojas, Killen et al. 1998; Colby, Tiffany et al. 2000). Results from a brain imaging study suggest that adolescent light smokers (1-5 cigarettes/day) responded to smoking-related cues in a similar manner to heavier smoking adolescents and adults; adolescent never-smokers did not show reactivity to smoking-related cues (Rubinstein, Luks et al. 2011). Together the findings suggest that while adolescence may represent a period of relatively low cigarette consumption, many adolescent smokers are nicotine dependent.

As there are differences in smoking behaviours between adolescents and adults, it is possible that there are age-related differences in how nicotine metabolism variation influences smoking. However, slow nicotine metabolism is associated with greater smoking cessation in both adults (Gu, Hinks et al. 2000; Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009) and
adolescents (see chapter 1). To further support the notion that altered nicotine metabolism may affect smoking behaviours similarly in adolescents and adults, lower cigarette consumption among slow metabolizers has been observed in both dependent adolescent and adult smokers ((O'Loughlin, Paradis et al. 2004; Schoedel, Hoffmann et al. 2004; Audrain-McGovern, Al Koudsi et al. 2007; Wassenaar, Dong et al. 2011); also see Appendix D).

Overall, nicotine pharmacokinetics are similar in adolescents and adults, suggesting that differences in nicotine pharmacokinetics do not explain age-related differences in smoking frequency and the level of cigarette consumption (O'Loughlin, DiFranza et al. 2003; Reid, Hammond et al. 2014). As described previously, adolescents and adults are thought to have similar rates of nicotine metabolism, measured in vitro in human liver microsomes (Parkinson, Mudra et al. 2004; Al Koudsi, Hoffmann et al. 2010), and in vivo in smokers receiving nicotine patch therapy (Gourlay, Benowitz et al. 1997). In healthy adults and children given coumarin, a CYP2A6 probe substrate, the level of 7’hydroxycoumarin excreted in urine was similar, further suggesting CYP2A6 activity toward coumarin also does not differ as a function of age (Pasanen, Rannala et al. 1997).

While differences in nicotine pharmacokinetics likely do not explain age-related differences in smoking behaviours, it is possible that differences in neural circuitry between adolescents and adults could lead to variable smoking behaviour. The adolescent period is marked by rapid changes in brain development, with the prefrontal cortex, implicated in cognitive control and goal-directed behaviour, being one of the last brain regions to mature (Crews, He et al. 2007; Casey, Jones et al. 2008). The limbic system, which includes the nucleus accumbens and amygdala, matures more rapidly than the prefrontal cortex, and is associated with risk-taking behaviour (Galvan, Hare et al. 2006; Casey, Jones et al. 2008; Bava and Tapert 2010). Reduced prefrontal cortex functionality in adolescence compared to adulthood, together with earlier maturation of the limbic system (Galvan, Hare et al. 2006), is thought to contribute to the greater impulsivity and lower goal-directed behaviour observed among adolescents; these factors likely contribute to substance abuse in adolescents (Warner, Kessler et al. 1995; Casey, Jones et al. 2008). As discussed in the previous section, the strength of functional connectivity within striatal-cortical pathways was affected by the rate of nicotine metabolism in adult smokers, with
faster nicotine metabolizers showing greater functional connectivity strength (Sufang Yi et al. unpublished findings). It is not known whether these nicotine metabolism-related differences in functional connectivity are also present in adolescent smokers. However, given that slow nicotine metabolizers are more likely to quit than normal metabolizers in both adulthood and adolescence, it is possible that the modulating effect of nicotine metabolism on the neural circuits implicated in drug use are similar in adolescents as in adults; if these differences in neural circuitry contribute to relapse risk, this may explain, at least in part, the effect of nicotine metabolism on smoking abstinence in both age groups.

Investigating whether these nicotine-mediated changes in neural circuitry occur early in smoking history, during adolescence, may provide mechanistic insight into early processes of nicotine dependence. Performing a resting state fMRI study in adolescent current smokers to examine functional connectivity strength within brain reward regions would clarify whether the rate of nicotine metabolism is associated with neuroplastic changes soon after smoking initiation. If the functional connectivity strength differs as a function of nicotine metabolism rate in adolescent current smokers, a follow-up resting state fMRI study examining adolescent former smokers may clarify whether nicotine metabolism-associated neuroplastic changes are reversed upon the cessation of smoking. Adolescent smokers who have undergone resting state fMRI analysis could also be followed over time to the end of adolescence to examine whether nicotine metabolism rate differences in functional connectivity influence smoking abstinence outcomes. A parallel longitudinal study could also be performed in adult smokers to examine whether abstinence outcomes in adulthood are modulated by differences in functional connectivity strength. These types of studies may help clarify whether the differences in abstinence outcomes associated with variation in the rate of nicotine metabolism are influenced by differences in functional connectivity. To investigate relationships between variation in the rate of nicotine metabolism, functional connectivity strength, and relapse, statistical mediation analysis could be performed; it is possible that greater functional connectivity strength in brain reward regions mediates the relationship between faster nicotine metabolism and higher relapse rates. If greater functional connectivity strength is independently associated with higher rates of relapse in all smokers, resting state fMRI, which is a relatively non-invasive procedure, may also be useful for
identifying sub-groups of smokers who are at higher relapse risk, regardless of their rate of nicotine metabolism.

6.4. Using CYP2A6 activity/the nicotine metabolite ratio to increase smoking cessation rates and better understand smoking behaviour

6.4.1. Inhibiting CYP2A6 activity

As slow nicotine metabolizers are more likely to quit smoking in both adolescence (see chapter 1) and adulthood (Gu, Hinks et al. 2000; Lerman, Tyndale et al. 2006; Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009; Schnoll, Patterson et al. 2009; see also chapter 4), mimicking slow metabolism through inhibiting CYP2A6 activity in smokers may lead to higher quit rates. If future studies demonstrate that the inhibition of nicotine metabolism increases quit rates in normal metabolizers, a reasonable treatment strategy may involve the assessment of CYP2A6 genotype and/or NMR, and treating normal nicotine metabolizers with a CYP2A6 inhibitor and NRT (Fig. 16). It is possible that inhibiting CYP2A6 activity may also increase the effectiveness of existing NRTs and oral nicotine as a form of NRT, and reduce CYP2A6-mediated bioactivation of tobacco-specific nitrosamines (Sellers, Tyndale et al. 2003; Zhu, Binnington et al. 2013). Compared to reversible inhibitors, mechanism-based inhibition of CYP2A6, causing irreversible and prolonged enzyme inactivation (Lin and Lu 1998), is likely to more closely approximate slow CYP2A6 activity. Thus, mechanism-based inhibitors of CYP2A6 may show greater promise in reducing cigarette consumption and promoting cessation; this may occur in the absence of cessation pharmacotherapy or while on NRT.
Figure 16 | Research design to test potential treatment strategies for optimizing quit rates in smokers. If CYP2A6 inhibitors are shown to increase quit rates in normal metabolizers, treatment of normal metabolizers with a CYP2A6 inhibitor, followed by NRT, may prove to be one useful approach for increasing cessation rates in this group. Abbreviation: NMR, nicotine metabolite ratio; NRT, nicotine replacement therapy. Based on findings from Sellers, Tyndale et al. 2003 and Lerman, Schnoll et al. 2015.

In addition to lower cigarette consumption and increased cessation rates, slow (versus normal) CYP2A6 activity is associated with a lower relative risk of lung cancer (Wassenaar, Dong et al. 2011), consistent with a lower level of smoking and tobacco-specific nitrosamine bioactivation (Zhu, Binnington et al. 2013); thus, CYP2A6 inhibitors, through their effects on reducing cigarette consumption, may also lower lung cancer risk over time if these reductions in consumption are sustained. A study investigating the duration of CYP2A6 inhibitor treatment required to increase quit rates or reduce the level of cigarette consumption would help establish whether long-lasting inhibitor treatment is required for promoting sustained abstinence or reducing cigarette consumption. Extending the duration of inhibitor treatment may be an effective strategy for increasing quit rates. For the nicotine patch, extending the duration of treatment to 24 weeks (versus 8 weeks for standard therapy) increased the overall rate of point-prevalence abstinence at 24 weeks by more than 50% (31.6% vs. 20.3%, respectively; P=0.002)
In slow metabolizers, where nicotine patch therapy is particularly efficacious for smoking cessation (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009), extending therapy to 24 weeks increased point-prevalence abstinence at 24 weeks to nearly 50%, compared to ~25% for standard therapy (Lerman, Jepson et al. 2010).

Methoxsalen, a potent mechanism-based inhibitor of CYP2A6 in vitro (Draper, Madan et al. 1997; Koenigs, Peter et al. 1997; Kharasch, Hankins et al. 2000), and a significant inhibitor of CYP2A6 activity in vivo (Kharasch, Hankins et al. 2000; Sellers, Kaplan et al. 2000), has shown promise in laboratory-based studies as a means to reduce smoking. Methoxsalen led to increased nicotine exposure following oral and subcutaneous nicotine administration in kinetic studies (Sellers, Kaplan et al. 2000; Sellers, Ramamoorthy et al. 2003), and lower smoking in an experimental study in adult nicotine dependent smokers (Sellers, Kaplan et al. 2000). Smokers that received methoxsalen and oral nicotine before a 60-minute abstinence period and 90-minute free smoking period had lower cigarette consumption, a longer latency between the first and second cigarette smoked, and fewer number of puffs taken compared to smokers that received placebo plus placebo (Sellers, Kaplan et al. 2000). These findings that demonstrate a reduction in smoking during the 90-minute free smoking period in those that underwent nicotine preloading are similar to those from a prior study using nicotine gum; nicotine gum pre-treatment led to significant reductions in the number of cigarettes smoked and number of puffs taken compared to placebo pre-treatment (Nemeth-Coslett, Henningfield et al. 1987). Methoxsalen treatment in smokers instructed to smoke a consistent number of cigarettes was also associated with higher levels of 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butan-1-one (NNAL) and NNAL-glucuronide in the urine, suggestive of lower bioactivation of the tobacco-specific nitrosamine 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Sellers, Ramamoorthy et al. 2003). Thus, the inhibition of CYP2A6 activity in smokers may be an effective strategy to treat tobacco dependence, and reduce the level of exposure and activation to carcinogens when lapses occur during the process of smoking cessation. While methoxsalen is a potent inhibitor of CYP2A6, it is non-selective and also strongly inhibits CYP1A2 (Zhang, Kilicarslan et al. 2001). Methoxsalen also has a short half-life of ~1.5 hours (de Wolff and Thomas 1986), and may act more as a competitive inhibitor in vivo, as opposed to a mechanism-based inhibitor (Kharasch, Hankins et
Theoretically, the use of mechanism-based inhibitors with longer half-lives may hold greater promise in promoting sustained inhibition of CYP2A6 activity.

