The interaction between genetics and tobacco consumption in light smokers

by

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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 is the largest preventable cause of death globally. In North America, although the overall smoking prevalence has been declining, an increasing number of smokers consume less than 10 cigarettes per day and are referred to as light smokers. Today, light smokers represent more than 30% of the smoking population and are at risk for smoking related mortality and morbidity. For example, light smokers have approximately 15 times higher risk of developing chronic obstructive pulmonary disease and approximately 20 times higher risk of developing lung cancer compared to non-smokers. Yet despite the high prevalence and substantial negative health consequences, light smokers are generally excluded from smoking related studies. Genetic variants in CYP2A6, encoding for the major nicotine-metabolizing enzyme, and CHRNA5-A3-B4, encoding for α5, α3 and β4 nicotinic receptors, are associated with altered tobacco consumption in heavy smokers, yet little is known about the influence of CYP2A6 and CHRNA5-A3-B4 genetic variants on tobacco consumption and smoking cessation in light smokers. In a cross sectional study of 400 Alaska Native individuals, we demonstrated that light smokers had similar nicotine exposure (as indicated by their urinary total nicotine equivalents) as heavy smokers despite self-reporting lower number of cigarettes per day. Like heavy smokers, these
light smokers titrated their tobacco consumption according to their CYP2A6 activity and CHRNA5-A3-B4 genotype. In addition, gene variants in CYP2A6 and CHRNA5-A3-B4 acted in combination to modify tobacco consumption. Furthermore, our biomarker analyses showed that variation in CYP2A6 metabolic activity, such as those that exist between CYP2A6 genotypes or the sexes, altered cotinine removal to a greater extent than cotinine formation. As a result, cotinine accumulates in individuals with lower CYP2A6 activity resulting in substantially higher cotinine levels for a given tobacco exposure. Overall, the characterization of tobacco consumption levels and the genetic contribution to tobacco consumption in light smokers improved our understanding of smoking behaviors in this increasingly prevalent population and could aid the development of more efficacious smoking cessation strategies.
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Conference Presentations


Symposium Presentation: ZHU, A.Z. et al., Hydroxybupropion concentration, mediated by CYP2B6 activity, is a major determinant of bupropion efficacy for smoking cessation. March 2012 -- Annual Meeting for Society of Nicotine & Tobacco Research, Houston, TX USA. Symposium 5E

Symposium Presentation: ZHU, A.Z. et al., Reduced CYP2A6 activity increases tobacco consumption and NNAL levels: a study in Alaska Native smokers and smokeless tobacco users. March 2012 -- Annual Meeting for Society of Nicotine & Tobacco Research, Houston, TX USA. Symposium 2D

Oral Presentation: ZHU, A.Z. et al., Hydroxybupropion level is a major determinant of bupropion’s smoking cessation efficacy- the pharmacogenetic role of CYP2B6. Addiction Research Seminar: Pharmacogenetics of tobacco addiction, treatment and related illness, Toronto, ON


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Abstract Presented

Poster Presentation: ZHU, A.Z. et al., The Ability of Plasma Cotinine to Predict Tobacco Exposure is Altered by Differences in CYP2A6: the influence of genetics and sex -- Annual Meeting for Society of Nicotine & Tobacco Research, Boston, MA USA. Pos3-148


Poster Presentation: **ZHU, A.Z.** et al., Common CYP2B6 Genetic Variants Alter the Rates of Nicotine C-Oxidation POS5-68 – Feb 2011-17th Annual Meeting for Society of Nicotine & Tobacco Research, Toronto ON, Canada

Poster Presentation: **ZHU, A.Z.** et al., “African Americans Have Unique Genetic Variation in CYP2B6: Implications for Smoking Cessation”. July 2010-Canadian Society of Pharmacology Meeting, Toronto, ON, Canada


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<table>
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<tbody>
<tr>
<td>3HC</td>
<td>3'-hydroxycotinine</td>
</tr>
<tr>
<td>3HC/COT</td>
<td>Ratio of 3'-hydroxycotinine to cotinine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CPD</td>
<td>Cigarettes per day</td>
</tr>
<tr>
<td>Cre</td>
<td>Creatinine</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Cytochrome P450 2A6</td>
</tr>
<tr>
<td>CYP2A6 NM</td>
<td>CYP2A6 Normal Metabolizers</td>
</tr>
<tr>
<td>CYP2A6 RM</td>
<td>CYP2A6 Reduced Metabolizers</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FMO3</td>
<td>Flavin-containing monooxygenase 3</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid Chromatography–Mass Spectrometry</td>
</tr>
<tr>
<td>MAF</td>
<td>Minor Allele Frequency</td>
</tr>
<tr>
<td>nAChRs</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NMR</td>
<td>Nicotine Metabolite Ratio</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylN-nitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylN-nitrosamino)-1-(3-pyridyl)-butanone</td>
</tr>
<tr>
<td>NNN</td>
<td>N-nitrosonornicotine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>TNE</td>
<td>Total nicotine equivalents</td>
</tr>
<tr>
<td>TSNA</td>
<td>Tobacco Specific Nitrosamines</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate-glucuronosyltransferases</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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STATEMENT OF RESEARCH PROBLEM

Tobacco smoking results in more than 6 million premature deaths each year, representing one of the greatest public health challenges of all time. World Health Organization predicted that smoking will kill one billion people over the course of the 21\textsuperscript{st} century. Today, \textasciitilde 20\% of adults smoke in North America, resulting in more than $100 billion in health care costs annually. In recent years, a large proportion of smokers have started to smoke fewer cigarettes per day or have switched to “less harmful” tobacco products such as smokeless tobacco. Today, more than 30\% of the current smokers in North America are light smokers. Light smokers are still at increased risk for smoking related mortality and morbidity. For example, they have approximately 15 times higher risk of developing chronic obstructive pulmonary disease and approximately 20 times higher risk of developing lung cancer than non-smokers. Yet despite their increased prevalence and still elevated disease risks, light smokers are generally excluded from clinical trials and most types of epidemiological studies.

Nicotine is the main psychoactive compound in tobacco. Twin studies have indicated that genetics play an important role in determining tobacco consumption and the ability to quit smoking. Previously, genetic variants in \textit{CYP2A6}, encoding the main nicotine-metabolizing enzyme, and \textit{CHRNA5-A3-B4}, encoding three nicotinic receptors in the brain, have been associated with altered tobacco consumption and smoking cessation in Caucasian heavy smokers; but their influences on tobacco consumption have not been demonstrated in light smokers or smokeless tobacco users of other ethnicities. This lack of association could be due to both the lack of research in light smokers and smokeless tobacco users as well as the accuracy of the tobacco consumption biomarkers used in previous studies. The studies presented in this thesis utilized a theoretically stronger biomarker of nicotine consumption, total nicotine equivalents (TNE), to investigate the contribution of genetics to tobacco consumption in light smokers and smokeless tobacco users. Using TNE, we will assess the impact of genetic variants in \textit{CYP2A6} and \textit{CHRNA5-A3-B4} on tobacco consumption, alone or in combination, in light smokers and smokeless tobacco users. These investigations will provide insight into the levels, and mechanisms, of nicotine dependence in light smokers and smokeless tobacco users.
MAIN RESEARCH OBJECTIVES

Tobacco smoking is one of the largest public health challenges of our time. While the contribution of genetics to smoking behaviors has been extensively studied in heavy smokers, little is known about the influence of genetic variation in nicotine metabolism on tobacco consumption in light smokers and smokeless tobacco users, despite their increasing prevalence. The first objective of this thesis was to characterize the phenotypic influence of CYP2A6 gene variants on nicotine metabolic profiles, smoking behaviors and carcinogen exposures in light smokers. By studying the influence of CYP2A6 gene variants on tobacco consumption in light smokers, we discovered that reduced function CYP2A6 gene variants were associated with lower urinary TNE and higher plasma cotinine. The opposite effects on urinary TNE and plasma cotinine were in agreement with the findings of a large body of literature. Together, these data suggested that cotinine might not be an accurate biomarker of tobacco consumption. The second objective of this thesis was to provide a mechanistic explanation for the systematic variation in cotinine levels observed in different smoking populations.

In addition to CYP2A6, gene variants in CHRNA5-A3-B4, encoding for the α5, α3, and β4 nicotinic receptors, also influence tobacco consumption in heavy smokers. Progress has been made in identifying the independent CHRNA5-A3-B4 signals associated with tobacco consumption in Caucasian heavy smokers. However, these associations have not been investigated in light smokers. The third objective of this thesis was to determine whether CHRNA5-A3-B4 gene variants influence tobacco consumption in light smokers as seen in heavy smokers. Furthermore, we also investigated whether CYP2A6 variants and CHRNA5-A3-B4 variants act in combination to alter tobacco consumption. These studies allowed us to improve our understanding of the mechanisms of nicotine dependence in light smokers with the long-term goal of improving prevention and cessation approaches for this prevalent, but understudied population. Some of our pharmacogenetic data regarding smoking cessation in light smokers are attached in the Appendices.
1 GENERAL INTRODUCTION

1.1 Epidemiology of Tobacco Smoking

1.1.1 Prevalence

Currently, there are approximately 1.3 billion smokers, and nearly 6 million smoking-induced deaths each year worldwide (Wipfli et al. 2009). Global smoking related mortality is higher than tuberculosis, HIV/AIDS and malaria combined, making it the largest cause of preventable death. Adult smoking prevalence in North America had been decreasing from the mid 1960s to the mid 1990s (e.g. from ~50% to 25%), but has stabilized at ~20% for the last ten years (Centers for Disease Control and Prevention 2012). Today, smoking prevalence differs substantially between sexes, ethnicities and socioeconomic classes. In the United States, 21.6% of adult men smoke whereas only 16.5% adult women smoke (Centers for Disease Control and Prevention 2008). In addition, smoking prevalence is 32% in American Indians/Alaska Natives, 20% in Non-Hispanic Whites and African Americans, and 10% in Asian Americans. Variation in smoking prevalence is also noted between socioeconomic classes. Roughly 29% of adults who live below the poverty level smoke in the United States compared with the 18% in adults who live above the poverty level (Centers for Disease Control and Prevention 2009; Centers for Disease Control and Prevention 2012).

1.1.2 The Health Consequences

Tobacco smoking has numerous negative health effects and is the largest cause of preventable death globally (Fig. 1). The World Health Organization estimates that smoking has killed 100 million people in the 20th century and will kill 1 billion more in the 21st century (assuming the current smoking prevalence) with about 80% of these deaths occurring in developing countries (World Health Organization et al. 2008). In North America, smoking accounts for one in five deaths each year (Benowitz 2010). Even more striking, for every smoking related death, another 20 smokers suffer from smoking related diseases (U.S Department of Health and Human Services 2010). It has been estimated that, in the United States alone, tobacco smoking costs society an estimated $168 billion in health care expenditures and productivity loss annually (Office of National Drug Control Policy. 2004).
Tobacco use is a major cause of cancer, cardiovascular disease and pulmonary disease. It is also a contributing factor to infections of the respiratory tract, adverse postoperative events and delayed wound healing (United States Public Health Service. Office of the Surgeon General. 2004). One of the difficulties in studying the health consequences of tobacco smoking is that it may take 40 to 50 years for the negative health impacts of tobacco smoking to mature. Therefore, the most convincing epidemiological data about the health consequences of smoking are generally from cohorts, which follow a large number of participants over many years. One of the most cited of these is the British Doctors study where the health information for male doctors in the United Kingdom was collected repeatedly over a 50-year period from 1951 to 2001 (Doll et al. 1954; Doll et al. 2004). In this study, the smokers generally smoked regularly for more than 40 years, which gave a good estimation of the health impacts of persistent smoking (Doll et al. 2004). The probability of a smoker dying prematurely (before the age of 70) was 43% compared to the 20% observed in the never smokers (Fig. 2). Overall, smoking resulted in a leftward shift of approximately 10 years in life expectancy (Fig. 2) (Doll et al. 2004). The findings of the British Doctors study were replicated subsequently by studies with more diverse participant composition. For example, a cohort study that followed more than 1.5 million participants in the United States showed that the probability of dying before the age of 80 was 62% for smokers compared with 30% in people who had never smoked, which represented an approximately 11 year reduction in life expectancy among smokers (Jha et al. 2013). The Global Adult Tobacco Survey showed that of the 30 most common causes of death in developed counties, 23 were increased substantially in smokers with the most substantial increase (21.4 times higher) seen with lung cancer (Pirie et al. 2013). This study estimated that the probability of dying before the age of 80 was 53% in smokers compared with 22% in the never smokers, which also represented an 11 year reduction in life expectancy (Pirie et al. 2013).
Figure 1 | Smoking contributes substantially to the top eight causes of mortality globally. Hatched areas represent the proportion of death that was attributed to smoking. Adapted from World Health Organization’s report on the global tobacco epidemic (World Health Organization et al. 2008).

Figure 2 | The effects of smoking on the survival of British doctors. Study of British doctors indicated that smoking resulted in a 10 year average loss of life expectancy. Adapted from Doll et al., 2004 and Peto et al., 2010 (Doll et al. 2004; Peto et al. 2010).
1.1.2.1 Cancers

Smoking can lead to a spectrum of negative health consequences (Fig. 3); cancers are among the most notable of these. There are over 4000 chemicals in cigarette smoke, and more than 60 of them are carcinogenic (Hecht 2003). In American smokeless tobacco or unburned tobacco, there are at least 16 known human carcinogens (Hecht 2003). In addition, hundreds of other toxic chemicals, including tumor promoters, co-carcinogens, oxidants, free radicals and inflammatory agents are also delivered to the body during the smoking process (Hecht 2003). Once in the body, the carcinogens and their metabolites bind to DNA, resulting in covalent DNA adducts and mutations in the genome. When these mutations affect the function of crucial genes, such as tumor suppressors and oncogenes, normal cellular growth is altered, resulting in the development of cancer (Centers for Disease Control and Prevention 1989; Hecht 2003; United States Public Health Service. Office of the Surgeon General. 2004; Hecht et al. 2009; Hecht 2012). Smoking and smokeless tobacco use induce a diverse array of different cancers including those of the lungs, oral cavity, nasal cavity, larynx, oropharynx, hypopharynx, esophagus, stomach, bladder, ureter, kidney, cervix, and myeloid leukemia (United States Public Health Service. Office of the Surgeon General. 2004).
The most notable smoking induced cancers is lung cancer. The association between smoking and lung cancer was first conclusively demonstrated during the 1950s by the combination of powerful epidemiologic evidence from the British Doctors study as well as experimental evidence from animals treated with cigarette tar (Wynder et al. 1953; Doll et al. 1954; Doll et al. 1955; Wynder et al. 1953).
1956). Today, lung cancer is the second most common cancer and accounts for 12% of all new cases of cancer globally with smoking contributing to up to 90% of these new lung cancer cases (Centers for Disease Control and Prevention 1989; United States Public Health Service. Office of the Surgeon General. 2004; van Meerbeeck et al. 2011). The relative risk of developing lung cancer is 21 to 25 times higher in smokers compared to never smokers (Pirie et al. 2013; Thun et al. 2013), and the risk is directly proportioned to cigarette per day (CPD) and with duration of smoking (Brownson et al. 1992). Of note, this risk of developing lung cancer decreases after smoking cessation compared with those who continued to smoke (Khuder et al. 2001). Even with all of the therapeutic advances in the last 50 years, lung cancer remains one of the most deadly types of cancer with a five-year survival rate of only 15%. It results in roughly 160,000 deaths in the United States each year (Jemal et al. 2011).