Selegiline, a mechanism-based inhibitor of CYP2A6 (Siu and Tyndale 2008), as well as a monoamine oxidase-B inhibitor (Youdim, Edmondson et al. 2006), has been previously studied for its potential utility in smoking cessation. Selegiline was originally investigated for smoking cessation due to the role of dopamine in nicotine dependence (George, Vessicchio et al. 2003); monoamine oxidase-B inhibitors block the metabolism of dopamine. Harman and norharman are monoamine oxidase inhibitors present in cigarette smoke, and are thought to contribute to the reinforcing properties of cigarette smoking (Herraiz and Chaparro 2005). The administration to treatment-seeking smokers of a monoamine oxidase-B inhibitor, such as selegiline, would theoretically reduce dopamine catabolism within the brain and may help compensate for the lack of nicotine-mediated dopaminergic signaling experienced by abstinent smokers. A preliminary investigation found a significant benefit of oral selegiline over placebo at enhancing end-of-treatment abstinence rates in a pilot study of 40 treatment-seeking smokers (George, Vessicchio et al. 2003), however a later study did not replicate this finding in a larger population (N=101) of treatment-seeking smokers (Weinberger, Reutenauer et al. 2010). Compared to the pilot investigation, eight-week quit rates on selegiline were lower (16% vs. 45%), while eight-week quit rates on placebo were higher (20% vs. 15%), in the larger study (George, Vessicchio et al. 2003; Weinberger, Reutenauer et al. 2010). Both samples of smokers were highly motivated to quit and had experienced similar numbers of failed prior quit attempts (~5-9) (George, Vessicchio et al. 2003; Weinberger, Reutenauer et al. 2010), suggesting differences in the motivation to quit or ‘hardening of the target’ likely do not explain the discordant findings. It is possible that differences in statistical power may have partially contributed to the differences in the findings between studies.

In a smoking cessation clinical trial where selegiline was administered using a transdermal system, there was no difference in quit rate between participants who received a selegiline patch compared to those who received a placebo patch (Kahn, Gorgon et al. 2012). In a separate trial in which all smokers received the nicotine patch for eight weeks, there was a trend toward higher quit rates at one year in smokers that were also treated with oral selegiline (vs. oral placebo) for
six months (25% and 11%, respectively); selegiline plus nicotine patch (versus placebo plus nicotine patch) treatment was also associated with lower craving (Biberman, Neumann et al. 2003). Thus, it appears that treatment with the selegiline patch alone may not promote cessation, perhaps due to insufficient inhibition of monoamine oxidase-B and/or CYP2A6. The increase in quit rates with selegiline patch plus nicotine patch (vs. selegiline patch alone) could be attributable to an inhibitory effect of selegiline on CYP2A6, which would mimic slow nicotine metabolism, leading to higher nicotine patch-derived nicotine levels and improved quit rates (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). It is possible that selegiline treatment may improve smoking cessation outcomes on the nicotine patch in normal nicotine metabolizers through its inhibition of CYP2A6 activity; future studies could assess quit rates according to nicotine metabolism group in smokers receiving nicotine patch therapy, where selegiline is additionally administered to half of the participants. It is possible that selegiline treatment in normal metabolizers would lead to quit rates on the nicotine patch comparable to those observed in slow metabolizers receiving nicotine patch alone.

Two novel inhibitors of CYP2A6 were recently discovered that are also potent inhibitors of CYP2A6 with improved selectivity (Tani, Juvonen et al. 2014). These compounds will be further developed as lead compounds; whether their CYP2A6 inhibitory activity leads to reductions in cigarette consumption and higher quit rates remains to be determined. Following pre-clinical testing using animal models as well as safety screening in humans, the ability of novel CYP2A6 inhibitors (Tani, Juvonen et al. 2014) to promote cessation on NRT could theoretically be investigated using early (i.e., one week) abstinence to screen for potential efficacy; early abstinence outcomes on patch and NRT are associated with prolonged abstinence at six months (Ashare, Wileyto et al. 2013). Daily cigarette consumption could also be measured throughout the one-week period, with reductions in consumption among those unable to quit being another favourable outcome; some benefits to health are noted among those who reduce their level of consumption (but do not quit) versus those who continue to smoke the same number of cigarettes (Benowitz, Jacob et al. 1986; Jimenez-Ruiz, Kunze et al. 1998; Pisinger and Godtfredsen 2007). To determine whether novel or established CYP2A6 inhibitors reduce cigarette consumption and increase quit rates, a mechanism-based CYP2A6 inhibitor or matched placebo could be administered to treatment-seeking smokers with or without the nicotine patch. Participants in the
four treatment groups (placebo + placebo patch, placebo + nicotine patch, inhibitor + placebo patch, and inhibitor + nicotine patch) could be followed for one week after the target quit date, recording their cigarette consumption on each day of the study to allow for timeline follow-back analyses of cigarette consumption during the seven days of active patch treatment. The nicotine metabolite ratio could be assessed at study baseline and included as a covariate in analytic models. One-week abstinence and cigarette consumption outcomes could be compared according to CYP2A6 inhibitor (yes/no) and nicotine patch (yes/no) status; those receiving both the CYP2A6 inhibitor and nicotine patch may have larger reductions in cigarette consumption as well as higher abstinence rates at one week, compared to those receiving the inhibitor or nicotine patch alone. The level of reduction in cigarette consumption and abstinence rates would presumably be lowest in those receiving placebo inhibitor + placebo patch. It is possible that the effect of CYP2A6 inhibition and nicotine patch treatment on reducing smoking and increasing abstinence would occur to a similar degree in normal and slow nicotine metabolizers, however overall quit rates may be higher in slow metabolizers compared to normal metabolizers.

Pharmacokinetic analyses could be conducted in those wearing nicotine patches to determine whether CYP2A6 inhibition altered patch-derived nicotine levels. Both oral and transdermal formulations of the CYP2A6 inhibitor could be tested; if proven to be efficacious in reducing smoking, an oral pill could be co-formulated with nicotine for further testing as a cessation aid (see below). In an acute setting (i.e., short-term, one-week abstinence), higher plasma nicotine levels may aid abstinence through relief of withdrawal symptoms; however, nicotine patch-derived plasma nicotine levels did not predict cessation outcomes at end-of-treatment (Lerman, Tyndale et al. 2006), highlighting the need for additional studies that investigate nicotine pharmacodynamic influences on smoking cessation outcomes.

The use of CYP2A6 inhibitors may also improve the bioavailability of oral nicotine; in mice, the methoxsalen-mediated inhibition of CYP2A5 (i.e., murine ortholog of CYP2A6) led to significant increases in nicotine’s half-life and area under the curve from an oral nicotine dose, suggestive of improved nicotine bioavailability with methoxsalen pre-treatment (Alsharari, Siu et al. 2014). The development of an oral nicotine capsule that contains both a CYP2A6 inhibitor as well as nicotine would likely increase the plasma concentration of nicotine by reducing the large first pass effect on nicotine metabolism (Zins, Sandborn et al. 1997), leading to enhanced
nicotine bioavailability and overall exposure. Following verification of enhanced nicotine bioavailability in animal models, pharmacokinetic studies could be performed in healthy adults to examine drug concentration-time curves for the co-formulated CYP2A6 inhibitor with nicotine, and to confirm that the plasma nicotine concentration resulting from the co-formulated nicotine is higher compared to that achieved by the administration of nicotine alone. To determine whether nicotine co-formulated with a CYP2A6 inhibitor reduces cigarette consumption, a laboratory-based study in smokers could be conducted that incorporates a 90-minute free smoking period following treatment with the co-formulated nicotine, similar to the design of the laboratory-based study examining the effect of methoxsalen and nicotine pre-treatment on smoking behaviour (Sellers, Kaplan et al. 2000). If this novel nicotine co-formulation were to show promise in reducing cigarette consumption in a laboratory-based study in human smokers, this may warrant its future investigation as a potential smoking cessation aid.

6.4.2. Tailoring smoking cessation therapy and finding new treatments

Knowledge of smokers’ CYP2A6 activity may also improve strategies for tailoring smoking cessation therapy. In adult heavy smokers (≥10 cigarettes/day), at end-of-treatment varenicline was more efficacious than the nicotine patch for aiding smoking cessation in normal nicotine metabolizers; in slow nicotine metabolizers, quit rates were similar on varenicline and the nicotine patch (Lerman, Schnoll et al. 2015). In addition, compared to normal metabolizers, slow metabolizers experienced higher summary side effects on varenicline (versus placebo) (Lerman, Schnoll et al. 2015). Thus, a reasonable treatment strategy to optimize quit rates and minimize negative side effects might be to treat normal metabolizers with varenicline, and slow metabolizers with the patch (Fig. 16). Whether this is also a viable treatment plan for adolescent smokers remains to be determined. In a nicotine patch trial in adolescents, treatment with the nicotine patch significantly increased quit rates at end-of-treatment in adolescent smokers (18% vs. 2.5%, respectively) (Moolchan, Robinson et al. 2005). However, these end-of-treatment quit rates on the nicotine patch are comparatively lower than those observed in adult smokers, where meta-analyzed data suggest that ~27% of adult smokers receiving the nicotine patch are abstinent at end-of-treatment (Fiore, Smith et al. 1994). In the sample of adolescent smokers receiving patch therapy, a substantial proportion (~75%) had co-existing psychiatric conditions such as
oppositional defiant disorder and conduct disorder (Moolchan, Robinson et al. 2005). In clinical trials in adult smokers, individuals with psychiatric comorbidities are often excluded; the high prevalence of psychiatric comorbidities in the sample of adolescent smokers may have led to their lower quit rates on nicotine patch compared to adult smokers (Moolchan, Robinson et al. 2005). Future clinical investigations in adolescent smokers that assess the efficacy and safety of the nicotine patch and varenicline, and incorporate assessments of NMR, are required to determine whether NMR interacts with treatment to influence abstinence in adolescents, as in adults (Lerman, Schnoll et al. 2015).

In adults, the enhanced efficacy of varenicline over nicotine patch in normal metabolizers was also apparent at one week post-quit (OR=1.52 for varenicline vs. patch, P=0.049). Also in agreement with end-of-treatment outcomes, there was no significant benefit of varenicline over patch in slow metabolizers at one week (OR=1.38 for varenicline vs. patch, P=0.11). In chapter 4, we showed an overall association between slow metabolism and an increased likelihood of abstinence at one week, after controlling for treatment effects (i.e., varenicline vs. placebo, and nicotine patch vs. placebo) and factors associated with NMR variation we had identified in chapter 3 (OR=1.43 for slow vs. normal metabolism, P=0.004). In a second model where the effect of varenicline versus nicotine patch was controlled for, NMR was also associated with one-week abstinence (OR=1.44 for slow vs. normal nicotine metabolism, P=0.01); in a model that included NMR as the only independent variable, the OR for quitting among slow versus normal metabolizers was 1.32 (P=0.02). Revised protocols for drug development in tobacco dependence that incorporate short-term efficacy screening in early phase II (Perkins and Lerman 2014) should likely include assessments of smokers’ NMR. This protocol compares the number of days of abstinence achieved in a week on placebo to that on medication using a within-subjects crossover design. While the inclusion of NMR in our one-week abstinence model did not alter any of the treatment-abstinence associations, NMR did have an overall effect on early abstinence (see chapter 4). At the very least, NMR should be included in analytic models as a covariate when evaluating efficacy of new treatments on early abstinence; slow metabolizers quit better in general and may show elevated abstinence rates on placebo compared to normal metabolizers, as has been observed in several phase III clinical trials (Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009). Even for drugs that are not substrates of CYP2A6, NMR
may aid in their development plan if researchers are interested in identifying characteristics of treatment responders, and in making comparisons to those for whom the treatment was not successful. In smokers that received varenicline, which is not a substrate of CYP2A6, differences in quit outcomes were observed across NMR groups (Lerman, Schnoll et al. 2015), further suggesting that nicotine metabolism differences in how brain neural circuits are remodeled through smoking (Sufang Yi, et al. unpublished findings) may influence smoking cessation success long after nicotine is no longer present in the body.

6.4.3. Influence of CYP2A6 activity on acquisition, cigarette consumption, the level of nicotine dependence, and smoking-related disease risk

In addition to its influence on cessation, variation in CYP2A6 activity, measured by CYP2A6 genotype or NMR, is associated with the rate of tobacco dependence acquisition (O'Loughlin, Paradis et al. 2004); also see Appendix D), escalation in the level of dependence (Audrain-McGovern, Al Koudsi et al. 2007), cigarette consumption (Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Wassenaar, Dong et al. 2011) and the level of nicotine dependence in some (Wassenaar, Dong et al. 2011; Sofuoglu, Herman et al. 2012; Schnoll, George et al. 2014) but not all (Benowitz, Pomerleau et al. 2003; Johnstone, Benowitz et al. 2006; Lerman, Tyndale et al. 2006; Kandel, Hu et al. 2007; Patterson, Schnoll et al. 2008; Ho, Mwenifumbo et al. 2009; Schnoll, Patterson et al. 2009) studies. In a cohort of adolescents followed from age 12 to 18, CYP2A6 slow nicotine metabolizers displayed an increased rate of conversion to tobacco dependence compared to normal metabolizers (See CYP2A6 and tobacco dependence acquisition paper in Appendix D). Once dependent, CYP2A6 slow metabolizers smoked fewer cigarettes compared to normal metabolizers (~6 vs. ~10 cigarettes/day, respectively; P<0.05). CYP2A6 activity is also associated with the level of cigarette consumption in adult heavy smokers, where slow metabolizers smoke fewer cigarettes compared to normal metabolizers (Schoedel, Hoffmann et al. 2004; Malaiyandi, Lerman et al. 2006; Wassenaar, Dong et al. 2011).

In contrast to adolescent light smokers (<10 cigarettes/day), there do not appear to be any differences in the level of cigarette consumption in adult light smokers as a function of CYP2A6 activity, although there could be differences in terms of how each cigarette is smoked (i.e., smoking topography), leading to differences in the level of nicotine exposure (Zhu, Binnington et
In heavier smokers, CYP2A6 slow metabolism was associated with a lower depth of inhalation (i.e., lower puff volume) compared to normal metabolism (Strasser, Malaiyandi et al. 2007). Emerging evidence from studies in adults suggests that ‘light smokers’ consume similar amounts of tobacco as heavy smokers. The levels of urinary total nicotine equivalents (TNE) were similar between African American, Alaska Native, and Caucasian smokers, despite marked differences in the number of cigarettes smoked/day (Zhu, Binnington et al. 2013; Zhu, Zhou et al. 2013), suggesting that light-smoking populations smoking fewer than 10 cigarettes/day (e.g., African Americans and Alaska Natives) extracted more nicotine per cigarette than heavier smoking populations (e.g., Caucasians). Urinary TNE, the gold standard of tobacco dose exposure, comprises up to ~90% of a nicotine dose (Benowitz, Jacob et al. 1994), and represents the molar sum of nicotine and its metabolites, including cotinine, 3’hydroxycotinine, and their respective glucuronides, among others (Zhu, Binnington et al. 2013). In a controlled pharmacokinetic study in non-smokers receiving a known dose of nicotine, urinary TNE was more highly related than plasma cotinine level to total nicotine dose (Benowitz, Dains et al. 2010). In addition, unlike cotinine, the utility of TNE as a measure of nicotine exposure is not affected by differences in CYP2A6 activity or genotype, because nicotine and its metabolites are collected as one value (Zhu, Renner et al. 2013). Thus, evaluating TNE in smokers, which also accounts for differences in smoking topography and represents a more accurate (vs. cotinine or cigarettes/day) biomarker of tobacco dose, suggests that adult ‘light smokers’ (<10 cigarettes/day) may be exposed to similar levels of cigarette smoke and tobacco-related carcinogens as adult heavy smokers. While TNE was not assessed in the Nicotine Dependence in Teens cohort (see Appendix D), we measured salivary cotinine levels at survey 22, when the mean age of participants was 24 years. Among White self-reported current smokers (n=162), the average (SD) cotinine level was 139 ng/ml (129.7). These young adult smokers smoked an average of ~246 cigarettes/month (i.e., ~8 cigarettes/day). In a sample of White adult smokers (mean age ~32 years) consuming nearly twice as many cigarettes (~14 cigarettes/day), the level of cotinine was only modestly higher than in our young adult sample (177 ng/ml vs. 139 ng/ml, respectively) (Perez-Stable, Herrera et al. 1998). In addition, the level of cotinine per cigarette (mean cotinine level divided by mean cigarettes per day), a surrogate of smoking intensity, was higher in our sample than in the previous investigation (~17 ng/ml vs. ~13 ng/ml, respectively) (Perez-Stable, Herrera et al. 1998); these data suggest that while the young adults in our sample...
reported a relatively low level of cigarette consumption (~8 cigarettes/day), they may have been smoking each cigarette more intensively.

A study investigating TNE in adolescent and young adult smokers would provide a more accurate measure (relative to self-reported cigarette consumption and measured cotinine level) of the real intake level of nicotine. A smoking topography study in adolescent heavy smokers (mean = 19 cigarettes/day) revealed an association between faster NMR and higher puff volume in males only (Moolchan, Parzynski et al. 2009). Males with higher NMR also displayed a lower number of puffs and shorter puff duration compared to males with lower NMR (Moolchan, Parzynski et al. 2009). Whether these differences in topography lead to an overall effect on total tobacco exposure (and thus disease risk) could be assessed through evaluating TNE in adolescent and young adult smokers. It would be optimal to assess TNE in both adolescent and young adult light and heavy smokers, as “light”-smoking youth (determined via cigarettes/day) may consume comparable amounts of tobacco as heavier-smoking youth, as has been observed in adults (Zhu, Binnington et al. 2013; Zhu, Zhou et al. 2013). However, a smoking topography study comparing adolescent non-daily (~19 cigarettes/week) and daily smokers (~73 cigarettes/week or ~10 cigarettes/day) revealed no differences in the number of puffs per cigarette, average puff volume, or total puff volume, suggesting non-daily and daily adolescent smokers may be exposed to similar levels of toxicants on a per-cigarette basis (Corrigall, Zack et al. 2001). Future studies in adolescent and young adult smokers that incorporate TNE will help clarify whether “light” and heavy smokers achieve similar doses of tobacco, and whether cigarettes/day is a comparatively crude measure of tobacco dose in younger smokers as in many adult smoking populations.

From a disease prevention perspective, it would be worthwhile to extend studies examining TNE in adolescent smokers to determine whether NMR modulates the risk of tobacco-related disease in adolescents and as they enter into young adulthood. While certain tobacco-related diseases such as lung cancer take years to develop, reduced lung function is apparent soon after smoking initiation in adolescents that smoke (CDC 2012a). In addition, an increased risk for atherosclerosis of the abdominal aorta is apparent in young adult smokers (CDC 2012a). Adolescents who smoke (vs. non-smokers) may also be susceptible to developing abdominal obesity, a risk factor for cardiovascular disease, as young adults (age ~ 24 years) (Saarni,
Pietilainen et al. 2009). In adults, while smoking is typically associated with lower body weight and BMI, those who smoke have higher waist circumference and waist-to-hip ratios compared to non-smokers (Bamia, Trichopoulou et al. 2004; Canoy, Wareham et al. 2005). Variation in CYP2A6 was shown to modulate the association between heavy smoking and abdominal obesity in adults (Liu, David et al. 2011). In CYP2A6 poor nicotine metabolizers (<25% CYP2A6 activity compared to wild type), the odds of abdominal obesity were ~4-fold higher in heavier smokers compared to lighter smokers (Liu, David et al. 2011); whether nicotine metabolism modulates abdominal obesity risk in adolescent smokers is not known. A study examining potential associations between adolescent smoking, NMR, and the development of abdominal obesity in young adulthood could be performed using data from the Nicotine Dependence in Teens cohort, where participants were followed until they were ~24 years old (OLoughlin, Dugas et al. 2014). We utilized data from the Nicotine Dependence in Teens cohort in chapter 1 and Appendix D to examine associations between nicotine metabolism variation and smoking behaviours in adolescents. A study such as this would help determine whether CYP2A6 or NMR variability moderates the association between smoking in adolescence and the development of abdominal obesity in young adults.

6.5. Identification and analysis of sources of variation in CYP2A6 activity/the nicotine metabolite ratio

A better understanding of the sources of variability in NMR would likely improve the interpretation of findings from studies that use NMR to assess differences in smoking behaviours and smoking-related diseases. In chapters 2 and 3, we showed that a number of potential sources of variation in CYP2A6 activity/NMR were relatively minor, and likely do not need to be considered when assessing relationships between NMR and smoking behaviours. In chapter 2, variation in FMO3 E158K (rs2266782) and POR A503V (rs1057868) did not substantially affect nicotine AUC (i.e., a proxy for nicotine exposure and clearance) or NMR, respectively. Among CYP2A6 genotyped reduced nicotine metabolizers, we observed a trend toward higher nicotine AUC, suggestive of slower nicotine clearance, in individuals with one or two copies of the reduced function FMO3 158K allele; there was no association between FMO3 E158K variation and nicotine AUC in CYP2A6 genotyped normal metabolizers. In chapter 2, we used a relatively heterogeneous definition of ‘CYP2A6 genotyped reduced metabolism’ (between ~0-75%
CYP2A6 activity); we anticipate that the impact of the FMO3 158K allele would be greater in individuals with poor CYP2A6 metabolism (i.e., lack of functional CYP2A6 alleles), as these individuals may rely more heavily on the FMO3 pathway for nicotine clearance (Yamanaka, Nakajima et al. 2004). In an exploratory analysis, we divided the CYP2A6 genotyped reduced metabolizers into CYP2A6 genotyped ‘intermediate’ and ‘slow’ metabolism groups, and tested the impact of having one or two copies of the FMO3 158K allele on nicotine AUC in these separate groups. We speculated that the influence of the FMO3 158K allele on increasing nicotine AUC (i.e., reducing the rate of nicotine clearance) would be greater in CYP2A6 genotyped slow metabolizers compared to intermediate metabolizers, as the slow metabolizers would have a relatively lower level of CYP2A6 activity. Although the sub-group analyses reduced power, the FMO3 158K allele was associated with 26% higher nicotine AUC in CYP2A6 genotyped intermediate metabolizers, and 29% higher nicotine AUC in CYP2A6 genotyped slow metabolizers, although neither of these findings was significant. Theoretically, the further impairment of nicotine clearance in FMO3 158K individuals with poor CYP2A6 metabolism might reduce the level of smoking beyond that caused by poor CYP2A6 activity, however this would represent a small segment of the population (i.e., ~2-3% of those with African ancestry (Ho, Mwenifumbo et al. 2008; Mwenifumbo, Al Koudsi et al. 2008; Al Koudsi, Ahluwalia et al. 2009)), and we were underpowered to test this in the current study.

In our analysis of the association between variation in POR A503V and CYP2A6 activity (i.e., NMR), we observed modestly higher NMR in CYP2A6 genotyped normal metabolizers with one or two copies of the POR 503V allele compared to CYP2A6 genotyped normal metabolizers with two copies of the POR A503 allele (P=0.03). No significant association between POR A503V and NMR was observed in CYP2A6 genotyped reduced metabolizers. The lack of association between POR A503V and NMR in CYP2A6 genotyped reduced metabolizers may be due to an effect of CYP2A6 reduced function variants on limiting CYP2A6 activity; thus, the influence of a POR variant on CYP2A6 activity may not be apparent. Another potential explanation for differences in the association between POR A503V and NMR in CYP2A6 genotyped normal and reduced metabolizers may be due to group differences in exposure to additional substrates, leading to competition for POR. In human liver, the level of POR protein is lower than that of P450 protein; thus, POR may be a limiting factor in microsomal P450 metabolic activities under
certain circumstances, such as when multiple substrates are present leading to competition for POR (Schmucker, Woodhouse et al. 1990; Tan, Patten et al. 1997; Gomes, Winter et al. 2009). Competition for POR activity between P450 enzymes can exist when several substrates are present that are metabolized by unique P450 enzymes (Tan, Patten et al. 1997), or when a large amount of one substrate is present that saturates one enzyme system; at high doses of nicotine, CYP2B6 is thought to be recruited to facilitate the metabolism of nicotine to cotinine (Yamazaki, Inoue et al. 1999). In a baculovirus expression system, the presence of a CYP2E1 substrate led to lower CYP2A6-mediated coumarin metabolism (Tan, Patten et al. 1997); it is possible that P450 competition for POR exists in vivo. If CYP2A6 genotyped reduced metabolizers had greater exposure to other P450 substrates, this may have led to POR recruitment to other P450s, causing negligible apparent impacts of POR variability on NMR.