Lung cancer can be classified into two histological subtypes. Roughly 20% of lung cancer cases are classified as small cell lung cancer, and the other 80%, which include adenocarcinoma, squamous cell carcinoma, large cell carcinoma and bronchoalveolar cell carcinoma, are collectively called non-small cell lung cancer (Travis et al. 2011). Historically, 95% of the cases of small cell lung cancer were directly attributed to tobacco smoking, whereas adenocarcinoma was considered to be minimally related to tobacco smoking (Doll et al. 1957). However, current evidence suggests that the recent increase in adenocarcinoma incidence is smoking related and may reflect the change in smoking behaviors over the recent decades (Thun et al. 1997).

Among the lung carcinogens in tobacco, tobacco specific nitrosamines (TSNA), such as 4-(methylnitrosamino)-1-(3)pyridyl-1-butaneone (NNK) and N-nitrosonornicotine (NNN), are consistently associated with the development of lung cancer, particularly adenocarcinoma, in animal models (Schuller et al. 1990; Hoffmann et al. 1996). TSNAs are prevalent and potent carcinogens derived from nicotine and related compounds (Hecht 2003). TSNAs induce tumors in every animal model tested, and can induce lung tumors independent of the route of administration in rodents (Hecht 2002). Once absorbed, TSNAs are bioactivated (metabolized) by CYPs to their α-hydroxyl metabolites, which are then spontaneously converted to diazonium ions, which can result in covalent DNA adduct formation (Hecht 1998). In the case of NNK, CYP2B6 exhibits the highest α-hydroxylation activity in vitro with an affinity roughly ten times higher (i.e. a lower K_m) than CYP2A6 (Jalas et al. 2005). In contrast, the α-hydroxylation of
NNN is thought to be primarily mediated by CYP2A6 (Kushida et al. 2000; Wong et al. 2005). Individuals vary extensively in their TSNA metabolism, possibly due to genetic variations in these and other carcinogen metabolizing enzymes (Harris et al. 1976; Hecht 2003; Stepanov et al. 2008; Ter-Minassian et al. 2012). This may contribute to the individual variability in cancer risk observed between smokers. Chapter 1 investigates the role of genetic variants in xenobiotic metabolizing enzymes on TSNA metabolism.

1.1.2.2 Respiratory and Cardiovascular Diseases

Although lung cancer is most often associated with smoking, a substantial amount of smoking related mortality is due to respiratory and cardiovascular diseases (Fig. 1). Smoking is the largest cause of chronic bronchitis and also increases the risk of dying from chronic bronchitis (Koop et al. 1982; United States Public Health Service. Office of the Surgeon General. 2004; Koop et al. 2006). Smoking is responsible for 75 to 90% of chronic obstructive pulmonary disease (COPD) related deaths (Schroeder 2013; Thun et al. 2013). Interestingly, the rate of death from COPD continues to increase among smokers in contrast to the reduction observed among never smokers (Thun et al. 2013), which is unlikely to be explained by the improved ability to diagnose COPD (because earlier detection of COPD should affect the disease prevalence rather than the rate of death). This suggests the possibility that the engineering modifications to cigarettes (to promote deeper inhalation) over the last 50 years might increase the lung parenchyma’s exposure to tobacco smoke and the severity of smoking related COPD (Thun et al. 2013).

Smoking is a major cause of cardiovascular disease. Smoking is responsible for 20 to 25% of coronary heart disease deaths and 18% of stroke deaths (Koop et al. 1982; United States Public Health Service. Office of the Surgeon General. 2004; Koop et al. 2006). Smoking is associated with a 6.5 times higher risk of death from aortic aneurysm, 4.5 times higher risk of death from coronary heart disease, 3.0 times higher risk of death from cerebrovascular disease, and 1.5 times higher risk of death from venous thromboembolism; smoking is also a significant risk factor for atherosclerosis (Koop et al. 1982; United States Public Health Service. Office of the Surgeon General. 2004; Koop et al. 2006; Pirie et al. 2013). Additionally, smoking can multiplicatively interact with other cardiovascular disease risk factors (such as elevated cholesterol or hypertension). Observations from the Pooling Project (an international consortium of cohort studies) suggested that smoking, elevated cholesterol and hypertension were each associated with
an increase of ~30 cardiac events per 1000 individuals in a 10 year period. But the cardiac event risk was dramatically greater in individuals with all three risk factors (190 events per 1000 individuals in a period of 10 years)(Pooling Group of the American Heart Association 1978). Thus, there is a significant toxic effect of smoking on the homeostatic balance of the cardiovascular system and robust associations between smoking and cardiovascular diseases.

1.1.2.3 Other Health Consequences

Smoking also has deleterious effects on human reproduction. Female smokers have an increased risk of delayed conception and infertility (Augood et al. 1998). Even among the female smokers who are able to conceive, smoking during pregnancy is associated with increased risk of premature rupture of membranes, placental abruption, placenta previa and preterm delivery (Harger et al. 1990; Williams et al. 1992). Furthermore, infants born to mothers who smoked during pregnancy have lower birth weights than infants with mothers who did not smoke (Butler et al. 1972; Sexton et al. 1984; Brooke et al. 1989). The risk of stillbirth and neonatal death are also increased among the infants with mothers who smoked during pregnancy (Winbo et al. 2001; Kristensen et al. 2005).

Involuntary exposure to tobacco smoke (i.e. secondhand smoke) can lead to numerous undesirable health consequences, such as cancer and respiratory diseases in healthy non-smokers. The risk of developing lung cancer in non-smokers who are exposed to secondhand smoke is 20 to 30% higher than those who are not exposed. Secondhand smoking results in an estimate of 3400 lung cancer deaths in the United States each year (United States. Surgeon-General's Office. 2006). Together, current evidence suggests that smoking can lead to a large number of negative health consequences. The next section will briefly discuss the benefits of smoking cessation.

1.1.3 Benefits of Smoking Cessation

Smoking duration correlates positively with the risk of developing smoking related diseases. Smoking cessation at any age has substantial health benefits. For example, a study with more than 200,000 participants demonstrated that smokers who had quit between the ages of 25 to 34, 35 to 44 or 45-54 gained about 10, 9 and 6 years of life respectively compared with those who continued to smoke (Jha et al. 2013). Similarly, another meta-analysis with more than 500,000
participants demonstrated similar effects of smoking cessation (Thun et al. 2013). Smokers who quit smoking generally have a large reduction in their lung cancer risk compared to the smokers who continue to smoke (Centers for Disease Control and Prevention 1989; United States Department of Health and Human Services 1990). Even smoking cessation at 60 years of age can lower the cumulative risk of lung cancer from 16% to about 10% (United States Public Health Service. Office of the Surgeon General. 2004; Jha et al. 2013). Smoking cessation has also been associated with a significant reduction in cardiovascular disease risk (Critchley et al. 2003). The risk of developing cardiovascular disease is reduced by 50% after 1 year of smoking abstinence (United States Public Health Service. Office of the Surgeon General. 2004). After 15 years of smoking abstinence, former smokers’ risk of developing cardiovascular disease is similar to never smokers (United States Public Health Service. Office of the Surgeon General. 2004). Among people who have been diagnosed with cardiovascular disease, smoking cessation greatly reduced (often by 50%) the risk of death (United States Public Health Service. Office of the Surgeon General. 2004). Despite the significant benefits of smoking cessation, only 3% of smokers successfully quit each year (Benowitz 2010). Thus, there is a pressing need for more effective smoking cessation strategies. A better fundamental understanding of the mechanisms behind smoking will likely lead to more effective smoking cessation strategies.

1.2 The Clinical and Molecular Neurobiology of Nicotine Dependence

Nicotine is the main psychoactive ingredient of tobacco (Benowitz 2009). It is highly addictive via inhalation and is responsible for the reinforcing properties of tobacco (Isaac et al. 1972; Koop et al. 1982; Henningfield et al. 1983; McMorrow et al. 1983). Nicotine, in its pure form, is self-administered by a number of rodent species and non-human primate species (Jaffe et al. 1978; Henningfield et al. 1983; Henningfield et al. 1983; Corrigall et al. 1992). It is also intravenously self-administered by human smokers in laboratory settings with an inverted U-shaped dose response relationship as seen in animals (Henningfield et al. 1983; Harvey et al. 2004).

1.2.1 Clinical Features of Nicotine Dependence

Smokers smoke to obtain positive reinforcement (such as the enhancements of mood and mental functions) and to avoid the negative reinforcement of nicotine withdrawal (Dani et al. 1996).
1.2.1.1 Positive Reinforcements: The Psychoactive Effects of Smoking

Although there are many chemical additives in commercial cigarettes engineered to enhance their addictiveness (Henningfield et al. 2004), nicotine is the principle addictive component in tobacco. Smoking is a rapid route of nicotine administration. During smoking, the rapid rate of nicotine absorption into pulmonary circulation, and subsequently into the brain, cause a strong subjective feeling of “rush” and reinforcing effects. Clinically, smokers report that smoking induces pleasure and reduces stress and anxiety (Hatsukami et al. 2008; Benowitz 2010). Laboratory studies suggested that smoking can improve concentration, reaction time, and performance in many cognitive tests among smokers (Knott et al. 2009; Benowitz 2010).

The smoking process allows precise dosing of nicotine to obtain the desirable reinforcing effects (or relief of withdrawal) (Benowitz 2010). Nicotine is sometimes considered a relatively weak reinforcer compared to other drugs of abuse such as cocaine and psychostimulants (Caggiula et al. 2002). Many of smoking’s rewarding effects are mediated by smoking related cues (Caggiula et al. 2002). Smokers learn to associate the rewarding effects of smoking with smoking related cues, such as sight, taste, and smell of cigarettes as well as specific situations (e.g. a meal or with alcohol), mood (e.g. unpleasant mood and irritability), and environmental factors (e.g. a particular location) (Rose 2006). Nicotine enhances the behavioral responses to smoking related cues and makes them more salient (Olausson et al. 2004). The importance of smoking related cues in smoking behavior is supported by human brain imaging data. The presence of smoking related cues activates a number of cortical regions in the brain including the insula (Franklin et al. 2007). Smokers with damages to the insula, such as from brain trauma, were able to stop smoking more easily compared to smokers with brain trauma that did not affect the insula (Naqvi et al. 2007). This suggests that activation of the insula by smoking related cues may play an essential role in the maintenance of smoking in regular smokers. In agreement with the data from human smokers, the nicotine self-administration behaviors of animals are also highly dependent on the presence of contingent cues, such as light and sound (Caggiula et al. 2002). Together, existing data suggest that both nicotine’s pharmacological effects and smoking related cues contribute to the positive reinforcing effects of smoking.
1.2.1.2 Negative Reinforcements: Nicotine Withdrawal

The negative reinforcing effects of nicotine withdrawal also play an important role in nicotine dependence. Clinically, nicotine withdrawal is associated with anger, anxiety, depressed mood, difficulty concentrating, irritability, insomnia, and restlessness (Hughes et al. 1986; Hughes 2007). Some studies, but not others, have also suggested that nicotine withdrawal is associated with constipation, cough, dizziness, increased dreaming, mouth ulcers, nausea, and sore throat (Hughes 2007). Withdrawal symptoms typically peak during the first week of smoking abstinence and gradually decrease over the next 2 to 4 weeks (Hughes et al. 1990; Hughes 2007). Smokers report that smoking relieves the withdrawal symptoms by modulating arousal and mood, and improving concentration, reaction time and performance (Knott et al. 2011). The avoidance of these withdrawal symptoms is a major driving force behind the observed pattern of regular daily smoking and smoking relapse.

1.2.1.3 Titration of Smoking Behaviors

Smokers tend to smoke the same number of cigarettes at fixed intervals from day to day to maintain a desired level of nicotine in the body and to avoid nicotine withdrawal (McMorrow et al. 1983). When the availability of nicotine is altered, smokers alter their smoking behaviors to compensate for the change. For example, smokers smoke more cigarettes and take bigger puffs when switched from regular to low nicotine yield cigarettes (Herning et al. 1981; Benowitz et al. 1983). Conversely, they smoke fewer cigarettes when switched to nicotine-enriched cigarettes (Fagerstrom 1982; Scherer 1999). Also consistent with this, pharmacokinetic manipulations of nicotine clearance alter smoking behaviors. For example, urinary acidification, which ion traps nicotine and increases nicotine’s renal clearance, results in an 18% increase in daily nicotine consumption (Schachter et al. 1977; Benowitz et al. 1985). In contrast, urinary alkalization, which decreases nicotine’s renal clearance, reduces smoking (Benowitz et al. 1985). Pre-treatment with nicotine (via transdermal patch) greatly reduces subsequent cigarette consumption and increases the latency to smoking the next cigarette during ad libitum smoking (Benowitz et al. 1998; Scherer 1999). Together this suggests that smokers try to maintain a desirable plasma nicotine level (and presumably brain levels) by altering smoking behaviors in response to the availability of nicotine.
1.2.2 Measurements of Nicotine Dependence

Nicotine dependence is a complex, multifactorial disorder. In order to measure the severity of nicotine dependence quantitatively, a number of measures/scales have been developed. The Fagerstrom Test for Nicotine Dependence (FTND) is a widely used measure of nicotine dependence (Table 1)(Heatherton et al. 1991). The FTND focuses on physical dependence and is weighted heavily toward questions such as “How many cigarettes per day do you smoke?” and “How soon after you wake up do you smoke your first cigarette?” (Piper et al. 2006). The FTND is easy to administer, has good reproducibility over time and is predictive of smoking relapse during smoking cessation attempts (Piper et al. 2006). A shorter version of the FTND, Heaviness of Smoking Index (HSI), is also used to measure the level of nicotine dependence in smokers (Kozlowski et al. 1994). HSI only contains two questions, time to first cigarette and cigarettes per day, and has shown good concordance with the FTND (Perez-Rios et al. 2009). Other measures, such as the diagnostic criteria from Diagnostic Statistical Manual-IV and International Classification of Diseases-10 capture other aspects of nicotine dependence, such as the cognitive and behavioral effects, and these scales generally have low concordance with the FTND (Hughes et al. 2004). Recently, a few other scales, such as the Nicotine Dependence Syndrome Scale and Wisconsin Inventory of Smoking Dependence Motives, have been developed to more comprehensively cover the multidimensional nature of nicotine dependence (Piper et al. 2004; Piper et al. 2008), but these scales have not yet been widely adapted.
### Table 1 | The Fagerstrom test for nicotine dependence (Heatherton et al. 1991).
The maximum score is 10 with higher scores indicating greater nicotine dependence. A score of greater than 5 indicates high level of nicotine dependence (Moolchan et al. 2002).