It is also possible that any nicotine metabolism differences associated with POR and FMO3 variation are due to the route of delivery of nicotine in our study (oral), which exaggerates differences in hepatic metabolism between groups due to the high first pass effect on nicotine; consequently, these effects may not be evident when nicotine is consumed via smoking.

Consistent with this idea, we observed no differences in NMR according to POR A503V in African American smokers (i.e., obtain nicotine via smoking), and there was no association between FMO3 E158K and nicotine clearance in a pharmacokinetic study where African Americans received nicotine intravenously (Taghavi et al, 2015, unpublished data). Thus, we conclude from chapter 2 that the overall impact of CYP2A6 variation on nicotine metabolism and resulting variation in smoking behaviour is far greater than that of FMO3 E158K and POR A503V variation.

In chapter 3, we showed little overall influence of demographic/environmental sources of variation on CYP2A6 activity/NMR. However, some of these demographic sources of variation, e.g., gender and ethnicity, showed significant associations with smoking behaviours, including cessation, and should continue to be evaluated in studies of smoking behaviour. These relationships with cessation are discussed further below.
6.6. Relationships between demographic/environmental factors and smoking cessation outcomes

6.6.1. Gender and smoking cessation

Smoking cessation rates are typically lower in women than in men (Bjornson, Rand et al. 1995; Borrelli, Spring et al. 2001), for reasons that are not fully understood. In chapter 4 of this thesis, however, we observed a trend toward higher likelihood of one-week smoking abstinence among females compared to males, after controlling for treatment and other demographics. In addition, while we observed an overall association between lower NMR and higher likelihood of one-week abstinence, further analysis indicated that this effect was restricted to men (OR=1.64, P=0.002); in women, there was no association between NMR and abstinence (OR=1.06, P=0.74 for lower vs. higher NMR) (Fig. 17). Thus, it is possible that NMR variation does not contribute to early (i.e., one-week) abstinence outcomes in female smokers. Some literature suggests that compared to men, women smoke for reasons less related directly to nicotine reinforcement (Perkins, Gerlach et al. 2001), including for mood regulation. A recent PET imaging study provides insight into these sex-related differences; while smoking a cigarette, dopamine was rapidly activated in the ventral striatum of men, but not women (Cosgrove, Wang et al. 2014). As described in an earlier section, the majority of nicotine withdrawal symptoms return to pre-quit levels early after cessation (Hughes 1992). Thus, NMR, which has been shown to modulate early nicotine withdrawal symptoms (Kubota, Nakajima-Taniguchi et al. 2006; Rubinstein, Benowitz et al. 2008), may not influence nicotine withdrawal in women to the same degree who smoke more for non-nicotine related reasons; as withdrawal can influence early relapse, this may explain the lack of association between NMR and early abstinence in women. In chapter 4, there was also no significant association between NMR and the likelihood of abstinence at end-of-treatment and six months among women; the reason for this lack of association between NMR and early and later abstinence outcomes in women is also not clear. In chapter 1, however, there was no interaction between sex and CYP2A6 metabolism group on one-year abstinence in adolescent smokers, suggesting that the relationship between slow CYP2A6 metabolism and a higher likelihood of abstinence at one year was apparent in both male and female adolescents.
Figure 17 | Likelihood of one-week abstinence according to NMR group, stratified by sex.

Previous studies examining relationships between NMR and the efficacy of the nicotine patch in predominantly Caucasian adult smokers did not test for an interaction between sex and NMR on abstinence outcomes, however the association between lower NMR and higher abstinence was apparent in analytic models that included sex as a covariate (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). In a secondary analysis of NMR and treatment outcomes in a bupropion clinical trial, the sample was not large enough to test for possible gender interaction effects (Patterson, Schnoll et al. 2008). In a sample of African American light smokers, there was no significant interaction between sex and NMR on quit outcomes in the placebo and nicotine gum arms at end-of-treatment; however, this study population was predominantly female (~67%) and thus may have been underpowered to examine potential interactions between sex and NMR on quit outcomes. It appears that the relationship between lower NMR and higher end-of-treatment abstinence may have been stronger in females than in males (Ho, Mwenifumbo et al. 2009). Thus, the available data suggest that there is insufficient information to determine whether sex interacts with NMR to influence quit outcomes at end-of-treatment, and additional analyses
in larger studies are required to explicitly test this. As discussed, for earlier, one-week abstinence, the association between lower NMR and a higher likelihood of abstinence may be more apparent in males than in females (Fig. 17).

Many prior studies investigating the reasons for sex differences in quit rates have centered on the potential role of estrogen, which varies in women according to menstrual cycle phase. Estrogen levels are lower at the beginning of the follicular phase, and then increase to peak at the point of ovulation, followed by a tapering. During the luteal phase, there is a second, smaller surge in estrogen levels, coupled with a drastic increase in progesterone (Carpenter, Upadhyaya et al. 2006; Guyton and Hall 2006) (Fig. 18).

![Figure 18](image)

**Figure 18 | Estradiol and progesterone levels according to day of menstrual cycle.** Adapted from Guyton & Hall, 2006.

In women, smoking cessation outcomes appear to be influenced by menstrual cycle phase. In a randomized study where women were assigned to quit smoking in either the follicular or luteal phase, women that quit smoking in the follicular phase (higher estrogen) experienced higher
relapse rates compared to women attempting to quit smoking in the luteal phase (lower estrogen) (Allen, Bade et al. 2008). Similarly, in women receiving bupropion to aid smoking cessation, those who chose to quit in the follicular phase were less successful in quitting than those who quit during the luteal phase (Mazure, Toll et al. 2011). In contrast to unaided quit attempts (Allen, Bade et al. 2008) and bupropion-aided quit attempts (Mazure, Toll et al. 2011), cessation rates on NRT were higher in the follicular phase (Franklin, Ehrman et al. 2008). It is possible that the level of activity of CYP2B6 (metabolizes bupropion) and/or CYP2A6 (metabolizes NRT) varies throughout the menstrual cycle phase, influencing cessation success on bupropion and NRT, respectively, as both of these enzymes are regulated by estrogen; estrogen induces CYP2A6 and CYP2B6 expression (Higashi, Fukami et al. 2007; Koh, Jurkovic et al. 2012). In the luteal phase, where estrogen levels are comparatively lower, cessation rates were higher on bupropion; this is in contrast to the lower predicted rates of CYP2B6-mediated metabolic activation of bupropion to hydroxybupropion (due to lower estrogen). Hydroxybupropion levels positively predict cessation success in smokers (Zhu, Cox et al. 2012), suggesting lower CYP2B6-mediated metabolism of bupropion to hydroxybupropion is likely to increase, rather than reduce, the risk for relapse. In the follicular phase, when estrogen levels are comparatively higher, cessation rates were higher on NRT (Franklin, Ehrman et al. 2008), again in seeming contradiction to predicted quit rates as faster CYP2A6 activity in general is associated with lower cessation success on NRT (Lerman, Tyndale et al. 2006; Schnoll, Patterson et al. 2009). However, a pharmacokinetic study in women found no differences in nicotine or cotinine clearance according to menstrual cycle phase (Hukkanen, Gourlay et al. 2005), suggesting CYP2A6 activity may not substantially vary throughout the menstrual cycle. These findings suggest that the differences in quit rates across phases of the menstrual cycle on NRT (Franklin, Ehrman et al. 2008), bupropion (Mazure, Toll et al. 2011), and in the absence of pharmacotherapy (Allen, Bade et al. 2008) are probably due to factors other than variation in rates of CYP2A6 or CYP2B6 metabolism as we currently understand it.

There appears to be a paucity of data comparing relapse rates between pre-menopausal and post-menopausal women. However, in the sample of female smokers from chapter 4, menopausal status (33% post-menopausal; 67% pre-menopausal) did not affect abstinence; the one-week abstinence rate was similar in pre-menopausal and post-menopausal women (53 and 56%,
respectively, \(P=0.59\). In addition, there were no significant differences in abstinence rates at end-of-treatment or six months between pre-menopausal and post-menopausal women. At end-of-treatment, quit rates were 24\% and 25\% in pre-menopausal and post-menopausal women, respectively (\(P=0.96\)), while quit rates at six months were 12\% and 16\% in pre-menopausal and post-menopausal women, respectively (\(P=0.18\)). Exogenous estrogen treatment, in the form of estrogen-containing birth control pills or estrogen-containing hormone replacement therapy, was also not associated with one-week smoking abstinence in this sample. In logistic regression models, there was also no relationship between exogenous estrogen treatment and quit rates at end-of-treatment or six months. After excluding women that were taking exogenous estrogen treatment, there was still no significant relationship between menopausal status and abstinence at one week, end-of-treatment, or six months, further suggesting that menopausal status and exogenous estrogen treatment did not influence cessation among women in this sample.

In a study of pregnant smokers, in whom the level of estrogen is higher compared to non-pregnant women (Guyton and Hall 2006), lower NMR was associated with a higher likelihood of abstinence at one month (Vaz, Coleman et al. 2015). There was no interaction between NMR and treatment (placebo patch vs. nicotine patch) on abstinence, suggesting that lower NMR was associated with higher abstinence in pregnant women receiving either placebo patch or nicotine patch (Vaz, Coleman et al. 2015). The lack of significant interaction between NMR and treatment on one-month abstinence also suggests that nicotine patch therapy, which was associated with higher one-month quit rates overall (Coleman, Cooper et al. 2012), works equally well in faster metabolizers and slower metabolizers (Vaz, Coleman et al. 2015). As reviewed previously, data from African American light smokers suggested that the relationship between NMR and end-of-treatment abstinence on nicotine gum and placebo might have been stronger in non-pregnant females than in males (Ho, Mwenifumbo et al. 2009), while clinical trial data from Chapter 4 (see also (Lerman, Schnoll et al. 2015)) suggested that NMR was not associated with end-of-treatment abstinence in non-pregnant smokers. Together the data suggest that among female smokers, NMR is associated with abstinence in pregnant, and in some populations of non-pregnant, women. While it is possible that the relationship between NMR and abstinence outcomes in women differs according to pregnancy, further studies are required to support this theory.
A relatively new idea in the literature is that cessation fatigue, described as being tired of trying to quit smoking, may play an important role in the discordance in cessation success between men and women (Liu, Li et al. 2013). Among treatment-seeking adult smokers, women were shown to be at higher risk than men for cessation fatigue; cessation fatigue, in turn, was associated with a higher risk for relapse, defined as the inability to achieve 6-month abstinence following a quit attempt (Liu, Li et al. 2013). Craving and negative affect were associated with the development of cessation fatigue; the relationship between craving and cessation fatigue was stronger in women than in men. In addition, cessation fatigue was reduced to a larger extent in women than in men with active pharmacotherapy (nicotine replacement therapy and bupropion) (Liu, Li et al. 2013).

Performing a study in female smokers incorporating assessments of cessation fatigue, which disproportionately affects women compared to men (Liu, Li et al. 2013), and menstrual cycle phase may help clarify differing relapse risks among pre-menopausal women. Using a between-subjects study design in treatment-seeking female smokers, half of the participants could be instructed to quit during the follicular phase, while the other half could be instructed to quit during the luteal phase. Cessation fatigue could be measured over the first two weeks of the quit attempt using ecological momentary assessments (Liu, Li et al. 2013), with relapse being assessed at one week and six months following the target quit date. To extend our findings using data from chapter 4 showing that NMR does not influence early or later abstinence outcomes among females, blood samples could be collected on the target quit date for NMR determination, and throughout the study to determine whether NMR varies according to menstrual cycle phase. If the findings from chapter 4 do not replicate and instead suggest that NMR is a significant predictor of abstinence in women at one week and/or six months, NMR could be used as a covariate in models examining the influence of, and potential interactions between, menstrual cycle phase and cessation fatigue on abstinence. Initially, this study could be performed in women receiving extended nicotine patch therapy, and may reveal that cessation fatigue modulates quit differences on NRT across menstrual cycle phases. As NRT in general does not appear to be as effective at treating nicotine dependence in women compared to men (Perkins and Scott 2008), future investigations could assess relationships between menstrual cycle phase
and cessation fatigue on abstinence outcomes in women receiving bupropion or varenicline therapy.

6.6.2. Ethnicity and smoking cessation

Compared to Caucasians, African Americans experience greater difficulty quitting smoking as indicated by lower quit ratios (percentage of ever smokers who are former smokers) (Giovino 2002). In chapter 4, we showed that the likelihood of one-week abstinence was lower among African American compared to Caucasian smokers.

While the high prevalence of menthol cigarette smoking among African Americans may play a role in their lower cessation success (Faseru, Nollen et al. 2013), other studies found no relationship between menthol status and quit rates (Hyland, Garten et al. 2002; Blot, Cohen et al. 2011). In chapter 4, we found no association between mentholated cigarette use and the likelihood of one-week abstinence in the overall model. We ran a separate logistic regression model that included only menthol status and ethnicity as predictors, with one-week abstinence as the outcome measure. After controlling for mentholated cigarette use, a trend toward higher likelihood of cessation among Caucasians (vs. African Americans) was observed (OR=1.31, P=0.07), suggesting mentholated cigarette use does not fully account for the lower cessation among African Americans.

A qualitative study in former and current smokers from ethnic minorities, including African Americans, revealed a number of potential reasons underlying their lower cessation rates (Fu, Burgess et al. 2007). Overall, few participants believed doctors could help them quit smoking; African American participants reported negative feelings about doctors, including a lack of trust (Fu, Burgess et al. 2007), consistent with previous findings (Boulware, Cooper et al. 2003). Many participants also felt that smoking cessation medications were expensive and inaccessible, consistent with few participants ever having used pharmacotherapy. However, the clinical trial participants in Chapter 4 received smoking cessation medications free of charge (Lerman, Schnoll et al. 2015), suggesting concern about treatment costs do not explain the lower one week abstinence rates among the African American trial participants. This lower level of cessation among African Americans relative to Caucasians was also observed at end-of-treatment, where
27% of Caucasians were abstinent compared to 22% of African Americans (P=0.049); this relationship was not substantially altered when we controlled for sex (P=0.052). In the aforementioned qualitative investigation, a number of former and current smokers were concerned about side effects in addition to treatment costs and believed that the side effects of medication were more serious than the health risks associated with smoking continuation (Fu, Burgess et al. 2007). It is possible these views may lead to lower adherence to smoking cessation medications by ethnic minorities. Previously, lower adherence to antipsychotic medication was observed for ethnic minorities compared to white patients (Opolka, Rascati et al. 2003). In the clinical trial participants from chapter 4, >60% of participants used at least 80% of the recommended number of nicotine patches or recommended varenicline dose (Lerman, Schnoll et al. 2015); whether smoking cessation medication compliance rates differed as a function of ethnicity would be an interesting future direction to explore. In addition, follow-up analyses to investigate whether side effects reduced compliance rates among certain groups of smokers would be warranted.

Socioeconomic status differences between African Americans and Caucasians may also contribute to differences in smoking cessation outcomes. Lower socioeconomic status, which disproportionately affects African Americans compared to Caucasians (Williams, Yan et al. 1997) is associated with lower cessation (Mohsin and Bauman 2005; Siahpush, Heller et al. 2005). Differences in socioeconomic status may partially explain the one-week and end-of-treatment quit differences between African American and Caucasian clinical trial participants. In terms of educational attainment, a higher proportion of African American relative to Caucasian participants received a high school education or less (42% vs. 24%, respectively; P<0.001). Moreover, only 17% of African American participants reported having an annual household income of greater than $US 50,000, compared to 51% of Caucasian participants (P<0.001). Thus, socioeconomic disparities may partially explain inter-ethnic differences in abstinence success among the clinical trial participants. Some evidence suggests that the effect of lower socioeconomic status on reduced cessation occurs via a lower use of smoking cessation resources (Honjo, Tsutsumi et al. 2006). Examining whether compliance rates differed as a function of ethnicity may offer additional insight regarding the relationship between educational attainment, income, and quit outcomes.
The belief held by some African American smokers that smoking continuation carries a lower risk to health compared to cessation treatment side effects (Fu, Burgess et al. 2007) may stem from their relatively lower level of cigarette consumption which may be perceived as a ‘safer’ level; compared to Caucasians, African Americans smoke fewer cigarettes per day (Lawrence, Fagan et al. 2007; Trinidad, Perez-Stable et al. 2009). However, compared to non-smokers, light or intermittent (i.e., non-daily) smokers still experience elevated risks of smoking-related diseases including cardiovascular disease and lung cancer (Schane, Ling et al. 2010). As previously discussed, TNE levels were similar between African American light smokers and Caucasian heavy smokers, suggestive of a similar level of tobacco exposure despite marked differences in the number of cigarettes smoked per day; these light smokers might be more aptly described as intermittent intensive smokers (Zhu, Zhou et al. 2013).

In general, there are few data concerning the efficacy of pharmacotherapies in African Americans, as many are lighter smokers; smokers consuming fewer than 10 cigarettes per day are typically excluded from smoking cessation clinical trials. In African American light smokers, neither nicotine gum nor bupropion therapy increased smoking cessation success (Ahluwalia, Okuyemi et al. 2006; Cox, Nollen et al. 2012). However, there was a significant main effect of counseling in promoting smoking cessation; those receiving health education compared to motivational interviewing were significantly more likely to be abstinent at six months (OR=2.2; P=0.0008) (Ahluwalia, Okuyemi et al. 2006). These data suggest that future studies aimed at boosting quit rates among African American smokers should incorporate health education counseling. In a separate study, increasing exposure to television advertisements that use emotionally evocative messaging was associated with an increased likelihood of quitting smoking among smokers in low- and mid-socioeconomic groups; this could also be tested further as a potential strategy to increase cessation among African American smokers (Durkin, Biener et al. 2009). Such a strategy may prove particularly useful for African American smokers such as those from Chapter 4, where comparatively lower levels of educational attainment and household income, suggestive of lower socioeconomic status, were noted compared to Caucasians.
6.7. Conclusions

Our findings contribute to the extant literature by examining genetic and non-genetic influences on NMR/CYP2A6 activity, and associations with variation in smoking behaviours including cessation. We first showed that slow CYP2A6 activity, measured by CYP2A6 genotype, was associated with an increased likelihood of smoking cessation in adolescents. These findings add to a growing literature examining risk and protective factors for smoking in teens. We further showed that specific variants in the FMO3 and POR genes contribute little to variation in nicotine metabolism in adults, and thus did not significantly influence smoking behaviours. Beyond the influence of variation in genes encoding enzymes involved in nicotine metabolism, environmental and/or demographic factors also influence NMR. We showed that many of these factors (e.g., gender, ethnicity, estrogen-containing hormonal therapy, alcohol consumption) represent relatively minor sources of NMR variability. Thus it is likely that the FMO3 and POR variants examined, as well as these environmental/demographic factors, need not be considered when NMR is used, for example, to examine differences in smoking behaviours and/or assign cessation treatments. This is a useful attribute of a genetically informed biomarker as it makes it robust to many of these minor influences. Finally we demonstrated that NMR is a predictor of one-week smoking abstinence in treatment-seeking adult smokers; NMR should likely be incorporated as a covariate in early screening studies that evaluate the efficacy of new compounds in development to treat tobacco dependence.

Our findings have generated several new ideas for future research. These include an imaging study in adolescents to examine the effects of variable nicotine metabolism on the strength of neural connections in brain reward regions, a study to investigate TNE in adolescent smokers to understand tobacco dose in heavy and light smoking adolescents, as well as a study in female smokers to help characterize the relationship between menstrual cycle phase, cessation fatigue, and short-term as well as prolonged abstinence. Finally, future studies investigating disparities in smoking cessation rates and smoking-related diseases between African American and Caucasian smokers are warranted and could focus on the role of menthol or medication compliance in cessation. Together these studies will help elucidate the mechanisms, and the role of variable nicotine metabolism, on smoking and cessation in adolescence and adulthood, and help inform
personalized treatment strategies that will reduce the health and economic burden associated with tobacco use.
7 BIBLIOGRAPHY


8 APPENDIX
Appendix A:
Description of the Nicotine Dependence in Teens (NDIT) cohort

Type of study: Prospective cohort study

Purpose of study: To investigate factors associated with cigarette smoking and nicotine dependence in adolescent smokers. Multiple sub-studies have also been conducted that examine determinants of physical activity, obesity, genetics, and blood pressure, among others.

Cohort characteristics: A total of 1,294 participants were recruited from 10 high schools in Montreal, Quebec. Sampling was restricted to all grade 7 classes. The majority of participants were born in Canada (92%), were Caucasian (82%), and had university-educated parents (58%). Participants had a mean age of 13 years at baseline. Forty-eight percent of participants were male, and 32% had ever smoked at baseline.

Data acquisition: Self-report questionnaires were administered every 3 months, from grade 7 to grade 11. A total of 20 survey cycles were administered during high school. Two additional waves were completed following high school, at which time participants had an average age of 20 and 24 years, respectively.

Primary outcomes: Smoking initiation, daily smoking, nicotine dependence, smoking discontinuation

Note: additional cohort information is provided in O’Loughlin, Dugas et al. 2014, as well as at www.nditstudy.ca
Appendix B: 
Descriptions of the Kick it at Swope (KIS) III clinical trial and the nicotine pharmacokinetic study

KIS III clinical trial

Type of study: Randomized, double-blind placebo-controlled clinical trial

Purpose of study: To determine whether bupropion, in combination with health education counseling, promotes cessation in African American light smokers

Inclusion/exclusion criteria: Eligible individuals self-reported African American ancestry, smoked for at least 3 years, and smoked ≤10 cigarettes per day. Participants were 18 years or older, and were willing to attend study visits and provide biological samples for genetic testing. Exclusion criteria included current use of smoking cessation treatments, psychoactive medications, or other tobacco products; consumption of ≥14 alcoholic drinks/week; history of seizures or eating disorders; current pregnancy or breast-feeding; recent myocardial infarction; or having another household member participating in the study. Additional criteria are described elsewhere (Cox, Nollen et al. 2012).

Interventions: Two hundred and seventy participants were randomized to receive bupropion SR, and 270 participants were randomized to receive placebo for 7 weeks. Up to six sessions of health education counseling were provided to all participants.

Participant characteristics: African American light-smoking adults were recruited from the metropolitan area of Kansas City, Missouri. Participants smoked 8 cigarettes/day on average. The mean age of participants was 46 years, and over half (~66%) of the participants were female. More than 60% of participants reported a monthly household income of <$1,800.
Primary outcome: Cotinine-verified (<15 ng/ml) 7-day point prevalence smoking abstinence at week 26.