<table>
<thead>
<tr>
<th>How soon after you wake up do you smoke your first cigarette?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within 5 min</td>
<td>3</td>
</tr>
<tr>
<td>6-30 min</td>
<td>2</td>
</tr>
<tr>
<td>31-60 min</td>
<td>1</td>
</tr>
<tr>
<td>After 60 min</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do you find it difficult to refrain from smoking in places where it is forbidden—e.g., in church, at the library, in cinema?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Which cigarette would you hate most to give up?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>The first one in the morning</td>
<td>1</td>
</tr>
<tr>
<td>All others</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>How many cigarettes per day do you smoke?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤10</td>
<td>0</td>
</tr>
<tr>
<td>11-20</td>
<td>1</td>
</tr>
<tr>
<td>21-30</td>
<td>2</td>
</tr>
<tr>
<td>≥31</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do you smoke more frequently during the first hours after waking than you do during the rest of the day?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do you smoke if you are so ill that you are in bed most of the day?</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>

#### 1.2.3 Mechanisms of Nicotine Dependence

During smoking, particles carry nicotine into the lungs, where it is absorbed into the blood circulation. It is then quickly transported to the brain where it activates nicotinic acetylcholine receptors (nAChR). Many brain circuits, particularly the mesolimbic dopamine reward pathway which projects from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and nucleus accumbens (NAc), are involved in nicotine addiction (Koob 2006). The activation of mesolimbic dopamine neurons by nicotine is mediated both directly by the activation of nAChR on dopamine neurons in the VTA, and indirectly by modulating GABA and glutamate inputs onto dopamine neurons (Laviolette et al. 2004). The ability of nicotine to modulate the mesolimbic dopamine...
pathway to signal for pleasurable experiences is critical for the reinforcing effects of smoking (Corrigall et al. 1992; Le Foll et al. 2006). Here, the role of neuronal nAChRs in the activation of mesolimbic dopamine pathway will be discussed.

1.2.3.1 Molecular Biology of Nicotinic Receptors

nAChRs are pentameric (5 subunits) structures with a centrally located cation channel (Changeux 2010). Twelve different neuronal nAChR subunit genes have been identified, including nine alpha genes (α2-α10) and three beta genes (β2-β4) (Changeux 2010). With some exceptions, most brain nAChRs are located presynaptically and modulate the release of other neurotransmitters (Gotti et al. 2009). Nicotine and tobacco research has historically focused on the high affinity, slow desensitization heteromeric α4β2 nAChRs expressed in the mesolimbic dopamine pathway and the low affinity, high Ca2+ permeability homomeric α7 nAChRs expressed in the hippocampus and cortex regions (Changeux 2010). More recently, genome wide association studies and subsequent animal studies have suggested the potential importance of nAChRs consisting of the α5, α3 and β4 subunits in governing tobacco consumption and other smoking behaviors (Amos et al. 2008; Hung et al. 2008; Thorgeirsson et al. 2008; Saccone et al. 2009; Wu et al. 2009; Jackson et al. 2010; Saccone et al. 2010; Fowler et al. 2011).

1.2.3.1.1 The Role of α4β2 nAChR in Nicotine Dependence

The activation of α4β2 nAChRs on the mesolimbic dopamine neurons plays an essential role in the acquisition and maintenance of nicotine dependence. Genetically modified mice lacking either the α4 or β2 subunits did not self-administer nicotine; the nicotine self-administration behavior was restored when α4 or β2 subunits were re-expressed (using viral vectors) in the VTA (Picciotto et al. 1998; Epping-Jordan et al. 1999; Maskos et al. 2005; Pons et al. 2008). This is unlikely to be a result of generally altered learning behaviors as β2 knockout mice readily self-administered cocaine, but stopped their self-administration behavior when the cocaine was switched to nicotine (Picciotto et al. 1998; Epping-Jordan et al. 1999). In agreement with the self-administration data, β2 knockout mice did not show nicotine conditioned place preference or nicotine discrimination (Walters et al. 2006). Conversely, the overexpression of the α4 gene results in nicotine hyper-sensitive mice, which displayed nicotine conditioned place preference at 50 times lower dose compared to wild type mice (Tapper et al. 2004). In addition, the current
FDA approved pharmacotherapies to promote smoking cessation (nicotine replacement therapy, bupropion and varenicline) all demonstrate some affinity toward the α4β2 nAChRs with varenicline displaying the highest selectivity toward this receptor combination (Slemmer et al. 2000; Gonzales et al. 2006). Together, these suggest that α4β2 nAChRs play an essential role in nicotine dependence.

1.2.3.1.2 The Role of α7 nAChR in Nicotine Dependence

The low affinity, high Ca\(^{2+}\) permeability homomeric α7 nAChRs may not be directly involved in nicotine’s rewarding effects, but may play a role in modulating the motivation to obtain nicotine. Genetically modified mice lacking functional α7 did not exhibit altered nicotine self-administration behaviors, nicotine induced conditioned place preference or nicotine discrimination (Stoleruman et al. 2004; Walters et al. 2006). However, local infusions of α-conotoxin, a selective α7 antagonist, into the nucleus accumbens shell or anterior cingulate cortex increased the breakpoints of nicotine self-administration under a progressive ratio schedule, suggesting that a reduction in α7 function in these brain regions increases the motivation to obtain nicotine (Brunzell et al. 2012). In agreement, varenicline, a full agonist at α7 nAChRs, is an effective treatment to promote smoking cessation in humans and generally decreases the motivation to obtain nicotine (Gonzales et al. 2006; Mihalak et al. 2006; Siu et al. 2007). Together, these data suggest that the α7 subunit is not directly involved in nicotine’s rewarding effects, but may play an important role in modulating the motivation to obtain nicotine.

1.2.3.1.3 The Role of α5, α3 and β4 nAChRs in Nicotine Dependence

The role of the α5, α3 and β4 subunits in nicotine dependence is relatively unknown. This is partially due to the fact that the α5 and α3 subunits are obligate accessory subunits and cannot form functional nAChRs by themselves (Berrettini et al. 2012). However, a number of genome wide association studies published in 2008 suggested a strong association between genetic variants in the CHRNA5-A3-B4 gene cluster, which encodes the α5, α3 and β4 subunits, with CPD and lung cancer risk (Amos et al. 2008; Hung et al. 2008; Thorgeirsson et al. 2008)(details in later sections). Subsequent research showed that genetically modified animals lacking the α5 subunit and animals with overexpressed β4 subunit (using a viral vector) displayed enhanced
nicotine’s reinforcing effects (Fowler et al. 2011; Frahm et al. 2011).

1.3 Nicotine Pharmacokinetics

Nicotine pharmacokinetics is an important determinant of smoking behaviors due to a smoker’s tendency to maintain a desired nicotine level. Smoking is a fast, efficient and highly addictive route of nicotine administration. In comparison, nicotine replacement therapies, used to promote smoking cessation, deliver nicotine slowly and have low abuse liability (Houtsmuller et al. 2003).

This section will focus on the absorption of nicotine via tobacco smoking, the distribution of nicotine in the body, and the metabolism and renal excretion of nicotine.

1.3.1 Absorption and Distribution

About 10 to 14 mg of nicotine is found in a typical cigarette and 1 to 1.5 mg is absorbed into the body during the smoking process (Benowitz et al. 1984; Kozlowski et al. 1998). Nicotine has a weakly basic pKa of 8.0 and its absorption kinetics are highly pH dependent since essentially only unionized nicotine can cross membranes and be absorbed. During smoking, nicotine is carried by tar droplets (also known as particulate matter), which have a pH between 6.0 to 7.8, into the respiratory tract (Pankow et al. 2003). At this pH range, 1% to 36% of nicotine is in the unionized form and can readily cross the biological membrane in the small airways and alveoli of the lungs and be rapidly absorbed into the blood stream (Pankow et al. 2003). After a puff, it takes about 10 to 20 seconds for nicotine to reach the brain and activate the nAChRs on the mesolimbic dopamine neurons and other brain regions (Henningfield et al. 1993; Rose et al. 2010). This rapid absorption of nicotine into the brain makes smoking one of the most reinforcing routes of nicotine administration (Henningfield et al. 1993). In addition, the short time needed for nicotine to reach the brain allows smokers to precisely control the depth of inhalation, puff volume, and number of puffs per cigarette to reach a desirable nicotine level (Herning et al. 1983).

In contrast to smoking, chewing (in the case of smokeless tobacco) is a comparatively slower way of delivering nicotine. The absorption of nicotine from smokeless tobacco is also highly pH
dependent. Therefore, chewing tobacco generally has a very basic pH to reduce ionization and maximize the absorption of nicotine across the buccal mucosa (Benowitz 1988). On average, a typical size smokeless tobacco chew delivers about 4.5 mg of nicotine to the body and a typical size snuff delivers on average 3.6 mg of nicotine to the body, whereas only 1.0 mg nicotine is delivered to the body from smoking a cigarette (Benowitz 1988). Nicotine’s absorption from smokeless tobacco occurs more slowly than the absorption from smoking, but the peak venous concentrations are very similar (Benowitz 1988; Benowitz et al. 1988; Benowitz 1997). In contrast to the rapidly falling nicotine concentrations after smoking, the nicotine concentrations were stable during, and after, the use of smokeless tobacco reflecting the continued absorption of nicotine to the systemic circulation even after the smokeless tobacco is removed from the mouth (Benowitz et al. 1988; Benowitz 1997). Thus, nicotine exposure is prolonged after smokeless tobacco use. **Fig. 4** illustrates the pharmacokinetic profile of nicotine after cigarette smoking (top left) and chewing tobacco (bottom left).

![Figure 4](image)

**Figure 4 | Blood nicotine concentration versus time profiles after cigarette smoking, oral snuff, chewing tobacco, and nicotine gum.**

The shaded bars represent the duration of tobacco consumption. Adapted from Benowitz et al., 1998.
After absorption, nicotine is distributed into a number of different body tissues, which is reflected by the large volume of distribution (2.6 L/kg) (Benowitz et al. 1991; Benowitz et al. 1994). Nicotine is not protein bound in the plasma (< 5%), and the distribution half-life is around 8 minutes in humans (Benowitz et al. 1991). The arterial concentration of nicotine after consuming a cigarette is usually between 20 to 60 ng/ml (Armitage et al. 1975; Henningfield et al. 1993; Rose et al. 1999; Lunell et al. 2000). The venous concentration of nicotine after consuming a cigarette generally ranges from 10 to 50 ng/ml (Benowitz 1990; Schneider et al. 2001). The mean increase in venous nicotine concentration after consuming a cigarette is around 11 ng/ml (Fig. 4)(Benowitz et al. 1988; Patterson et al. 2003), but this can be as high as 30 ng/ml depending on how a cigarette is smoked (Lunell et al. 2000; Schneider et al. 2001).

1.3.2 Metabolism

1.3.2.1 The Primary Metabolites of Nicotine

In humans, the majority of absorbed nicotine is metabolized before excretion. Nicotine is metabolized to a number of primary metabolites including cotinine, nicotine N’-oxide, nornicotine, and nicotine N’-glucuronide (Fig. 5), and minor metabolites such as nicotine isomethonium ion and 2’-hydroxynicotine.
Figure 5 | Nicotine’s metabolic profile in humans.

The percentage below each metabolite is an estimate of the average excretion of this metabolite as a percentage of total urinary nicotine equivalents or the nicotine dose. This figure represents broad estimations as the data were gathered by different studies using various methods. Bold indicates compound of interest (Brandange et al. 1979; Byrd et al. 1992; Cashman et al. 1992; Benowitz et al. 1994; Nakajima et al. 1996; Nakajima et al. 1996; Messina et al. 1997; Benowitz et al. 2002; Hukkanen et al. 2005; Yamanaka et al. 2005; Chen et al. 2007; Kaivosaari et al. 2007; Berg et al. 2010).

Nicotine is the main metabolite of nicotine in humans. Approximately 70 to 80% of the absorbed nicotine is metabolized to cotinine (Benowitz et al. 1994; Mwenifumbo et al. 2010; Zhu et al. 2013). The metabolism of nicotine to cotinine is a two-step process. The first step is a CYP mediated oxidation of nicotine to nicotine-Δ^{1(5)}-iminium ion (Murphy 1973; Peterson et al. 1987; Obach et al. 1990), and the second step involves the conversion of nicotine-Δ^{1(5)}-iminium
ion to cotinine by aldehyde oxidase I (Brandange et al. 1979; Gorrod et al. 1982). The second step is generally not a rate-limiting step in cotinine formation (Brandange et al. 1979; Obach et al. 1990).

In humans, CYP2A6 is responsible for 90% of the nicotine C-oxidation to cotinine (Nakajima et al. 1996; Messina et al. 1997; Yamazaki et al. 1999). A number of in vitro metabolism studies demonstrated that the rate of nicotine C-oxidation correlated highly with hepatic CYP2A6 protein levels and coumarin 7-hydroxylase activity (a phenotypic marker for CYP2A6 activity) in human liver microsomes (Berkman et al. 1995; Nakajima et al. 1996; Messina et al. 1997). In addition, cDNA expressed CYP2A6 enzyme demonstrates the highest rate of cotinine formation among all CYPs (Nakajima et al. 1996), and CYP2A6 inhibitory antibodies and chemical inhibitors prevent cotinine formation from nicotine (Nakajima et al. 1996; Messina et al. 1997). The essential role of CYP2A6 in nicotine C-oxidation is also supported by in vivo studies. Individuals with two copies of the CYP2A6 deletion allele have very low plasma cotinine levels after nicotine administration (Nakajima et al. 2000; Kwon et al. 2001; Nakajima et al. 2001; Xu et al. 2002; Dempsey et al. 2004; Mwenifumbo et al. 2010), and excrete very small amounts of cotinine or cotinine metabolites in urine (Kitagawa et al. 1999; Yang et al. 2001; Zhang et al. 2002). Methoxsalen, a chemical inhibitor of CYP2A6, can significantly reduce the metabolism of nicotine in vivo (Sellers et al. 2000; Sellers et al. 2003).

In addition to CYP2A6, other CYPs may also play a small role in nicotine C-oxidation. For example, cDNA expressed CYP2B6 can catalyze nicotine C-oxidation, but with an affinity that is 10 times lower than CYP2A6 (i.e. higher Km) (Yamazaki et al. 1999). This observation is also supported by data from human liver bank analysis. No correlation between nicotine C-oxidation activity and CYP2B6 protein levels was observed after adjusting for CYP2A6 protein levels in human livers (Al Koudsi et al. 2010). CYP2A13 can also mediate nicotine C-oxidation in vitro (Bao et al. 2005; Murphy et al. 2005). However, the contribution of CYP2A13 to systemic nicotine C-oxidation is small due to the limited hepatic expression of this enzyme (Koskela et al. 1999; Su et al. 2000).

In humans, 4 to 7% of nicotine is eliminated in urine as nicotine N’-oxide (Benowitz et al. 1994), and the formation of nicotine N’-oxide is thought to be mediated by flavin-containing
monooxygenase 3 (FMO3, Fig. 5) (Cashman et al. 1992; Park et al. 1993). In humans, less than 1% of nicotine is eliminated as nornicotine in urine (Byrd et al. 1992; Benowitz et al. 1994). In human liver microsomes, the rate of nicotine $N$-demethylation is biphasic with contributions from CYP2B6 to the high affinity site and CYP2A6 to the low affinity site (Yamanaka et al. 2005).

In addition to the oxidative pathways, nicotine can also undergo phase II glucuronidation to nicotine $N'$-β-glucuronide (Byrd et al. 1992; Benowitz et al. 1994). In humans, about 3 to 5% of nicotine is eliminated as nicotine $N'$-β-glucuronide in urine (Benowitz et al. 1994). However, this percentage may be higher in individuals with reduced nicotine C-oxidation (Yamanaka et al. 2004). The rates of nicotine and cotinine glucuronidation are highly correlated, suggesting that the same uridine 5'-diphosphoglucuronosyl transferase (UGT) isoform mediates the conjugation of both nicotine and cotinine (Benowitz et al. 1994; Nakajima et al. 2002). Recent evidence suggests that nicotine and cotinine glucuronidation are largely mediated by UGT2B10 (Chen et al. 2007; Kaivosaari et al. 2007; Berg et al. 2010).

Other minor nicotine metabolic pathways include the 2'-hydroxylation of nicotine to produce 4-(methylamino)-1-(3-pyridyl)-1-butanone (Hecht et al. 2000). This pathway is interesting as 4-(methylamino)-1-(3-pyridyl)-1-butanone can be converted to carcinogenic nitrosamines (Hecht et al. 2000). However, the formation of nitrosamines (from nicotine) is not detected in individuals who were treated for an extended period of time with nicotine transdermal patch (Stepanov et al. 2009), suggesting that this may not be a pathway with much clinical relevance.

1.3.2.1.1 Pharmacology of Nicotine Metabolites

Some of nicotine’s primary metabolites have pharmacological activity and some are essentially inactive. Cotinine generally does not have much activity and is unlikely to contribute to nicotine’s pharmacological actions. Administration of high doses of cotinine (10-times higher than those observed in smokers) did not result in any notable physiological changes (i.e. changes in heart rate, blood pressure, and skin temperature) or subjective changes (i.e. increased tension or anxiety) (Benowitz et al. 1983; Hatsukami et al. 1997; Zevin et al. 2000). Cotinine also does not activate nAChRs to evoke dopamine release (Dwoskin et al. 1999). An in vitro study suggested that cotinine can activate the PI3K/AKT pathway and promote lung tumorigenesis by
its anti-apoptotic effects (Nakada et al. 2012). However, the contribution of cotinine to smoking’s carcinogenic effects has not been demonstrated in vivo.

In contrast to cotinine, nornicotine, which is a minor nicotine metabolite (<1%), binds to nAChRs with high affinity and can evoke dopamine release in rat brain slices (Reavill et al. 1988; Green et al. 2001). Rats also self-administer nornicotine (Bardo et al. 1999). In vivo studies suggest that nornicotine can accumulate in rat brain following repeated nicotine exposure due to its long half-life (8 hours) (Kyerematen et al. 1990; Ghosheh et al. 2001; Hukkanen et al. 2005). However, the pharmacological effects of nornicotine in humans have not been demonstrated. Therefore, it is hard to quantify the contribution of nornicotine to nicotine’s pharmacology in humans.

### 1.3.2.2 The Secondary Metabolites of Nicotine

Although the majority of nicotine is metabolized to cotinine, only a small percentage of cotinine is excreted unchanged (Benowitz et al. 1984; Benowitz et al. 1994). In humans, most of the cotinine is metabolized to 3’-hydroxycotinine (3HC). This results in approximately 40 to 50% of absorbed nicotine being excreted as 3HC and its glucuronide in urine (McKennis et al. 1964; Benowitz et al. 1994; Neurath 1994). The formation of 3’-hydroxycotinine from cotinine is catalyzed exclusively by CYP2A6 in humans as demonstrated both in vitro and in vivo. cDNA expressed CYP2A6 has a very high cotinine 3’ hydroxylase activity, and in human liver microsomes cotinine 3’-hydroxylase activity is highly correlated with CYP2A6 protein levels and with coumarin 7’-hydroxylase activity (Nakajima et al. 1996). Both inhibitory antibodies, and chemical inhibitors of CYP2A6, inhibit 3HC formation from cotinine in human liver microsomes (Nakajima et al. 1996) and no 3HC is detected in individuals who are homozygous for the CYP2A6 deletion allele (Dempsey et al. 2004; Mwenifumbo et al. 2010).

**1.3.2.3 Using the 3HC to COT Ratio as A Marker of CYP2A6 Activity**

The ratio of 3’-hydroxycotinine to cotinine (3HC/COT ratio, also known as the nicotine metabolite ratio or NMR) is often used as a non-invasive probe for CYP2A6 activity and nicotine clearance in vivo. Cotinine has a long half-life (16 hours) and plasma cotinine levels show little fluctuation in regular smokers (Lea et al. 2006). 3HC has a much shorter half-life compared to cotinine (5 hours) (Benowitz et al. 1983; Benowitz et al. 2001), and at steady state,
the elimination rate of 3HC is essentially formation-limited. Therefore, the NMR is stable over time and highly reproducible in regular smokers (Lea et al. 2006; St Helen et al. 2012).

As a phenotypic marker, the NMR has high predictive validity and several technical advantages. The NMR is highly predictive of total nicotine clearance \textit{in vivo}, making it useful as a surrogate measure of total nicotine clearance (Dempsey et al. 2004). In contrast to most of the phenotypic probes for enzymes, the NMR can be assessed from a single smoker’s plasma sample without the administration of a probe drug and subsequent assessments of metabolites at a specified time (Levi et al. 2007). The NMR also shows little temporal fluctuation, samples can be taken at any time of the day (Lea et al. 2006; Levi et al. 2007).

1.3.2.4 Non-Genetic Sources of Variation in Nicotine Pharmacokinetics

A number of non-genetic factors can alter nicotine pharmacokinetic in humans (Fig. 6). These factors generally alter nicotine metabolism via either altering CYP2A6 activity or changing hepatic blood flow (Fig. 6). Some common non-genetic factors’ contributing to the variability in nicotine pharmacokinetics will be discussed here.

![Diagram showing factors influencing nicotine metabolism](image)

**Figure 6 | Factors influencing nicotine metabolism in vivo.**
A number of factors, including genetics, gender, pregnancy, age, meals, diet, smoking and xenobiotics can influence nicotine metabolism. These factors generally affect nicotine
metabolism by either influencing hepatic blood flow or CYP2A6 mediated nicotine metabolism. Adapted from Zhu et al., 2012.

To understand the variability in nicotine pharmacokinetics, it is important to note that nicotine is a high extraction drug, in contrast to its primary metabolite cotinine (Benowitz et al. 1983). The intrinsic clearance ($V_{\text{max}}/K_{\text{m}}$) of nicotine is 1.69 µL/min/mg protein in human liver microsomes, which is 10 times faster than the 0.16 µL/min/mg protein of cotinine (Nakajima et al. 1996; Nakajima et al. 1996). For a high extraction drug, such as nicotine, its metabolism is relatively more dependent on hepatic blood flow and less dependent on drug-metabolizing enzyme activity (Hukkanen et al. 2005). In comparison, the metabolism of a low extraction drug, such as cotinine, is highly dependent on drug-metabolizing enzyme activity and less dependent on hepatic blood flow. Therefore, comparing the effects of a factor (i.e. gender or age) on nicotine metabolism relative to cotinine metabolism can help to determine the underlying process that this factor alters. Factors that alter CYP2A6 activity are likely associated with greater alterations in cotinine metabolism than nicotine metabolism. In contrast, factors that alter hepatic blood flow are likely associated with greater alterations in nicotine metabolism than cotinine metabolism.

1.3.2.4.1 Non-Genetic Factors Contribute to Variation in Nicotine Pharmacokinetics by Altering CYP2A6 Activity

The influences of several physiological and environmental factors on CYP2A6 activity and nicotine metabolism will be discussed, with particular focus on the contribution of estrogen to the gender difference in nicotine metabolism and the influence of CYP2A6 inducers and inhibitors on in vivo nicotine metabolism.

1.3.2.4.1.1 Gender

Females, on average, have faster nicotine and cotinine metabolism compared to males (Benowitz et al. 2006). The fact that metabolism of both nicotine and cotinine are higher in females suggests that this increase is due to an alteration in CYP2A6 activity, as alterations in hepatic blood flow would only affect nicotine metabolism but not cotinine metabolism. In support of this hypothesis, females have a higher 3HC/COT ratio (suggesting faster CYP2A6 mediated 3HC formation) compared to males (Johnstone et al. 2006; Ho et al. 2009), and livers from females have significantly higher CYP2A6 mRNA, protein, and activity compared to livers from males (Al Koudsi et al. 2010).
The higher CYP2A6 activity in females is mediated by their comparatively higher estrogen levels. Estrogen can increase the transcription of CYP2A6 in vitro via an estrogen receptor dependent transcriptional up-regulation (Higashi et al. 2007). Estrogen-containing oral contraceptives increase CYP2A6 activity and the clearance of both nicotine and cotinine (Krul et al. 1998; Benowitz et al. 2006), while progesterone-containing contraceptives do not (Benowitz et al. 2006). Furthermore, menopausal or postmenopausal women, who have low estrogen levels, have similar nicotine and cotinine clearance compared to men (Benowitz et al. 2006). Yet the comparatively low nicotine and cotinine clearance was enhanced by estrogen containing hormone replace therapy (Benowitz et al. 2006). Together, these data suggest that the increase in nicotine and cotinine metabolism in females is due to the higher estrogen levels resulting in higher CYP2A6 activity.

1.3.2.4.1.2 Xenobiotics

CYP2A6 activity and nicotine metabolism can also be altered by the exposure to xenobiotics. CYP2A6 activity is induced by a number of prototypical CYP inducers, such as rifampicin, dexamethasone and phenobarbital (Kyerematen et al. 1990; Maurice et al. 1991; Sotaniemi et al. 1995; Mattes et al. 1997; Rae et al. 2001; Madan et al. 2003; Al Koudsi et al. 2010). In particular, rifampicin treatment increases both CYP2A6 mRNA and activity in human primary hepatocytes by 2 to 8 folds (Meunier et al. 2000; Rae et al. 2001; Madan et al. 2003). Interestingly, rifampicin has also been shown to act as an inhibitor of CYP2A6 (Xia et al. 2002), which may explain some of the variability observed in CYP2A6 activity upon rifampicin treatments. Phenobarbital, another prototypical CYP inducer, can also induce CYP2A6 (Madan et al. 2003; Al Koudsi et al. 2010). Liver microsomes from patients who are exposed to phenobarbital exhibit higher levels of CYP2A6 protein than unexposed patients (Cashman et al. 1992). Experiments in primary hepatocytes suggest that phenobarbital treatment can significantly induce nicotine C-oxidation activity in as little as two days (Kyerematen et al. 1990). The induction of nicotine metabolism by xenobiotics such as rifampicin and phenobarbital is mediated by nuclear receptor dependent transcriptional activation of CYP2A6 (Donato et al. 2000; Rae et al. 2001; Maglich et al. 2002). Rifampicin and phenobarbital bind to, and activate, the pregnane X receptor and the constitutive active androstane receptor respectively. These nuclear receptors then translocate to the nucleus where they dimerize with the retinoid X receptor.
α, and bind to the DR4 sites upstream of the CYP2A6 gene to increase its transcription (Itoh et al. 2006).

Xenobiotics can also inhibit CYP2A6 activity and nicotine metabolism. A number of compounds such as methoxsalen, menthol, tryptamine, coumarin, tranylcypromine and selegiline, can inhibit nicotine C-oxidation in vitro with some selectivity for CYP2A6 (Nakajima et al. 1996; Messina et al. 1997; Le Gal et al. 2001; Taavitsainen et al. 2001; Zhang et al. 2001; MacDougall et al. 2003; Siu et al. 2008). Clinically, oral methoxsalen administration inhibits the first pass metabolism of oral nicotine (Sellers et al. 2000), and decreases the clearance of nicotine following subcutaneous nicotine injections (Sellers et al. 2003). Methoxsalen administration can also reduce the number of cigarettes smoked, indicating titration of tobacco consumption to compensate for lower nicotine clearance (Sellers et al. 2003).

1.3.2.4.2 Non-Genetic Factors Contribute to Variation in Nicotine Pharmacokinetics by Altering Hepatic Blood Flow

Variation in hepatic blood flow can also contribute significantly to the variation in nicotine pharmacokinetics. The influence of factors altering hepatic blood flow on nicotine metabolism is discussed in this section with a particular focus on the influence of age and circadian rhythm.