Note: additional study information is provided in Cox, Nollen et al. 2012.

**Nicotine pharmacokinetic study**

Type of study: Experimental study

Purpose of study: To investigate associations between sex, age, smoking status, and current alcohol and marijuana use, with CYP2A6 activity and nicotine pharmacokinetics in African Canadian smokers and non-smokers. An additional objective was to determine whether CYP2A6 activity was associated with cigarettes per day in the African Canadian smokers.

Inclusion/exclusion criteria: Eligible individuals were men and women aged 18 to 60 years with at least three grandparents of black African descent, and were in good health. Both non-smokers and smokers were recruited. Exclusion criteria included known contraindications to nicotine administration and current use of medications that may alter drug metabolizing enzymes. Additional criteria are described elsewhere (Mwenifumbo, Sellers et al. 2007).

Interventions: Oral capsules containing 4 mg of nicotine were administered on test day to non-smokers and 12-hour abstinent smokers (confirmed by CO level <10 ppm). One gram of ammonium chloride was administered orally one hour prior to nicotine treatment, in order to reduce variation in nicotine renal clearance.

Participant characteristics: Individuals of black African descent were recruited from a community-based sample in Toronto, Ontario. For analyses contained within this thesis (see Chapter 2), nicotine kinetic data from the non-smokers were utilized. The mean age of the sample was 32 years (range 20-59), and over half (61%) were female.
Primary outcomes: The nicotine metabolite ratio at 270 minutes, as well as the estimated nicotine area under the concentration versus time curve over 360 minutes, following oral nicotine administration

Note: additional study information is provided in Mwenifumbo, Sellers et al. 2007.
Appendix C:
Description of the Pharmacogenetics of Nicotine Addiction Treatment (PNAT) 2 clinical trial

Type of study: Randomized, double-blind placebo-controlled clinical trial

Purpose of study: To determine whether the nicotine metabolite ratio prospectively predicts smoking cessation outcomes on varenicline or the nicotine patch

Inclusion/exclusion criteria: Eligible participants were between the ages of 18 and 65, smoked at least ten cigarettes/day for at least 6 months, and had a CO level >10 ppm. Individuals were excluded from the study if they used other tobacco products or smoking cessation medications, had a history of substance abuse, were pregnant, had a medical contraindication (e.g., cancer, kidney, liver, or heart disease) or history of psychiatric disorder, or used psychoactive drugs or medications known to affect CYP2A6 activity. Additional criteria are provided in Lerman, Schnoll et al. 2015.

Interventions: A total of 1246 smokers were randomized to treatment arm (placebo, nicotine patch, varenicline) based on their nicotine metabolite ratio. A total of 408 participants received placebo, while 418 and 420 received the nicotine patch and varenicline, respectively. All smokers received four sessions of telephone counseling.

Participant characteristics: Participants were recruited from four clinical sites (University of Toronto/CAMH, University of Pennsylvania, SUNY at Buffalo, and MD Anderson Cancer Center). Over half (56%) of the participants were Caucasian, while 37% and 7% of the participants reported African American or other ancestry, respectively. Participants’ mean age was 46 years, 44% of the participants were female, and 37% were unemployed. The average level of smoking at baseline was 18 cigarettes/day.
Primary outcome: CO-verified (≤8 ppm) 7-day point prevalence abstinence at end of treatment (week 11).

Note: additional study information is provided in Lerman, Schnoll et al. 2015
Appendix D:

Associations between variation in *CYP2A6* and *CYP2B6* and tobacco dependence throughout adolescence and in young adult smokers.
Abstract (247/250)

**Background:** Smoking is influenced by genetic factors including variation in *CYP2A6* and *CYP2B6*, which encode nicotine-metabolizing enzymes. In early adolescence, *CYP2A6* slow nicotine metabolism was associated with higher dependence acquisition, but reduced cigarette consumption. Here we extend this work by examining associations of *CYP2A6* and *CYP2B6* with tobacco dependence acquisition in a larger sample of smokers followed throughout adolescence.

**Methods:** White participants from the Nicotine Dependence in Teens cohort that had ever inhaled (n=421) were followed frequently from age 12-18 years. Cox’s proportional hazards models compared the risk of ICD-10 tobacco dependence acquisition (score ≥3) between *CYP2A6* and *CYP2B6* metabolism groups. At age 24, we assessed concordance between self-reported cigarette consumption and salivary cotinine, as well as tobacco dependence status between *CYP2B6* metabolism groups.

**Results:** In those who initiated inhalation during follow-up, *CYP2A6* slow (vs. normal) metabolizers were at greater risk of developing dependence (hazards ratio (HR)=2.3; 95% CI=1.1, 4.7; P=0.03)); *CYP2B6* slow (vs. normal) metabolizers had non-significantly greater risk (HR=1.5; 95% CI=0.9, 2.7; P=0.13). Among dependent smokers, *CYP2A6* slow (vs. normal) metabolizers reported lower mean cigarette consumption at end of follow-up (192 vs. 300 cigarettes/month, respectively; P<0.05). Self-reported cigarette consumption was associated with salivary cotinine (B=0.37; P<0.001), a biomarker of tobacco exposure, suggesting accurate self-reporting. At age 24, 45% of *CYP2B6* slow metabolizers were dependent, compared to 29% of *CYP2B6* normal metabolizers (P=0.05).

**Conclusions:** Our findings extend previous work indicating that *CYP2A6*, and perhaps *CYP2B6*, slow nicotine metabolism confers biological vulnerability to tobacco dependence throughout adolescence.
1. Introduction

Approximately 90% of smokers begin smoking in adolescence (O'Loughlin et al., 2014b; U.S. Department of Health and Human Services, 2012). Twin studies indicate that a substantial proportion (~40-75%) of multiple smoking behaviours are influenced by genetics (Broms et al., 2006; Vink et al., 2005). CYP2A6 inactivates nicotine, the principle psychoactive compound in cigarette smoke, to cotinine (Nakajima et al., 1996). Genetic variation in CYP2A6 that reduces the rate of nicotine metabolism has been associated with lower cigarette consumption (Malaiyandi et al., 2006; Wassenaar et al., 2011), nicotine dependence scores (Schnoll et al., 2014; Sofuoglu et al., 2012; Wassenaar et al., 2011), brain response to smoking cues (Tang et al., 2012), and a higher likelihood of quitting smoking (Gu et al., 2000; Lerman et al., 2006; Schnoll et al., 2009), even in adolescence (Chenoweth et al., 2013). In adolescents, CYP2A6 slow nicotine metabolism was also associated with an increased risk of tobacco dependence acquisition at young ages (from age 12 to 16 years) (Al Koudsi et al., 2010; O'Loughlin et al., 2004), but slower escalation in nicotine dependence (Audrain-McGovern et al., 2007) and reduced cigarette consumption (O'Loughlin et al., 2004). In young adults, CYP2A6 slow metabolizers were less likely than normal metabolizers to be smokers (Schoedel et al., 2004). Together these findings suggest that while CYP2A6 slow nicotine metabolism is associated with an increased risk of becoming a smoker in younger adolescence, slow metabolism also increases the likelihood of cessation, as well as reduces cigarette consumption in dependent smokers. However, it is not known whether CYP2A6 slow metabolism increases smoking acquisition in later adolescence, a period during which a substantial amount of smoking uptake occurs (O'Loughlin et al., 2014b).

A small proportion (~10%) of the inactivation of nicotine to cotinine occurs via a second enzyme, CYP2B6 (Al Koudsi and Tyndale, 2010). The CYP2B6*6 allele, a prevalent haplotype (~25% frequency in Whites (Rotger et al., 2007)) containing two amino acid changes (Q172H and K262R), was associated with lower CYP2B6 hepatic protein levels (Al Koudsi and Tyndale, 2010) and slower CYP2B6-mediated metabolism of bupropion and efavirenz (reviewed in (Thorn et al., 2010)). In adult smokers, CYP2B6*6 was associated with lower abstinence rates in the placebo arm of a bupropion smoking cessation clinical trial; 15% of individuals with one or two copies of CYP2B6*6 achieved abstinence, compared to 32% of CYP2B6*1/*1 individuals (Lee et al., 2007a). In a separate study, the CYP2B6*6 allele was found to be more frequent in
individuals with nicotine dependence compared to those that were not dependent (32% vs. 22%, respectively) (Riccardi et al., 2015). Whether CYP2B6*6 also influences the risk for acquiring nicotine dependence in adolescence is not known.

Here we examined associations for CYP2A6 and CYP2B6 with the acquisition of tobacco dependence in a larger (n>400) sample of adolescent smokers assessed frequently (four times each year) across the entire period of adolescence (from age 12-18 years). We hypothesized that CYP2A6 slow metabolizers would be at increased risk of developing dependence compared to CYP2A6 normal metabolizers, and that CYP2B6 slow metabolizers (i.e., individuals with one or two copies of CYP2B6*6) would be at increased risk of acquiring dependence compared to CYP2B6 normal metabolizers. We also assessed the level of cigarette consumption at the end of follow-up among dependent smokers to determine whether CYP2A6 slow metabolizers smoke fewer cigarettes than CYP2A6 normal metabolizers in later adolescence, as observed in earlier adolescence. We also hypothesized that CYP2B6 slow metabolizers would be more likely to be dependent as young adults (i.e., age 24), consistent with a recent study (Riccardi et al., 2015). Finally, an adjunct biochemical analysis to assess the validity of the self-reported cigarette consumption data was undertaken. We examined the construct-related validity of self-reported cigarette consumption against salivary cotinine, which is widely used as an objective biomarker of tobacco consumption (Connor Gorber et al., 2009), and also assessed its relationships with nicotine dependence and withdrawal scores.

2. Methods

2.1. Study population and data collection

As previously described (O’Loughlin et al., 2014a), 1294 adolescents from 10 secondary schools in Quebec were recruited in 1999 for the Nicotine Dependence in Teens (NDIT) cohort study (Montreal, Quebec, Canada). Self-report questionnaires were administered every three months during the 10-month school year over the five years of secondary school (grade 7-11), for a total of 20 survey cycles. Data from these 20 surveys for n=421 ever smoking Whites were included in the current analyses of tobacco dependence acquisition and cigarette consumption. Previous analyses in this population included data only up to survey cycle 16 (age 15-16 years) for only 281 smokers (O’Loughlin et al., 2004). Two additional surveys (survey cycles 21 and 22) were administered three and six years, respectively after high school graduation (O’Loughlin
et al., 2014a). Data from survey 22, completed when participants were aged 24 years on average, were used, along with salivary cotinine, for additional analyses. Parents or guardians provided written informed consent and participants provided assent at baseline. Participants (who had attained legal age) provided informed consent during post-high school survey cycles. The study was approved by McGill University (Quebec, Canada), the Centre de recherche du Centre hospitalier de l'Université de Montréal (Quebec, Canada), and the University of Toronto research ethics board (Toronto, Canada).

2.2. Determination of $CYP2A6$ and $CYP2B6$ genotype

DNA was extracted from saliva or blood samples, and participants were genotyped for four $CYP2A6$ alleles which are found at relatively high frequency (~1-8%) in Whites and have an established impact on reducing nicotine metabolizing activity: $CYP2A6^*2$, $CYP2A6^*4$, $CYP2A6^*9$, and $CYP2A6^*12$ (Benowitz et al., 2006; Chenoweth et al., 2013). Participants were grouped into $CYP2A6$ normal, intermediate, or slow nicotine metabolism groups based on the predicted metabolic impact of each $CYP2A6$ variant allele (Chenoweth et al., 2013). Participants were also genotyped for the $CYP2B6^*6$ allele, using a haplotyping method as described previously (Lee et al., 2007a; Mwenifumbo et al., 2005); individuals with the $CYP2B6^*1/ *1$ genotype were grouped as normal metabolizers, while those with one or two copies of the $CYP2B6^*6$ alleles were grouped as slow metabolizers. For genetic analyses, the sample was restricted to White ever-smokers in order to minimize possible effects of population stratification. $CYP2A6$ genetic data were available for 421 White ever-smokers, while $CYP2B6$ genetic data were available for 391 White ever-smokers.