1.3.2.4.2.1 Age and Circadian Rhythm

Age does not alter nicotine metabolism in adolescents and adults (Shimada et al. 1994; Gourlay et al. 1996; Al Koudsi et al. 2010). However, slower nicotine metabolism is observed in neonates and the elderly. The half-life of nicotine is longer in neonates than in adults, but the half-life of cotinine is similar in neonates and adults (Leong et al. 1998; Dempsey et al. 2000; Dempsey et al. 2013). Since cotinine clearance is less dependent on hepatic blood flow compared to nicotine and is unaltered in neonates, the reduction of nicotine metabolism in neonates is more likely explained by a reduction in hepatic blood flow (Gow et al. 2001). This is supported by the observation that hepatic CYP2A6 protein levels are only slightly lower in neonates compared with adults (Tateishi et al. 1997). In adolescents and adults, no correlation between age and CYP2A6 protein level and activity has been observed (Parkinson et al. 2004; Al Koudsi et al. 2010), suggesting a limited impact of age on nicotine metabolism. Furthermore, no differences in steady state nicotine plasma concentrations are observed between different adult age groups.
receiving the same dose of nicotine (Gourlay et al. 1996; Mwenifumbo et al. 2007). In agreement with this, the rate of coumarin metabolism is similar across a wide age range (Shimada et al. 1994; Pasanen et al. 1997), suggesting CYP2A6 activity does not change with age. In the elderly, the clearance of nicotine is slower (Molander et al. 2001), most likely due to a reduction in hepatic blood flow (Hukkanen et al. 2005), since no age-associated reduction in the CYP2A6 protein or \textit{in vitro} nicotine metabolism have been reported (Messina et al. 1997; Al Koudsi et al. 2010).

Circadian variation in nicotine metabolism has also been reported (Gries et al. 1996). Nicotine metabolism increases after a meal as hepatic blood flow increases (Lee et al. 1989; Gries et al. 1996), and decreases during sleep as hepatic blood flow decreases (Hukkanen et al. 2005). Consequently, nicotine clearance can vary by as much as 17% between 7:00 PM and 3:00 AM (Gries et al. 1996).

\subsection*{1.3.3 Renal Clearance of Nicotine}

The renal clearance of nicotine is a relatively minor route of nicotine clearance (\textbf{Fig. 5}); only 8 to 10\% of an intravenously administered nicotine dose is excreted unchanged, whereas the majority of nicotine is metabolized before excretion (Neurath 1994; Hukkanen et al. 2005). Nicotine is excreted from the body by glomerular filtration and tubular secretion. The normal nicotine renal clearance rate is highly dependent on urinary pH, but is normally in the range of 35 to 123 ml/min (Benowitz et al. 1999; Benowitz et al. 2000; Hukkanen et al. 2006). In acidic urine, where nicotine is highly ionized and little tubular reabsorption occurs, nicotine renal clearance rates can be as high as 600 ml/min (Benowitz et al. 1985). In contrast, in basic urine where nicotine is largely unionized and tubular reabsorption is increased, the renal clearance of nicotine can be as low as 17 ml/min (Benowitz et al. 1985). Twin studies suggest a significant genetic contribution to the variation in nicotine renal clearance (Benowitz et al. 2008). However, the molecular mechanism is largely unknown. \textit{In vitro} experiments suggested that variation in the polymorphic organic cation transporter 2 might play a role (Benowitz et al. 2008).
1.4 Markers of Tobacco Consumption

1.4.1 Self-Report Cigarettes Per Day

Self-report number of cigarettes smoked per day (CPD) is the most commonly used tobacco and carcinogen exposure indicator in epidemiological studies. Like many self-report measurements, CPD has significant limitations. CPD exhibits a notable degree of number preference -- smokers are generally biased to report CPD in packs (i.e. half a pack per day or a pack per day)(Klesges et al. 1995). Additionally, CPD does not reflect how much of a cigarette is smoked nor the depth of smoking inhalation or puff volumes. In fact, the levels of nicotine and carcinogen intake per cigarette are inversely related to CPD, which is likely due to the fact that heavier smokers reduce the intensity of smoking/depth of inhalation compared to lighter smokers (Joseph et al. 2005). This makes CPD a particularly weak indicator of tobacco consumption in light smokers as the greatest variation in nicotine intake per cigarette is observed in light smokers (Malaiyandi et al. 2006; Ho et al. 2009; Benowitz et al. 2011). Thus, objective markers of tobacco consumption generally provide better estimations of nicotine intake than CPD.

1.4.2 Cotinine

Cotinine is used as an objective index of tobacco and tobacco-derived carcinogen exposure. The use of cotinine is particularly useful because it overcomes the issue of the wide inter-individual variability in depth of inhalation, and number of puffs per cigarette (Benowitz et al. 2011). A number of studies in heavy smokers have observed significant correlations between plasma cotinine levels and CPD with correlation coefficients ranging from 0.3 to 0.8 (Perez-Stable et al. 1995; Domino et al. 2002; Mustonen et al. 2005; Scherer et al. 2007). A weaker correlation between plasma cotinine levels and CPD is observed in light smokers with only 17% of the variation in plasma cotinine level accounted for by CPD (Ho et al. 2009).

In addition to being a tobacco consumption biomarker, plasma cotinine levels are used extensively to distinguish smokers from non-smokers. Since non-smokers can sometimes be exposed to low levels of nicotine via secondhand smoke and have detectable plasma cotinine levels (Jarvis et al. 1987; 2002; Benowitz et al. 2009), a plasma cotinine cutoff of 14 ng/mL is used to distinguish smokers from non-smokers; this cut off can also be used to verify smoking status in light smokers (Ho et al. 2009).
Despite its popularity, plasma cotinine has some significant limitations as a biomarker of tobacco consumption; the correlation between plasma cotinine levels and nicotine intake differs among different groups of smokers. For example, the findings with plasma cotinine sometimes contradict the findings with other indicators of tobacco exposure in genetic association studies. Caucasian heavy smokers with reduced function \textit{CYP2A6} alleles smoke fewer CPD compared with those without any reduced function \textit{CYP2A6} alleles, reflecting smokers’ tendency to titrate tobacco consumption to obtain a desired level of nicotine in the body (see later sections) (Schoedel et al. 2004; Wassenaar et al. 2011). Yet despite smoking fewer CPD, smokers with reduced function \textit{CYP2A6} alleles have similar plasma cotinine levels as those without any reduced function \textit{CYP2A6} alleles (Strasser et al. 2011). Another example is found in studies of African American light smokers, where similar levels of CPD are reported between those with reduced function \textit{CYP2A6} alleles and those without any reduced function \textit{CYP2A6} allele, but significantly higher plasma cotinine levels are observed in those with reduced function \textit{CYP2A6} alleles (Ho et al. 2009). Furthermore, gene variants at the \textit{CYP2A6} loci, which are associated with decreased lung cancer risk, are paradoxically associated with increased plasma cotinine levels (Timofeeva et al. 2011). Plasma cotinine levels also vary between the sexes. The male participants of the National Health and Nutrition Examination Surveys have significantly higher plasma cotinine levels compared to the female participants even after adjusting for CPD, machine-determined nicotine delivery of cigarettes, race, age, body mass index, poverty status, and the use of either menthol or regular cigarettes (Gan et al. 2008). Furthermore, notable variation in cotinine levels is also observed between races. African American smokers generally have higher plasma cotinine levels compared to Caucasian smokers after adjusting for CPD and the machine-determined nicotine delivery of cigarettes (Wagenknecht et al. 1990), which could be partially due to the slower cotinine clearance in African Americans compared to Caucasians (Perez-Stable et al. 1998). Overall, the observed systemic variation in plasma cotinine levels among \textit{CYP2A6} genotypes, the sexes and races suggests that using plasma cotinine levels as means of assessing tobacco consumption has significant flaws and may result in systemic bias. Chapter 2 will discuss the precise molecular mechanism of this phenomenon.
1.4.3 Total Nicotine Equivalents

Total nicotine equivalents (TNE) is the urinary molar sum of nicotine and 8 of its major metabolites, including nicotine, nicotine glucuronide, cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, nicotine \(N'\) oxide, nornicotine, and cotinine \(N'\) oxide (St Charles et al. 2006)(see Fig. 5). Together, these 9 analytes account for more than 90% of a transdermally administered nicotine dose (Benowitz et al. 1994). A 6 analytes version of TNE (without nicotine \(N'\) oxide, nornicotine, and cotinine \(N'\) oxide) is sometimes used in the literature, and generally correlates highly with the 9 analytes version (Zhu et al. 2013). The creatinine corrected spot urinary TNE is highly predictive of the nicotine dose (Benowitz et al. 2010). The correlation between creatinine corrected TNE and administered nicotine dose is stronger than the correlation observed between plasma cotinine levels and administered nicotine dose (Benowitz et al. 2010). Urinary TNE also has some additional advantages compared to plasma cotinine. Analytically, the urinary concentrations of nicotine metabolites are generally higher (often four to five-fold higher) than that found in plasma, which make analytical detection more sensitive. Most importantly, variability in the metabolism of nicotine and its metabolites has little effect on the validity of TNE as a biomarker of nicotine dose due to its inclusion of all nicotine metabolites (Benowitz et al. 2010). Due to these advantages, we used TNE as a reference biomarker of tobacco consumption in the thesis chapters.

1.4.4 NNAL

In addition to TNE, the urinary levels of NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), which is a reductive metabolite of the highly carcinogenic NNK, is also used as a biomarker of tobacco and tobacco related carcinogen exposure. As discussed in earlier sections, TSNA, particularly NNK, is a class of strong human carcinogens which can induce tumors of the pancreas, nasal cavity, liver and lungs in experimental animals (Hecht 1998). The direct measurement of NNK levels in humans is difficult due to its extensive metabolism to NNAL that results in low NNK levels. Therefore, urinary levels of NNAL and NNAL glucuronide are used as an indicator of NNK and tobacco smoke exposure. Urinary NNAL is a more precise predictor of lung cancer risk than self-report smoking history (Yuan et al. 2009). The NNAL levels are strongly associated with the risk of developing lung cancer in smokers (Church et al. 2009; Yuan et al. 2009). Those smokers who were in the highest tertile of urinary NNAL levels had 9-fold
higher risk of developing lung cancer compared to those in the lowest tertile of urinary NNAL levels, even after adjusting for smoking history and tobacco consumption (Yuan et al. 2009).

As a biomarker, urinary NNAL has a number of desirable characteristics and some limitations. It is highly specific to tobacco exposure and is found only in people who use tobacco or are exposed to secondhand tobacco smoke (Yuan et al. 2009). NNAL also has a long half-life in humans (7-10 days), which makes it a desirable biomarker of tobacco exposure especially in light smokers (Benowitz et al. 2010; Goniewicz et al. 2011; Ter-Minassian et al. 2012). A limitation of NNAL is that variable NNK metabolism may alter NNAL levels without altering tobacco consumption (Ter-Minassian et al. 2012). However, the contribution of variable NNK metabolism to the observed NNAL levels in smokers is still unclear. Urinary NNAL levels will only be used in the thesis chapters to represent exposure to TSNA.

1.5 Genetic Influence of Smoking Behaviors in Heavy Smokers

1.5.1 Heritability of Smoking Behaviors

A large body of evidence, including twin and adoption studies, demonstrates a significant genetic contribution to ad libitum smoking behaviors and the ability to quit smoking. The heritability estimations for the genetic contribution to daily tobacco consumption are usually higher than 50% (Swan et al. 1997; True et al. 1997; Kendler et al. 2000; Vink et al. 2004; Ho et al. 2007), and the heritability of smoking cessation is approximately 50% (Xian et al. 2003). Variations in a number of genes can influence smoking behaviors in heavy smokers. Most of these genes can be classified into either nicotine pharmacodynamic genes or in nicotine’s pharmacokinetic genes.

1.5.2 Variants in Nicotine Pharmacodynamic Genes Alter Tobacco Consumption in Heavy Smokers

Variations in many genes involved in nicotine’s pharmacodynamic pathways are associated with alterations in tobacco consumption and nicotine dependence as assessed primarily in heavy smokers. Candidate gene studies suggested that the genes encoding for the α4 and β2 nAChR subunits, DOPA decarboxylase, GABA receptor subunits, and catechol-O-methyltransferase are significantly associated with tobacco consumption (Feng et al. 2004; Beuten et al. 2005; Li et al. 2005; Ma et al. 2005; Beuten et al. 2006; Lou et al. 2006; Yu et al. 2006). However, most of
these gene loci are not consistently replicated in genome wide association studies in Caucasians, which involve more than 100,000 subjects. This discrepancy is likely due to small sample sizes, small effect sizes and genetic heterogeneity of the original studies (Consortium 2010; Liu et al. 2010; Thorgeirsson et al. 2010).

A number of genome wide association studies have identified significant associations between the chromosome 15q25 CHRNA5-A3-B4 gene cluster (which encodes for α5, α3, and β4 nAChR subunits) and lung cancer risk in Caucasians (Amos et al. 2008; Hung et al. 2008; Thorgeirsson et al. 2008). Subsequent analyses have suggested that this association between CHRNA5-A3-B4 and lung cancer risk is at least partially mediated though altered tobacco consumption (Bierut 2009; Keskitalo et al. 2009; Saccone et al. 2009; Munafo et al. 2012). Genetic variants in CHRNA5-A3-B4 will be used here to illustrate the contribution of nicotine pharmacodynamic genes on smoking behaviors.

1.5.2.1  CHRNA5-A3-B4 and Nicotine Dependence

1.5.2.1.1  CHRNA5-A3-B4 and Tobacco Consumption

A significant association between CHRNA5-A3-B4 gene variants and nicotine dependence (i.e. FTND scores) was first observed by Saccone et al. (Saccone et al. 2007). In this study, the minor allele ‘A’ of rs1051730 (which correlates highly with rs16969968 in Caucasians, $r^2>0.98$) was significantly associated with increased FTND scores (Saccone et al. 2007). This association between rs1051730/rs16969968 and smoking behaviors was subsequently replicated in a genome wide association study, which also demonstrated significant associations between rs1051730/rs16969968 and smoking related diseases (lung cancer, COPD and peripheral arterial disease) (Thorgeirsson et al. 2008; Siedlinski et al. 2011). Since then, rs1051730/rs16969968 has been consistently associated with nicotine dependence and tobacco consumption in Caucasian heavy smokers (Keskitalo et al. 2009; Saccone et al. 2010; Thorgeirsson et al. 2010; Chen et al. 2012). At least three independent loci in CHRNA5-A3-B4 are associated with tobacco consumption in Caucasian heavy smokers. The most commonly implicated locus is rs1051730/rs16969968 (sometimes referred to as ‘Bin A’ or ‘Locus 1’). Each copy of the ‘A’ allele of rs16969968 is associated with an increase of roughly 1 CPD, 24-100 ng/mL in cotinine, and a 1.3 fold higher odds ratio of developing lung cancer (Le Marchand et al. 2008; Keskitalo et
The association between rs1051730/rs16969968 and objective tobacco consumption biomarkers is generally stronger than the association observed between rs1051730/rs16969968 and CPD. For example, rs1051730/rs16969968 accounted for five times greater variance in cotinine levels than in CPD (Keskitalo et al. 2009). In addition to rs1051730/rs16969968, two loci in *CHRNA5-A3-B4* are also independently associated with tobacco consumption in Caucasian smokers. Those include rs588765 and correlated SNPs (sometimes referred to as ‘Bin B’ or ‘Locus 3’), and rs578776 and correlated SNPs (sometimes referred to as ‘Bin C’ or ‘Locus 2’) (Saccone et al. 2010; Chen et al. 2012).

Associations between *CHRNA5-A3-B4* gene variants and other aspects of smoking behaviors, such as smoking initiation and smoking cessation, have also been observed. However, those associations are less consistently replicated compared to the associations observed with tobacco consumption. In terms of smoking initiation, rs1051730/rs16969968 has been associated with a higher risk of being a regular smoker, which is partially attributed to the more positive first smoking experience in individuals with the minor ‘A’ allele (Sherva et al. 2008). However, subsequent research failed to replicate this association between rs1051730/rs16969968 and smoking initiation (Lips et al. 2010; Kaur-Knudsen et al. 2011). This discrepancy may be due to inconsistent definitions of smoking initiation between the studies. A more precise definition of smoking initiation in a prospective cohort design could clarify the association between *CHRNA5-A3-B4* gene variants and smoking initiation.

With regards to smoking cessation, significant associations between *CHRNA5-A3-B4* gene variants and smoking cessation have been reported. However, it is not clear whether the influence of *CHRNA5-A3-B4* gene variants on smoking cessation is independent of the type of pharmacological treatments (i.e. cessation in general, or just a particular pharmacological therapy). One study suggested that the association between *CHRNA5-A3-B4* variants rs16969968 (Bin A) or rs680244 (Bin B) and smoking cessation is primarily observed among smokers treated with the placebo treatment, and is not observed among those who received active pharmacological therapy (Chen et al. 2012). In contrast, another study reported a significant association between *CHRNA5-A3-B4* variants rs16969968 and smoking cessation outcomes in smokers who received nicotine replacement therapy with little effect observed in the placebo arm.
A recent study reported significant associations between *CHRNA5-A3-B4* variants and smoking cessation outcomes in both placebo and nicotine replacement therapy arms but the effects were in opposite directions when compared with the earlier studies (Bergen et al. 2013). Gene variants in *CHRNA5-A3-B4* have also been associated with altered varenicline side effect profiles (King et al. 2012). Some of these discrepancies could be due to variation in clinical trial design (i.e. sample sizes, inclusion/exclusion criteria, statistical adjustments for covariates, the use of behavior counseling and different follow up periods). Therefore, the precise pharmacogenetic impacts of *CHRNA5-A3-B4* gene variants on smoking cessation are not yet clear.

### 1.5.2.1.2 *CHRNA5-A3-B4* and Health Consequences of Smoking

*CHRNA5-A3-B4* gene variants are associated with increased risk for smoking related diseases such as bladder cancer, upper aerodigestive tract cancers (including lung cancer) and COPD (Young et al. 2008; Pillai et al. 2009; Lambrechts et al. 2010; Lips et al. 2010; Kaur-Knudsen et al. 2011). The most robust association between *CHRNA5-A3-B4* gene variants and smoking related diseases is observed with lung cancer. Rs1051730/rs16969968 is associated with lung cancer across a range of histological types (adenocarcinoma, squamous cell carcinoma, large cell and small cell carcinoma) and ethnicities (Caucasians, African Americans and Asians) (Amos et al. 2008; Hung et al. 2008; Sherva et al. 2008; Spitz et al. 2008; Shiraishi et al. 2009; Amos et al. 2010; Lips et al. 2010; Saccone et al. 2010; Truong et al. 2010; Jaworowska et al. 2011; Kaur-Knudsen et al. 2011; Sakoda et al. 2011; Timofeeva et al. 2011). After adjusting for rs1051730/rs16969968, two additional loci in *CHRNA5-A3-B4*, rs588765 (Bin B) and rs578776 (Bin C) are also significantly associated with lung cancer risks in Caucasians (Saccone et al. 2010). Despite the consistent association, it is still not clear whether the association between *CHRNA5-A3-B4* gene variants and lung cancer is (at least partially) mediated directly via altering cell proliferation and survival (Brennan et al. 2011) or exclusively mediated indirectly via altering tobacco consumption (Munafo et al. 2012). Some studies observed associations between *CHRNA5-A3-B4* gene variants and lung cancer even after adjusting for smoking quantity (Kaur-Knudsen et al. 2011; Wassenaar et al. 2011), which suggests that at least some variants may directly alter cell proliferation and survival. Others failed to observe a significant association between *CHRNA5-A3-B4* gene variants and lung cancer in never smokers, which
suggests that these variants alter lung cancer risk by altering tobacco consumption (Girard et al. 2010). Some of these discrepancies may be due to the difficulty in correctly classifying former smokers and never smokers using self-report data. A large lung cancer study of correctly classified never smokers exposed to environmental tobacco smoke and other environmental lung carcinogens may be needed to convincingly demonstrate whether CHRNA5-A3-B4 gene variants directly contribute to lung carcinogenesis.

1.5.2.1.3 CHRNA5-A3-B4 and Body Weight

In addition to its role in modulating tobacco consumption and the health consequences of smoking, CHRNA5-A3-B4 gene variants may also play a role in modulating body weight. Tobacco smoking regulates body weight in smokers due to nicotine’s ability to increase metabolic rate and suppress the compensatory increase in appetite and feeding (Audrain-McGovern et al. 2011). Smokers usually weigh less than nonsmokers and typically gain 4 to 5 kg when they stop smoking (Seeley et al. 2011). Tobacco’s ability to regulate body weight is partially mediated by the activation of α3β4 nicotinic receptors located on the POMC (Pro-opiomelanocortin) neurons in the arcuate nucleus of the hypothalamus by nicotine (Audrain-McGovern et al. 2011; Mineur et al. 2011). Since CHNRA5-A3-B4 encodes for the α3β4 nicotinic receptors, it is possible that CHRNA5-A3-B4 gene variants can modulate nicotine’s ability to regulate body weight by altering α3β4 nicotinic receptor function. A CHRNA5-A3-B4 variant, rs1051730, which alters nicotine intake, is associated with altered body weight (Freathy et al. 2011). Yet it is not clear whether this association is an indirect effect of rs1051730 on altering nicotine intake, or a direct effect of rs1051730 on altering nicotine’s effect on body weight by modulating nicotinic receptor function (Freathy et al. 2011). This topic will be the subject of investigation in Chapter 3.

1.5.3 Variants in Nicotine Pharmacokinetic Genes Alter Tobacco Consumption in Heavy Smokers

1.5.3.1 CYP2A6 and Nicotine Pharmacokinetics

There is a significant heritable (~60%) contribution to nicotine clearance and metabolism (Swan et al. 2005). Genetic variation in the main nicotine-metabolizing enzyme, CYP2A6, contributes extensively to this high heritability as outlined below.
1.5.3.2 CYP2A6 Gene Variants and Nicotine Metabolism

A substantial amount of inter-individual variation is observed in hepatic CYP2A6 mRNA levels, protein levels and *in vitro* catalytic activity (Dempsey et al. 2004; Mwenifumbo et al. 2007; Al Koudsi et al. 2010). The human CYP2A6 gene contains 9 exons and spans 6700 base pairs on chromosome 19 (Hoffman et al. 1995). Currently, 38 numbered and two duplication alleles have been identified ([http://www.cypalleles.ki.se/cyp2a6.htm](http://www.cypalleles.ki.se/cyp2a6.htm)).

CYP2A6 variant alleles with lower activity can be classified into either loss of function alleles or reduced function alleles based on both *in vitro* and *in vivo* metabolism data ([Table 2](#)). CYP2A6 slow metabolizers are defined as individuals with one or more copies of loss of function alleles or two copies of reduced function alleles. Individuals with one copy of a reduced function allele are considered intermediate metabolizers (Schoedel et al. 2004; Mwenifumbo et al. 2008; Ho et al. 2009). In some studies, slow and intermediate metabolizers are grouped together as reduced metabolizers to maximize statistical power. The most profound effects of CYP2A6 genetic variation on nicotine metabolism are observed in individuals with two loss of function CYP2A6 alleles. For example, individuals with two copies of the CYP2A6*4 allele, which is the CYP2A6 whole gene deletion allele (Nunoya et al. 1998; Nunoya et al. 1999; Nunoya et al. 1999; Oscarson et al. 1999; Oscarson et al. 1999), have higher nicotine plasma levels, higher peak nicotine levels and lower oral nicotine clearance compared to those without any CYP2A6*4 alleles following an oral nicotine administration (Xu et al. 2002; Dempsey et al. 2004). Furthermore, CYP2A6*4/*4 individuals excrete no 3HC and only 11 to 15% of the cotinine that wild type individuals excrete after receiving the same amount of nicotine or following a similar amount of smoking (Kitagawa et al. 1999; Yang et al. 2001; Dempsey et al. 2004; Mwenifumbo et al. 2010).

Currently, while many of the variant alleles identified have a substantial impact on nicotine metabolism, only a small percentage of the inter-individual variation in nicotine metabolism can be explained by characterized CYP2A6 variants, and there is significant variability in nicotine metabolism among people without known CYP2A6 variants (Dempsey et al. 2004). Thus, the phenotypic marker, NMR, is useful as it can account for the known and unknown genetic variants, as well as the environmental influences on CYP2A6 activity *in vivo*. 
Table 2 | *CYP2A6* alleles, their allele frequencies and *in vitro* and *in vivo* impacts on nicotine clearance.

↑=Increased; ↓=Decreased.

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Allele Frequencies (%)</th>
<th>Allele description</th>
<th>In vitro activity</th>
<th>In vivo grouping</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYP2A6</em>1A</td>
<td>29-35 13 - 20 57 48-55 43-51</td>
<td>The reference allele</td>
<td></td>
<td></td>
<td>(Yamano et al. 1990; Oscarson et al. 1999; Gambier et al. 2005; Gyamfi et al. 2005; Nakajima et al. 2006; Nurfadhlina et al. 2006; Wang et al. 2006; Mwenifumbo et al. 2008)</td>
</tr>
<tr>
<td><em>CYP2A6</em>1B</td>
<td>25 18 9 0.5-1</td>
<td>Higher transcription by mRNA stabilization. Associated with faster nicotine clearance <em>in vivo</em>.</td>
<td>↑</td>
<td></td>
<td>(Yamano et al. 1990; Oscarson et al. 1999; Gambier et al. 2005; Gyamfi et al. 2005; Nakajima et al. 2006; Nurfadhlina et al. 2006; Wang et al. 2006; Mwenifumbo et al. 2008)</td>
</tr>
<tr>
<td><em>CYP2A6</em>1D</td>
<td>17 11 9 0.5-1</td>
<td>50% lower transcription <em>in vitro</em>. Unclear impact <em>in vivo</em>.</td>
<td>↓</td>
<td></td>
<td>(Pitarque et al. 2004; von Richter et al. 2004; Nakajima et al. 2006)</td>
</tr>
<tr>
<td><em>CYP2A6</em>2</td>
<td>1-4 0.3-1 0 0</td>
<td>Unstable, fails to incorporate heme and inactive protein <em>in vitro</em>. Inactive <em>in vivo</em>.</td>
<td>None</td>
<td>Loss</td>
<td>(Yamano et al. 1990; Benowitz et al. 1995; Oscarson et al. 1999; Benowitz et al. 2001; Schoedel et al. 2004)</td>
</tr>
<tr>
<td><em>CYP2A6</em>5</td>
<td>0-0.3 0 0.5 2 0.5-1</td>
<td>Unstable and inactive enzyme <em>in vitro</em>. Unclear impact <em>in vivo</em>.</td>
<td>None</td>
<td></td>
<td>(Oscarson et al. 1999; Schoedel et al. 2004; Gyamfi et al. 2005; Huang et al. 2005; Nakajima et al. 2006; Nurfadhlina et al. 2006)</td>
</tr>
<tr>
<td><em>CYP2A6</em>7</td>
<td>0 0 11 10-13 6-10</td>
<td>Unstable enzyme <em>in vitro</em>. Inactive towards nicotine <em>in vivo</em>.</td>
<td>↓</td>
<td>Loss</td>
<td>(Ariyoshi et al. 2001; Xu et al. 2002; Yoshida et al. 2002; Schoedel et al. 2004; Gambier et al. 2005; Gyamfi et al. 2005; Minematsu et al. 2006; Nakajima et al. 2006; Nurfadhlina et al. 2006)</td>
</tr>
<tr>
<td><em>CYP2A6</em>8</td>
<td>0 0 0-2 0-1 0-1</td>
<td>Unclear <em>in vivo</em> impact.</td>
<td></td>
<td></td>
<td>(Xu et al. 2002; Yoshida et al. 2002; Gyamfi et al. 2005; Mwenifumbo et al. 2005; Nakajima et al. 2006; Nurfadhlina et al. 2006)</td>
</tr>
<tr>
<td><em>CYP2A6</em>9</td>
<td>5-8 7-9 16 19-20 16</td>
<td>Lower transcription <em>in vitro</em>. Reduced activity <em>in vivo</em>.</td>
<td>↓</td>
<td>*1/*9:Reduced *9/*9:Loss</td>
<td>(Yoshida et al. 2002; Pitarque et al. 2004; Schoedel et al. 2004; Benowitz et al. 2006; Malayandi et al. 2006; Minematsu et al. 2006; Nakajima et al. 2006)</td>
</tr>
<tr>
<td><em>CYP2A6</em>10</td>
<td>0 0 1-4 2-3 2-4</td>
<td>Inactive <em>in vivo</em>.</td>
<td></td>
<td>Loss</td>
<td>(Xu et al. 2002; Yoshida et al. 2002; Gyamfi et al. 2005; Mwenifumbo et</td>
</tr>
<tr>
<td>CYP2A6*11</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.5</td>
<td>Lower activity <em>in vitro</em>. Unclear impact <em>in vivo</em>.</td>
</tr>
<tr>
<td>----------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>CYP2A6*12</td>
<td>0-3</td>
<td>0-0.4</td>
<td>0</td>
<td>0-1</td>
<td>0</td>
</tr>
<tr>
<td>CYP2A6*13</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>1</td>
<td>Likely to exhibit reduced activity as it has the same -48T&gt;G SNP as CYP2A9*9.</td>
</tr>
<tr>
<td>CYP2A6*14</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Unclear impact <em>in vivo</em>. Likely to exhibit normal activity.</td>
</tr>
<tr>
<td>CYP2A6*15</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>Likely to exhibit reduced activity as it has the same -48T&gt;G SNP as CYP2A9*9.</td>
</tr>
<tr>
<td>CYP2A6*16</td>
<td>0.3</td>
<td>0-2</td>
<td>0</td>
<td>0</td>
<td>Unclear impact <em>in vivo</em>.</td>
</tr>
<tr>
<td>CYP2A6*17</td>
<td>0</td>
<td>7-11</td>
<td>0</td>
<td>0</td>
<td>Lower activity <em>in vitro</em>. Inactive <em>in vivo</em>.</td>
</tr>
<tr>
<td>CYP2A6*18</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>Similar nicotine C-oxidation activity as the wild type <em>in vitro</em>.</td>
</tr>
<tr>
<td>CYP2A6*19</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>Lower nicotine C-oxidation activity <em>in vitro</em>. Likely to exhibit reduced activity as it has the same 6558T&gt;C SNP as CYP2A6<em>7 and CYP2A6</em>10.</td>
</tr>
<tr>
<td>CYP2A6*20</td>
<td>0</td>
<td>1-2</td>
<td>0</td>
<td>0</td>
<td>Truncated protein <em>in vitro</em>. Inactive <em>in vivo</em>.</td>
</tr>
<tr>
<td>CYP2A6*21</td>
<td>0.5-2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Similar activity toward nicotine <em>in vivo</em>.</td>
</tr>
<tr>
<td>CYP2A6*22</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Likely to exhibit reduced activity <em>in vivo</em> as it alters the same amino acid as CYP2A9*7.</td>
</tr>
<tr>
<td>Genotype</td>
<td>Frequency</td>
<td>Activity</td>
<td>Impact</td>
<td>Source(s)</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>----------</td>
<td>--------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*24</td>
<td>1</td>
<td>Similar nicotine C-oxidation activity as the wild type in vitro.</td>
<td></td>
<td>(Mwenifumbo et al. 2008; Ho et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*25</td>
<td>0.5-1</td>
<td>Similar nicotine C-oxidation activity as the wild type in vitro. Inactive in vivo.</td>
<td>Loss</td>
<td>(Mwenifumbo et al. 2008; Ho et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*26</td>
<td>0.7</td>
<td>No detectable protein in vitro. Associated with greatly reduced activity in vivo.</td>
<td>↓ Loss</td>
<td>(Mwenifumbo et al. 2008; Ho et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*27</td>
<td>0.7</td>
<td>No detectable protein in vitro. Inactive in vivo.</td>
<td>↓ Loss</td>
<td>(Mwenifumbo et al. 2008; Ho et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*28</td>
<td>1-2</td>
<td>Similar nicotine C-oxidation activity as the wild type in vitro. Inconsistent in vivo impact.</td>
<td></td>
<td>(Mwenifumbo et al. 2008; Ho et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*31</td>
<td>1</td>
<td>Associated with reduced activity</td>
<td>Loss</td>
<td>Unpublished observation</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*34</td>
<td></td>
<td>Likely exhibits lower CYP2A6 activity</td>
<td>Reduced? /Loss?</td>
<td>Unpublished observation</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*38</td>
<td>0.5</td>
<td>Unclear impact in vivo</td>
<td></td>
<td>(Bloom et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1X</td>
<td>0-1</td>
<td>Gene duplication. Unclear impact in vivo.</td>
<td></td>
<td>(Rao et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1X</td>
<td>0</td>
<td>Gene duplication. Unclear impact in vivo.</td>
<td></td>
<td>(Rao et al. 2000; Fukami et al. 2007)</td>
<td></td>
</tr>
</tbody>
</table>
1.5.3.3 **CYP2A6 and Interethnic Variability in Nicotine Metabolism**