2.3. Determination of salivary cotinine level

Cotinine levels were determined from saliva samples collected from current smokers at survey cycle 22, when participants were 24 years of age on average. The level of cotinine was assessed using liquid chromatography-tandem mass spectrometry (LC/MS-MS) (Chenoweth et al., 2014; St Helen et al., 2012; Tanner et al., 2015). In total, cotinine levels were available for $n=162$ White self-reported current smokers with genetic data. The limit of quantification for cotinine was 1 ng/ml. Four participants had cotinine levels below the limit of quantification; as
per convention, the cotinine value was replaced with the limit of quantification (1 ng/ml) divided by the square root of 2, yielding a value of 0.7 ng/ml (Kalkbrenner et al., 2010).

2.4. Study variables

Data on tobacco/nicotine dependence (measured by the International Classification of Diseases (ICD)-10 and the modified Fagerstrom Tolerance Questionnaire (mFTQ) were collected, as were data on nicotine withdrawal, other nicotine dependence symptoms, and self-medication scores (Chenoweth et al., 2013). Briefly, nicotine withdrawal, other nicotine dependence symptoms, and self-medication scores were measured in six, 14, and five individual items, respectively, and assessed symptoms of withdrawal including irritability, restlessness, anxiety, craving frequency, and endorsement of statements that smoking improves energy level, affect, and stress (Chenoweth et al., 2013). Scores were pro-rated if there were fewer than half the items missing for an individual score. If half or more of the items were missing, the participant was assigned a missing value. Three measures of cigarette consumption were used. The mean number of cigarettes smoked per month in the 3-month interval preceding each survey cycle was assessed by multiplying the average number of cigarettes smoked per day by the average number of days smoked per month in each of the three months and calculating the average monthly consumption (O'Loughlin et al., 2014c). The number of cigarettes smoked in the past week was assessed at survey cycle 22 by summing the number of cigarettes smoked on each of the preceding seven days. Finally, the number of cigarettes smoked in the past 24 hours was assessed at survey cycle 22, indicated by the number of cigarettes smoked on the day prior to administration of the survey.

2.5. Statistical analyses

**Analysis 1. Tobacco dependence acquisition and level of smoking in adolescence (Surveys 1-20).** Data from surveys 1-20 (age 12-18 years) were included in this analysis; surveys 21 and 22, both collected in young adulthood (ages 20 and 24 years, respectively), were not included in this analysis due to the much less frequent data collection interval. Using bootstrap-based multiple imputation to manage missing data, 10 imputed datasets were created as previously described (Chenoweth et al., 2013). Cox’s proportional hazards models were used to compare the risk of acquiring ICD-10 tobacco dependence (score of ≥3) in adolescence (age 12-
18 years) between CYP2A6 normal, intermediate, and slow nicotine metabolizers who had ever inhaled on a cigarette, and between CYP2B6 normal and slow metabolizers who had ever inhaled on a cigarette. Models were stratified according to whether the participant had inhaled at cohort inception (yes, no); separate models examined all inhalers (i.e., those who inhaled prior to cohort inception + those who inhaled during follow-up) and those who first inhaled during follow-up (i.e., incident inhalers), as data covering the entire smoking career were available in this latter group. Time was measured in days from time zero in all participants who ever inhaled on a cigarette, which corresponded to the day participants who had inhaled prior to cohort inception joined NDIT, or the day incident smokers first inhaled during NDIT follow-up. Participants were followed from time zero until they became dependent or were censored (i.e., were lost to follow-up or the follow-up period ended). Once smokers entered the analysis, all surveys were considered to be ‘at-risk’ periods. Past-month cigarette consumption at end of follow-up (i.e., each participant’s last available survey from surveys 1 to 20) was compared according to CYP2A6 genotype group and dependence status among incident inhalers for whom we had complete data; covariates included sex, age, and duration of smoking, and consumption was compared using one-way ANOVA and Tukey-Kramer post-test.

Analysis 2. Construct-related validity of self-reported smoking behaviour (cigarette consumption) against cotinine in young adults (Survey 22). Cotinine was not normally distributed. Therefore the strength of the association between self-reported cigarette consumption and cotinine level was assessed using Spearman’s rho and linear regression analysis. We also explored the relationships between cotinine and nicotine dependence scores (i.e., ICD-10, mFTQ dependence scores, withdrawal, other nicotine dependence symptoms and self-medication) and smoking indicators using Spearman’s rho and by conducting a separate univariate linear regression analysis for each smoking or nicotine dependence indicator.

Analysis 3. Association between CYP2B6 and tobacco dependence status in young adulthood (Survey 22). Using the same study population from analysis 2, we performed a chi-square test to compare the proportion of CYP2B6 normal and slow metabolizers that were ICD-10 tobacco dependent (score of ≥3).

3. Results
Participant characteristics at baseline are shown in Table 1. In all participants (n=421), 78.9%, 14.5%, and 6.7% were CYP2A6 normal, CYP2A6 intermediate, and CYP2A6 slow metabolizers, respectively. In those for whom CYP2B6 genetic data were available (n=391), 58.3% were CYP2B6 normal metabolizers (n=228), while 41.7% were CYP2B6 slow metabolizers (n=163). The frequency of the CYP2B6*6 allele was 24%, consistent with a previous report (Rotger et al., 2007). As ~6.7% of CYP2B6 slow metabolizers would be expected to have CYP2A6 slow metabolism (see CYP2A6 metabolism group distributions, above), this corresponds to ~11 individuals. Twelve of the participants had both CYP2A6 and CYP2B6 slow metabolism; this did not substantially deviate from the expected value.

The median follow-up time was 1574 days. We first examined the risk of tobacco dependence acquisition according to CYP2A6 metabolism group in incident inhalers and in all inhalers. In the incident inhalers, CYP2A6 slow metabolizers were more likely to acquire tobacco dependence than CYP2A6 normal metabolizers, with a hazards ratio (HR) of 2.3 (95% confidence interval (CI) = 1.1, 4.7; P=0.03) (Table 2). Similar findings were observed in all inhalers (HR = 1.8, 95% CI = 1.0, 3.3; P=0.04 for tobacco dependence acquisition in CYP2A6 slow vs. normal metabolizers) (Table 2). We then examined the risk of tobacco dependence acquisition according to CYP2B6 metabolism group in incident inhalers and in all inhalers. In the incident inhalers, CYP2B6 slow metabolizers were not significantly more likely than CYP2B6 normal metabolizers to acquire tobacco dependence (HR = 1.5, 95% CI = 0.9, 2.7; P=0.13). Similar findings were observed in all inhalers (HR = 1.2, 95% CI = 0.9, 1.8; P=0.24 for tobacco dependence acquisition in CYP2B6 slow vs. normal metabolizers) (Table 2). We were underpowered to test for potential interactions between CYP2A6 and CYP2B6 on tobacco dependence acquisition in adolescence.

We next investigated the level of cigarette consumption between CYP2A6 metabolism groups according to dependence status. In incident smokers who were tobacco dependent, mean monthly cigarette consumption differed according to CYP2A6 metabolism group (ANOVA P<0.001), with slow metabolizers smoking a mean of ~108 fewer cigarettes per month compared to normal metabolizers (Tukey-Kramer test P<0.05; Table 3). Mean monthly cigarette consumption was also lower by ~28 cigarettes in slow metabolizers in the total group (i.e., non-dependent and dependent smokers combined), but not in non-dependent smokers (Table 3).
We also undertook an adjunct analysis to determine the construct-related validity of using self-reported smoking behaviour as an indication of tobacco dose. We first focused on the relationship between self-reported cigarette consumption (number of cigarettes/month), and salivary cotinine level. The measures were strongly correlated (Rho=0.71, P<0.001) (Figure 1a). Using linear regression, self-reported cigarette consumption was strongly associated with salivary cotinine level (B=0.37, P<0.001). We next examined the relationship between past-week and past-day self-reported cigarette consumption and salivary cotinine level, and these measures were also strongly correlated (Rho=0.75 and 0.73, respectively; P<0.001). Both past-week and past-day self-reported cigarette consumption were also associated with cotinine in linear regression analyses (B=1.8 and 11.2, respectively, P<0.001). We also observed strong correlations between cotinine and nicotine dependence scores (i.e., ICD-10 and mFTQ dependence scores, withdrawal, other nicotine dependence symptoms and self-medication; Figure 1b-f; Rho=0.28-0.61, all P<0.001). In linear regression analyses, ICD-10 dependence (B=23.2, P=0.001), mFTQ dependence (B=37.6, P<0.001), withdrawal (B=7.9, P<0.001), other nicotine dependence symptoms (B=8.1, P<0.001), and self-medication scores (B=15.1, P=0.008) were all significantly associated with cotinine level.

Finally, we examined the association between CYP2B6 metabolism group and dependence status in young adult (age ~24 years) current smokers. Twenty-nine percent of CYP2B6 normal metabolizers (CYP2B6*1/*1; n=82) were ICD-10 dependent, compared to 45% of CYP2B6 slow metabolizers (CYP2B6*1/*6 + *6/*6; n=65) (P=0.05).

4. Discussion

In this longitudinal study in adolescent smokers, we have extended previous findings (O’Loughlin et al., 2004) of an increased risk for tobacco dependence acquisition among CYP2A6 slow nicotine metabolizers relative to CYP2A6 normal metabolizers, by demonstrating that this elevated risk occurs throughout the entire adolescent period. While the precise mechanism(s) underlying the association between CYP2A6 slow metabolism and increased risk for tobacco dependence acquisition is unknown, the effect of slower nicotine metabolism on nicotine-mediated reward was recently characterized in nicotine-naïve adult mice (Bagdas et al., 2014). Slower nicotine metabolism, achieved through methoxsalen-mediated inhibition of CYP2A5 (the murine ortholog of human CYP2A6), caused animals to display a preference for
nicotine in the conditioned place preference (CPP) paradigm (Bagdas et al., 2014), suggestive of greater reward (Tzschentke, 2007). In contrast, animals that did not receive methoxsalen treatment did not show this preference (Bagdas et al., 2014). Although this study was performed in adult animals, limiting comparisons to adolescents, the lack of prior exposure to nicotine and the use of a relatively low dose of nicotine (0.1 mg/kg s.c.) (Bagdas et al., 2014) may support its suitability as a model of first-time smoking in human adolescents. If these findings extend to novice smokers, those with slow nicotine metabolism may experience greater reward during initial smoking experiences, potentially due to greater nicotine exposure, increasing their risk for developing nicotine dependence.

Despite their higher risk of acquiring tobacco dependence, once dependent, CYP2A6 slow metabolizers smoked fewer cigarettes at the end of follow-up relative to dependent normal metabolizers (~192 vs. ~300 cigarettes/month, respectively), consistent with NDIT cohort data collected in earlier adolescence when participants had a maximum age of 15 or 16 at the end of follow-up (~13 vs. ~29 cigarettes/week, respectively) (O'Loughlin et al., 2004). The level of cigarette consumption was higher in the current analysis, suggesting heavier levels of smoking in later adolescence compared to earlier adolescence. Slow nicotine metabolism, measured by CYP2A6 genotype or NMR, is similarly associated with lower cigarette consumption among dependent adult smokers (Chenoweth et al., 2014; Malaiyandi et al., 2006; Wassenaar et al., 2011). Thus it is likely that dependent adolescent smokers, similar to adult dependent smokers, titrate their level of nicotine intake to maintain desirable levels (Ashton et al., 1979; Hill and Marquardt, 1980), with slower nicotine metabolizers needing to smoke fewer cigarettes to achieve these levels.