There is substantial interethnic variation in nicotine metabolism, which can be attributed to the genetic differences observed in CYP2A6. The frequency of CYP2A6 alleles varies substantially between ethnicities; loss of/reduced function CYP2A6 alleles are more prevalent in Asians, followed by African Americans, and then Caucasians (Malaiyandi et al. 2005; Mwenifumbo et al. 2007). The combined frequency of individuals with at least one copy of loss of/reduced function CYP2A6 alleles is 55%, 47% and 23% for Asians, African Americans and Caucasians respectively (Nakajima et al. 2006; Ho et al. 2009; Mwenifumbo et al. 2009; Lerman et al. 2010). The higher frequency of loss of/reduced function CYP2A6 alleles contributes to the lower CYP2A6 protein levels and activity in Asian liver microsomes compared to Caucasian liver microsomes (Shimada et al. 1996). These genetic findings are supported by the interethnic variation observed in *in vivo* nicotine pharmacokinetics. Asian Americans and African Americans metabolize nicotine and cotinine more slowly than Caucasian Americans (Benowitz et al. 1999; Benowitz et al. 2002). The interethnic variation in CYP2A6 may explain some of the differences in tobacco consumption and smoking related mortalities observed between different ethnicities (Benowitz et al. 2002).

1.5.3.4 **CYP2A6 and Tobacco Consumption in Heavy Smokers**

Tobacco consumption is closely regulated by CYP2A6 mediated nicotine clearance. Dependent smokers maintain a desired nicotine level in the body and cigarette craving is negatively associated with plasma nicotine concentration (Jarvik et al. 2000). Therefore, smokers with reduced CYP2A6 function tend to consume less tobacco to maintain desirable levels of nicotine in the body. Consistent with this, CYP2A6 slow metabolizers smoke fewer CPD, are less likely to smoke within 5 min after waking, have reduced inhalation depth, and are less nicotine dependent compared to CYP2A6 normal metabolizers (Pianezza et al. 1998; Gu et al. 2000; Schoedel et al. 2004; Malaiyandi et al. 2006; Strasser et al. 2007). On the other hand, individuals with faster CYP2A6 activity, such as those with duplication alleles or the gain of function CYP2A6*1B, have higher tobacco consumption, again suggesting that smokers titrate tobacco consumption according to their CYP2A6 activity and resulting nicotine clearance (Rao et al. 2000; Nakajima et al. 2001; Tyndale et al. 2002). Together, these data suggest that the rate of nicotine clearance could have a profound influence on smoking behaviors.
1.5.3.5  **CYP2A6 and Smoking Cessation Outcomes in Heavy Smokers**

Genetic variants in *CYP2A6* can significantly alter smokers’ ability to quit smoking and their response to smoking cessation pharmacotherapies. For example, slower nicotine metabolism (by NMR or by *CYP2A6* genotype) is associated with higher smoking cessation rates in placebo-treated individuals (i.e. without the aid of a pharmacotherapy)(Patterson et al. 2008; Ho et al. 2009). This is consistent with *CYP2A6* slow metabolizers’ higher likelihood of being former smokers compared with current smokers (Gu et al. 2000; Iwahashi et al. 2004; Schoedel et al. 2004). Furthermore, individuals with low NMR (indicative of slow CYP2A6 activity), or *CYP2A6* slow metabolizers by genotype, have significantly better smoking cessation rates on nicotine patch compared to individuals with normal CYP2A6 activity (Lerman et al. 2006; Malaiyandi et al. 2006; Lerman et al. 2010). This is likely due to the higher nicotine plasma concentrations in the *CYP2A6* slow metabolizers as well as the slow metabolizers’ higher unaided smoking cessation rates. Interestingly, no difference in quit rates was observed between *CYP2A6* slow metabolizers and normal metabolizers receiving nicotine nasal spray treatment (Lerman et al. 2006; Malaiyandi et al. 2006). This may be due to the fact that the subjects receiving nicotine nasal spray altered the amount of spray usage to titrate their plasma nicotine levels according to their different rates of nicotine metabolism (i.e. the *CYP2A6* slow metabolizers used less nicotine spray) (Malaiyandi et al. 2006). Moreover, no associations were found between CYP2A6 activity and the clinical response to bupropion (not a CYP2A6 substrate) therapy (Patterson et al. 2008). Together, these data strongly suggest that the rate of nicotine C-oxidation can have significant effects on smoking cessation outcomes.

1.5.3.6  **CYP2A6 and Health Consequences of Smoking**

Besides altering smoking behaviors, reduced CYP2A6 activity is associated with decreased risk for smoking related diseases. Specifically, reduced function *CYP2A6* gene variants have been associated with lower risk of developing lung cancer and COPD in smokers (Timofeeva et al. 2011; Wassenaar et al. 2011). The association between reduced function *CYP2A6* gene variants and decreased lung cancer risk is mediated by both reduced tobacco consumption and lower carcinogen metabolism. Reduced CYP2A6 mediated nicotine metabolism results in lower CPD and consequently lower carcinogen exposure (Schoedel et al. 2004; Strasser et al. 2011; Wassenaar et al. 2011). Additionally, the procarcinogenic TSNAs in tobacco require metabolic
activation by CYPs to form their reactive metabolites, which are then spontaneously converted to diazonium ions and react with DNA. The metabolic activation of NNN is mediated by CYP2A6 (Kushida et al. 2000; Wong et al. 2005). Therefore, individuals with reduced CYP2A6 activity have less activation of NNN as will be discussed in Chapter 1. CYP2A6 slow metabolizers have a decreased risk of lung cancer even after adjusting for the amount of cigarettes smoked (Fujieda et al. 2004; Wassenaar et al. 2011). Thus, reduced CYP2A6 activity decreases lung cancer risk by both decreasing tobacco consumption and TSNA activation.

1.6 Light Smokers

1.6.1 Prevalence

Historically, smoking and tobacco research has focused on heavy smokers. The United States National tobacco survey did not contain any questions about light smokers and non-daily smokers until 1992 (Shiffman 2009). Over the past 30 years, although the overall smoking prevalence has decreased, an increased proportion of smokers have started to smoke fewer CPD (Centers for Disease Control and Prevention 2008). The average CPD smoked by adult smokers in the United States decreased from 26 in 1989 to 16 in 2009 (Filion et al. 2012). The majority of the reduction occurred before the year 2000 with little change observed since 2000 (Duval et al. 2008). In the United States, about 30% of current smokers are light smokers who smoke less than 10 CPD (Kandel et al. 2000; Trinidad et al. 2009). Similar trends are reported in the Canadian population; the average tobacco consumption among Canadian smokers is 14.4 with nearly 40% light smokers (Health Canada 2011).

The prevalence of light smoking can vary widely by sex, age and ethnicity. Light smoking is particularly prevalent in young people, educated people, females and ethnic minorities (Hassmiller et al. 2003; Wortley et al. 2003). Although the percentage of light smokers increased among both sexes from 2001 to 2011, this increase is more pronounced in females. In fact, around 62.6% of adult Canadian female smokers are light smokers (Health Canada 2007; Health Canada 2008; Health Canada 2011). Variation in the prevalence of light smoking is also observed between ethnic populations. As much as 67% of African-Americans, 72% of Asian Americans, and 76% of Hispanic Americans are light smokers compared to the 40% in Non-Hispanic Whites (Trinidad et al. 2009). Light smoking is also highly prevalent globally. The
mean CPD in Indian male smokers is 6.1, and as much as two-thirds of the smokers in developing countries are light smokers (Giovino et al. 2012).

1.6.2 Health Consequences

Although the risks of many smoking related diseases are dose dependent (Garfinkel et al. 1988; Jimenez-Ruiz et al. 1998; Mucha et al. 2006), it is generally believed that there is no safe level of smoking. Women who smoke less than 10 cigarettes per day had a two times higher overall mortality rate compared to non-smokers (Pirie et al. 2013). Light smoking has been associated with a 20 times greater risk of developing COPD, a 15.5 times greater risk of developing lung cancer (see Fig. 7) (Thun et al. 1997), a 3 times greater risk of death from ischemic heart disease, a 2.1 times greater risk for myocardial infarction, as well as higher risks of developing gastrointestinal cancers compared to never smokers (Jimenez-Ruiz et al. 1998; Prescott et al. 2002; Bjartveit et al. 2005; Schane et al. 2010).

![Figure 7](image_url)  
**Figure 7** | Relative risks of lung cancer and chronic obstructive pulmonary disease in light smokers (<10 CPD) in comparison with heavy smokers.  
This figure was plotted using data Published by Thun et al., 2013.
1.6.3  Smoking Characteristics

Light smokers have distinct smoking characteristics compared with moderate to heavy smokers (Shiffman 1989; Shiffman et al. 1995). Light smokers do not exhibit the characteristic features of tobacco/nicotine dependence observed in heavy smokers and they generally score very low on the FTND scale (which is designed for heavy smokers) (Shiffman 1989; Shiffman et al. 1995; Okuyemi et al. 2002; Ho et al. 2009). In addition, light smokers tend to smoke their first cigarette later in the morning than heavy smokers, and generally do not smoke when ill or in places where smoking is prohibited (Shiffman 1989; Shiffman et al. 2006). They also do not report many withdrawal symptoms during smoking abstinence, suggesting nicotine withdrawal plays a relatively limited role in determining the smoking patterns of light smokers (Shiffman 1989; Shiffman et al. 2006). Heavy smokers smoke at regular intervals to maintain sufficient nicotine levels in the body and to avoid nicotine withdrawal. The low frequency of smoking in light smokers makes it difficult to maintain sufficient plasma nicotine levels to avoid nicotine withdrawal (Shiffman et al. 1992). It is possible that light smokers are either more sensitive to nicotine or clear nicotine more slowly than heavy smokers. However, this is not supported by clinical evidence. Light smokers generally show similar cardiovascular responses to cigarettes, and appear to eliminate nicotine at similar rates compared to heavy smokers (Shiffman et al. 1990; Brauer et al. 1996), suggesting that nicotine’s pharmacokinetics and pharmacodynamics are not significantly different in light smokers. Overall, it appears that, unlike heavy smokers, light smokers do not maintain stable plasma nicotine levels. This characteristic makes light smokers distinct from heavy smokers and a novel population in the study of the role of genetics in smoking behaviors.

1.6.4  Smoking Cessation

Despite the prevalence and health consequences associated with light smoking, there is little data on the efficacy of smoking cessation interventions for this group since most clinical trials exclude light smokers. Light smokers are more likely to perceive quitting as not very difficult compared to heavy smokers (Owen et al. 1995), and less likely to receive professional help from physicians to stop smoking (Kotz et al. 2013). Yet smoking cessation can be very difficult in light smokers, possibly more difficult than heavy smokers. Light smokers usually report repeated unsuccessful quit attempts, and the unaided smoking cessation rate is lower in light smokers.
compared to heavy smokers at 6 month (Ahluwalia et al. 2002; Ahluwalia et al. 2006). This could be due, at least in part, to the lower perception of risk associated with light smoking (Owen et al. 1995). Furthermore, a number of clinical trials also suggested that light smokers do not respond to smoking cessation pharmacotherapies, including nicotine replacement therapy and bupropion, as well as heavy smokers do (Ahluwalia et al. 2002; Ahluwalia et al. 2006; Cox et al. 2012; de Dios et al. 2012). This could be due to light smokers’ low adherence to smoking cessation medications (manuscript attached in Appendix B (Zhu et al. 2012)). Together, these data suggest that there is a pressing need for more efficacious smoking cessation treatments in light smokers. A better understanding of the mechanisms behind light smoking behaviors may lead to more efficacious smoking cessation strategies.