In a cross-sectional study of adolescent smokers (<5 cigarettes/day) aged 13 to 17 years (mean = 16 years), salivary NMR was negatively associated with mFTQ dependence scores and cigarette consumption, such that slower metabolizers displayed both higher dependence scores and higher cigarette consumption (Rubinstein et al., 2013). It is possible that this study captured an earlier time-point in smoking history than our study, where the CYP2A6 slow metabolizers were more likely to be dependent (and have higher dependence scores), but had not yet begun to titrate their nicotine intake according to their rate of nicotine metabolism (i.e., had not begun to smoke fewer cigarettes).
Many studies of adolescent smoking, including our own, utilize self-report questionnaire data (Ary and Biglan, 1988; Rigotti et al., 2000). Misclassification in self-report data can occur in epidemiological studies, particularly in studies of substance use behaviour and in younger cohorts (Brener et al., 2003; Clarke et al., 2014; Fendrich et al., 2005; Morral et al., 2000). There may be issues stemming from recall bias, as well as the desire to conform with social norms (Brener et al., 2003). In order to help minimize recall bias, we collected data frequently (every three months) in adolescence (O’Loughlin et al., 2014a), and assessed tobacco dependence acquisition in all inhalers as well as in those who initiated inhalation during follow-up (i.e., incident inhalers). We also wanted to assess the accuracy of self-reported cigarette consumption using an objective biomarker of tobacco consumption, salivary cotinine level, which was available at age 24. There was strong agreement between self-reported cigarette consumption and salivary cotinine, suggesting that self-reported cigarette consumption is concordant with objective measures of consumption/exposure, at least at age 24 (Connor Gorber et al., 2009), supporting the construct validity of the questionnaire data. The associations between cotinine and the other smoking and dependence measures (i.e., ICD-10, mFTQ, withdrawal, other nicotine dependence symptoms and self-medication scores) suggest that these measures are also related to tobacco dose. Associations between dependence measures and salivary cotinine levels have been previously demonstrated in young adolescent smokers with an average age of 15 years (Rubinstein et al., 2007). Dependence measures including the mFTQ, nicotine dependence syndrome scale, timing of craving in the morning, and self-rated level of addiction were all correlated with cotinine level (Rubinstein et al., 2007). In these young adolescent smokers, the mean cotinine level and cigarette consumption were both lower than in our sample at age 24 (~4 vs. ~139 ng/ml, and ~4 vs. ~8 cigarettes/day, respectively). Together these findings suggest that dependence measures are related to tobacco dose across a range of exposures and ages (Rubinstein et al., 2007).

In a recent study of young smokers, the influence of CYP2A6 variation on smoking outcomes varied throughout the 6-year follow-up period (Cannon et al., 2015). At age 16, CYP2A6 intermediate metabolism (vs. normal and slow metabolism) was associated with the highest smoking frequency (number of days smoked per month) and nicotine dependence syndrome scale (NDSS) score; in contrast, as young adults (age 22), CYP2A6 normal metabolizers had the highest smoking frequency and NDSS score (Cannon et al., 2015). We
performed a cross-sectional analysis in current smokers at survey 22 (participants aged ~24 years) to determine whether CYP2A6 normal metabolizers had higher cigarette consumption as well as an increased likelihood of ICD-10 tobacco dependence. There was no significant difference in the mean number of cigarettes smoked per month between CYP2A6 normal (240; standard deviation (SD)=211; n=125), intermediate (255; SD=230; n=24), and slow (310; SD=250; n=9) nicotine metabolism groups (P=0.64). In addition, there was no difference in the mean level of cotinine + 3’hydroxycotinine, a biomarker of tobacco dose, between CYP2A6 metabolism groups (184 ng/ml, 202 ng/ml, and 181 ng/ml in normal, intermediate, and slow metabolizers, respectively; P=0.59), suggesting a similar degree of tobacco exposure. Finally, no significant relationship between CYP2A6 metabolism group and ICD-10 tobacco dependence was observed; 35% of CYP2A6 normal metabolizers were dependent (score 3+), compared to 42% and 56% of intermediate and slow metabolizers, respectively (P=0.44). Differences between our findings and those from the longitudinal investigation (Cannon et al., 2015) may stem from differences in study design (cross-sectional vs. longitudinal), variation in the assessment of CYP2A6 activity (categorical grouping of metabolism activity vs. use of a metabolism metric), and/or differences in smoking phenotypes examined (cigarette consumption vs. smoking frequency; ICD-10 dependence status vs. nicotine dependence syndrome scale). Future longitudinal investigations will help to clarify the factors, including the role of CYP2A6, associated with smoking in adolescence, and as youth transition to adulthood. A better understanding of these factors may lead to targeted interventions that help reduce the risk for lifelong smoking.

While there was some indication that CYP2B6 slow metabolizers were at greater risk of acquiring tobacco dependence in adolescence, this did not reach significance. When we assessed the participants in young adulthood, we saw evidence suggesting that CYP2B6 variation may be associated with dependence status in this age group, as there were higher dependence rates in CYP2B6 slow (vs. normal) metabolizers. These findings are consistent with a recent report in Italians where the frequency of the CYP2B6*1/*6 genotype was significantly higher in individuals with nicotine dependence (FTND score 4+) compared to those who were not dependent (36% vs. 21%, respectively), which was not seen for those with the normal CYP2B6*1/*1 genotype (20% vs. 33%, respectively) (Riccardi et al., 2015). Together with our findings, these data suggest that the CYP2B6*6 allele is associated with a higher risk of nicotine...
dependence in adulthood; over adolescence we speculate that the risk conferred by \textit{CYP2B6} slow metabolism increases. Variation in \textit{CYP2B6} does not substantially alter the rate of peripheral nicotine metabolism (Lee et al., 2007b), however data from an animal model suggests variable brain CYP2B activity may influence nicotine metabolism within the brain, in turn affecting nicotine-mediated behaviours (Garcia et al., 2015). In rats, the inhibition of brain CYP2B activity through intracerebroventricular injection of a selective CYP2B inhibitor led to higher rates of acquisition of nicotine self-administration behaviour, without altering peripheral nicotine levels or metabolism (Garcia et al., 2015). It is possible that \textit{CYP2B6} variation within human brain may lead to altered central metabolism of nicotine, which may account for the observed differences in nicotine dependence (Riccardi et al., 2015) and cessation outcomes (Lee et al., 2007a).

Overall, we have shown that throughout adolescence, \textit{CYP2A6} slow nicotine metabolizers are at a higher risk of developing tobacco dependence, but as dependent smokers they consume fewer cigarettes relative to normal nicotine metabolizers. The role of genetic variation in \textit{CYP2B6} in smoking acquisition and dependence remains to be clarified, but may increase over time. The adjunct analysis that validates our measure of smoking behaviour supports these conclusions. The findings highlight the role of genetic risk factors in nicotine addiction in adolescence, a development period in which the contribution of genetics to smoking behaviours has been studied only infrequently to date.
Acknowledgments:
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Conflict of interest: In the past three years, Dr. Tyndale has consulted for Apotex. The remaining authors declare no conflicts of interest.
References:


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Tables

Table 1. Selected baseline characteristics of genotyped White ever-smokers, according to whether they had inhaled at cohort inception. NDIT 1999-2012.

<table>
<thead>
<tr>
<th></th>
<th>All inhalers</th>
<th>Incident inhalers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(inhaled before cohort inception or during follow-up)</td>
<td>n=214</td>
</tr>
<tr>
<td>Male, %</td>
<td>36.2-36.3</td>
<td>35.5</td>
</tr>
<tr>
<td>Mean age in years (SD)</td>
<td>12.7 (0.5)</td>
<td>12.6 (0.4)</td>
</tr>
<tr>
<td>Francophone, %</td>
<td>18.8-19.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Single parent family, %</td>
<td>9.3-9.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Parent(s) university/college educated, %</td>
<td>50.0-52.4</td>
<td>57.0-61.2</td>
</tr>
<tr>
<td>Parent(s) smoke, %</td>
<td>42.9-43.3</td>
<td>28.5-29.0</td>
</tr>
<tr>
<td>Friend(s) smoke, %</td>
<td>48.8-49.1</td>
<td>30.4-30.8</td>
</tr>
</tbody>
</table>

*Ranges in values represent the values derived from the 10 imputed datasets*
Table 2. Hazard ratios (HR) and 95% confidence intervals (CI) for ICD-10 tobacco dependence in adolescent smokers according to *CYP2A6* and *CYP2B6* metabolism groups. NDIT 1999-2012.

<table>
<thead>
<tr>
<th>Metabolism group</th>
<th>HR (95% CI)</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All inhalers(^a)</td>
<td>Incident inhalers</td>
</tr>
<tr>
<td><strong>CYP2A6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (reference)</td>
<td>1.0; n=330-333</td>
<td>1.0; n=162-165</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.8 (0.4, 1.3); n=60-62</td>
<td>0.9 (0.4, 2.1); n=29-31</td>
</tr>
<tr>
<td>Slow</td>
<td>1.8 (1.0, 3.3); n=27-28</td>
<td>2.3 (1.1, 4.7); n=20-21</td>
</tr>
<tr>
<td><strong>CYP2B6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (reference)</td>
<td>1.0; n=228</td>
<td>1.0; n=121</td>
</tr>
<tr>
<td>Slow</td>
<td>1.2 (0.9, 1.8); n=163</td>
<td>1.5 (0.9, 2.7); n=82</td>
</tr>
</tbody>
</table>

\(^a\)Includes prevalent (i.e., those who had inhaled before cohort inception) and incident (i.e., those who first inhaled during follow-up) inhalers
Table 3. Average past month cigarette consumption at end of follow-up among participants who initiated inhalation during follow-up, according to dependence status and CYP2A6 metabolism group. NDIT 1999-2012.

<table>
<thead>
<tr>
<th>CYP2A6 Metabolism group</th>
<th>All</th>
<th>ICD-10 Tobacco Dependent</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SE)²; n</td>
<td>Mean (SE)²; n</td>
<td>Mean (SE)²; n</td>
<td>Mean (SE)²; n</td>
</tr>
<tr>
<td>Normal</td>
<td>78.5 (12.8); 162-165</td>
<td>299.8 (47.9); 29-30</td>
<td>25.6 (8.6); 133-135</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>48.0 (30.0); 29-31</td>
<td>172.2 (234.6); 1-2</td>
<td>45.8 (18.9); 28-29</td>
<td></td>
</tr>
<tr>
<td>Slow</td>
<td>50.8 (36.5); 20-21</td>
<td>191.5 (109.4); 6</td>
<td>19.9 (26.5); 14-15</td>
<td></td>
</tr>
<tr>
<td>P value from ANOVA test</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>P value from Tukey-Kramer post-testᵇ</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: SE, standard error

ᵃThe mean of means and mean of SEs are reported, derived from 10 imputed datasets

ᵇComparing CYP2A6 normal and slow nicotine metabolizers
Figure 1. Self-reported smoking behaviours are strongly associated with salivary cotinine levels in young adults.
Figure Legends

Figure 1 Self-reported smoking behaviours are strongly associated with salivary cotinine levels in young adults. Correlation between self-reported cigarettes/month and cotinine, demonstrating construct-related validity, is shown in (a). Correlations between cotinine and ICD-10 (b) and mFTQ (c) dependence scores, withdrawal (d), other nicotine dependence symptoms (e), and self-medication (f) scores are also shown in all White self-reported current smokers (n=162).