### 1.7 Smokeless Tobacco

#### 1.7.1 Prevalence

In the United States, about 7% of men and 0.3% of women are regular smokeless tobacco users (Hatsukami et al. 2004; Minematsu et al. 2006; Phillips et al. 2009; Centers for Disease Control and Prevention 2010). In Canada, 8% of Canadian adults have tried smokeless tobacco products, but the prevalence of regular smokeless tobacco use is under 1% (Health Canada 2007; Health Canada 2008; Health Canada 2011). Like smoking, there are substantial gender, ethnic and socioeconomic differences in the prevalence of smokeless tobacco use. Particularly, smokeless tobacco use is more prevalent in males, young non-Hispanic Whites, those with less than high school education, those living in the southern United States, and those living in rural areas (Tomar 2003).

#### 1.7.2 Health Consequences

Although generally perceived as a safer alternative to smoking due to the lack of combustion, smokeless tobacco can cause serious health consequences. Smokeless tobacco in North America contains more than 30 carcinogens (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2007; Boffetta et al. 2008). Among these carcinogens, TSNA levels are higher than those found in cigarette products (Benowitz et al. 2012). Clinically, smokeless tobacco users have a higher risk of developing oral and lung cancers compared to people who do not use any tobacco products (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans
In a study that compared the risk of developing lung and oral cancers between smokers who had switched to North American brands of smokeless tobacco and smokers who had quit smoking completely, the relative risks of lung cancer and oral cancer were 1.5 and 2.6 times higher in the smokeless tobacco group respectively (Henley et al. 2007).

Smokeless tobacco is sometimes marketed as a harm reduction method for cigarette smokers who can not quit smoking (Hatsukami et al. 2004). This is largely supported by data from Sweden, where a significant reduction in tobacco related morbidity and mortality has been attributed to the high popularity of the low TSNA oral snuffs (Henley et al. 2007). However, it is important to note that the North American brands of smokeless tobacco are cured differently, resulting in a much higher nitrosamine content. Therefore, the utility of using smokeless tobacco as a harm reduction method in North American smokers is questionable.

### 1.8 Alaska Native Tobacco Users: Light Smoking and Smokeless Tobacco Use

This thesis focuses on Alaska Native tobacco users as an example population of light smokers and smokeless tobacco users. Tobacco use is prevalent in Alaska Native peoples. In fact, American Indians and Alaska Natives reported the highest rate of tobacco use of any ethnic groups in the United States (United States Public Health Service. Office of the Surgeon General. 1998; Behavioral Risk Factor Surveillance System 2008). The prevalence of smoking in Alaska Native individuals is 43.5% compared to the United States National average of 20%. The prevalence of light smoking is also high (85%) in Alaska Native smokers (Trinidad et al. 2009; Renner et al. 2013). Alaska Native light smokers share many characteristics compared to other populations of light smokers (e.g. young, lower incomes and without high school diploma) (Smith et al. 2010). In addition to light smoking, Alaska Native individuals, especially women, also exhibit a high prevalence of smokeless tobacco use in the forms of commercial chewing tobacco and iqmik (which is a homemade smokeless tobacco product containing fire-cured tobacco leaves with ash generated from burning the fungus)(Renner et al. 2005). A significant portion of Alaska Native individuals believe that the health risks associated with iqmik are lower compared with smoking and commercial chewing tobacco (Renner et al. 2005).
Due to the high prevalence of light smoking and smokeless tobacco use, and the similar smoking characteristics compared to other populations of light smokers, Alaska Native tobacco users serve as a useful representative population for studying the genetic influence on the smoking behaviors in light smokers and smokeless tobacco users.
1.9 STATEMENT OF RESEARCH HYPOTHESES

In Chapter 1 “Alaska Native smokers and smokeless tobacco users with slower CYP2A6 activity have lower tobacco consumption, lower tobacco-specific nitrosamine exposure and lower tobacco-specific nitrosamine bioactivation”. We hypothesize that light smokers and smokeless tobacco users with lower CYP2A6 activity will have lower tobacco consumption as measured by urinary TNE. We also hypothesize that the tobacco users with lower CYP2A6 activity will have lower urinary NNAL levels, reflecting lower tobacco consumption, and higher urinary NNN levels, reflecting reduced NNN metabolic activation.

In Chapter 2 “The ability of plasma cotinine to predict nicotine and carcinogen exposure is altered by differences in CYP2A6: the influence of genetics, race, and sex”. We hypothesize that lower CYP2A6 activity will decrease cotinine clearance and alter the quantitative relationship between plasma cotinine with nicotine or tobacco-derived carcinogen exposure in smokers.

In Chapter 3 “CHRNA5-A3-B4 genetic variants alter nicotine intake and interact with tobacco use to influence body weight in Alaska Native tobacco users”. We hypothesize that (1) genetic variants in CHRNA5-A3-B4 alter tobacco consumption and tobacco-derived carcinogen exposure in Alaska Native light smokers as seen in heavy smokers; (2) CHRNA5-A3-B4 gene variants are associated with altered body weight, even after controlling for nicotine intake.
2 CHAPTER 1: ALASKA NATIVE SMOKERS AND SMOKELESS TOBACCO USERS WITH SLOWER CYP2A6 ACTIVITY HAVE LOWER TOBACCO CONSUMPTION, LOWER TOBACCO-SPECIFIC NITROSAMINE EXPOSURE AND LOWER TOBACCO-SPECIFIC NITROSAMINE BIOACTIVATION


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DKH, APL, CCR, NLB and RFT designed the overall study. APL and CCR recruited the participants. NLB determined the levels of nicotine, cotinine and trans-3-hydroxycotinine in plasma. IS determined the levels of NNN. CSS and CHW determined the levels of urinary nicotine metabolites. AZZ performed the DNA extraction from blood and plated the DNA for genotyping. MJB and AZZ conducted the genotyping. AZZ merged and maintained a study database and performed all analyses and modeling. AZZ and RFT wrote the manuscript.
2.1 Abstract

Nicotine, the psychoactive ingredient in tobacco, is metabolically inactivated by CYP2A6 to cotinine. CYP2A6 also activates procarcinogenic tobacco-specific nitrosamines (TSNA). Genetic variation in CYP2A6 is known to alter smoking quantity and lung cancer risk in heavy smokers. Our objective was to investigate how CYP2A6 activity influences tobacco consumption and procarcinogen levels in light smokers and smokeless tobacco users. Cigarette smokers (n=141), commercial smokeless tobacco users (n=73), and iqmik users (n=20) were recruited in a cross-sectional study of Alaska Native peoples. The participants’ CYP2A6 activity was measured by both endophenotype and genotype, and their tobacco and procarcinogen exposure biomarker levels were also measured. Smokers, smokeless tobacco users and iqmik users with lower CYP2A6 activity had significantly lower urinary total nicotine equivalents (TNE) and NNAL levels (a biomarker of TSNA exposure). Levels of NNN, a TSNA metabolically bioactivated by CYP2A6, were higher in smokers with lower CYP2A6 activities. Light smokers and smokeless tobacco users with lower CYP2A6 activity reduce their tobacco consumption in ways (e.g. inhaling less deeply) that are not reflected by self-report indicators. Tobacco users with lower CYP2A6 activity are exposed to lower procarcinogen levels (lower NNAL levels) and have lower procarcinogen bioactivation (as indicated by the higher urinary NNN levels suggesting reduced clearance), which is consistent with a lower risk of developing smoking related cancers. This study demonstrates the importance of CYP2A6 in the regulation of tobacco consumption behaviors, procarcinogen exposure and metabolism in both light smokers and smokeless tobacco users.
2.2 Introduction

Smoking is the largest preventable cause of lung cancer. American Indians and Alaska Native people have the highest prevalence of tobacco usage among all ethnic groups in the U.S.A. (Behavioral Risk Factor Surveillance System 2008). On average Alaska Native smokers consume fewer cigarettes per day (CPD) compared to Caucasians (United States Public Health Service. Office of the Surgeon General. 1998), yet the incidence of lung cancer among Alaska Native people is higher than the U.S. national average (Centers for Disease Control and Prevention 2003; Centers for Disease Control and Prevention 2010). In addition to smoking, a significant proportion of Alaska Native people use commercial smokeless tobacco products and iqmik. The latter is a homemade smokeless tobacco product containing alkaline ash (Renner et al. 2005). We investigated whether genetic variation in \textit{CYP2A6}, a nicotine and tobacco specific nitrosoamine metabolizing enzyme, alters tobacco consumption and procarcinogen levels which could influence the risk of developing lung cancer in this population.

Nicotine is the main psychoactive ingredient in tobacco. Genetic variants influencing the pharmacokinetics and pharmacodynamics of nicotine are associated with altered tobacco consumption and lung cancer risk (Thorgeirsson et al. 2010; Wassenaar et al. 2011). In humans, the majority of nicotine is metabolized to cotinine (COT) by \textit{CYP2A6} (Nakajima et al. 1996). COT is then metabolized to \textit{trans}-3’-hydroxycotinine (3HC) exclusively by \textit{CYP2A6} (Nakajima et al. 1996). The human \textit{CYP2A6} gene is highly polymorphic: about 25% of Caucasians, 50% of African Americans, and 60% of Asians have at least one copy of a reduced function \textit{CYP2A6} allele (Mwenifumbo et al. 2007; Ho et al. 2009; Lerman et al. 2010). \textit{CYP2A6} genotype significantly alters nicotine clearance, and has been associated with altered tobacco consumption and tobacco related lung cancer risk (Benowitz et al. 2006; Wassenaar et al. 2011). In addition to \textit{CYP2A6} genotype, the ratio of 3HC to COT (also known as the nicotine metabolite ratio, NMR) is an \textit{in vivo} endophenotype of \textit{CYP2A6} activity (Dempsey et al. 2004). The NMR is stable throughout the day and correlates highly with \textit{in vivo} nicotine clearance (Dempsey et al. 2004; Lea et al. 2006). Caucasian smokers who have one or more reduced function \textit{CYP2A6} allele(s) (i.e. \textit{CYP2A6} reduced metabolizers, RM), or have lower plasma NMR, generally smoke fewer CPD and are less nicotine dependent compared to genotypical normal metabolizers (NM) or smokers with higher plasma NMR (Schoedel et al. 2004; Wassenaar et al. 2011). However, such
differences in CPD and nicotine dependence scores by CYP2A6 genotype or NMR are not observed in some light smoking populations, such as African Americans (Ho et al. 2009). This may be due to the limited sensitivity of tobacco consumption indicators like CPD and carbon monoxide in these light smokers (Mwenifumbo et al. 2007; Ho et al. 2009). The influence of CYP2A6 genetic variation on smokeless tobacco use has never been investigated.

CPD, carbon monoxide levels and plasma COT are widely used indicators of nicotine and tobacco consumption in smokers, however they have significant limitations. For example, CPD is subject to reporting bias and does not account for the inter-individual differences in the depth of smoke inhalation or other smoking topography measures. This is particularly relevant in light smokers as the level of nicotine intake per cigarette is inversely related to reported CPD (Benowitz et al. 2011). Carbon monoxide has a short half-life, which limits its utility in sporadic/light smokers (Ho et al. 2009), and it is not an indicator of smokeless tobacco use. Plasma COT is specific to nicotine exposure, has a relatively long half-life, and in heavy smokers correlates with tobacco consumption (Benowitz et al. 2011). However, since COT is both formed and removed at different rates by CYP2A6, reduced function CYP2A6 variants may alter COT clearance more substantially than nicotine clearance, thus limiting the utility of COT as a biomarker of nicotine exposure. Recently urinary TNE, which represents the summation of urinary nicotine and its metabolites (nicotine, nicotine glucuronide, COT, COT glucuronide, 3HC, 3HC glucuronide, nicotine-\(N\)-oxide, cotinine-\(N\)-oxide, and nornicotine), has been used as an alternative biomarker of nicotine consumption (Scherer et al. 2007; Le Marchand et al. 2008). TNE accounts for about 90% of a transdermally administered nicotine dose (Benowitz et al. 1994), and creatinine-adjusted (e.g. per mg Cre) spot urinary TNE correlates strongly with daily nicotine consumption (Benowitz et al. 2010). The advantage of urinary TNE is that metabolites from non-CYP2A6 enzymatic pathways are also accounted for, such that TNE is not influenced by the rate of metabolism via CYP2A6. In this study, urinary TNE was used as the primary biomarker of nicotine consumption to evaluate the influence of CYP2A6 activity on nicotine consumption in smokers and smokeless tobacco users.

Tobacco use is associated with an elevated risk of developing cancer (Hecht 2003). Nicotine itself is not carcinogenic, but the structurally related tobacco-specific nitrosamines (TSNA) such as 4-(methylnitrosamino)-1-(3)pyridyl-1-butane (NNK) and N-nitrosonornicotine (NNN) are
Once absorbed, these nitrosamines are bioactivated by cytochrome P450s to their α-hydroxyl metabolites, which are then spontaneously converted to diazonium ions and can result in DNA adducts. In the case of NNK, it is α-hydroxylated by CYP2B6 with an affinity roughly ten times higher (i.e. a lower \( K_m \)) than that of CYP2A6 (Jalas et al. 2005). In contrast, the α-hydroxylation of NNN is thought to be primarily mediated by CYP2A6 (Kushida et al. 2000; Wong et al. 2005). Individuals vary extensively in their procarcinogen exposure and metabolism (Harris et al. 1976; Hecht 2003; Stepanov et al. 2008). Urinary biomarkers can provide information on the intake and metabolism of procarcinogens. In the case of NNK, it is rapidly metabolized in humans and has not been detected in urine (Hecht et al. 1999). Hence total urinary (methylnitrosamino)-1-(3)pyridyl-1-butanol (NNAL), a reductive metabolite of NNK, is commonly used as a biomarker of NNK intake (Hecht 2003). NNAL has a long half-life, and urinary NNAL levels are stable and highly specific to tobacco exposure (Hecht 2003; Goniewicz et al. 2011). The long half-life (7 to 10 days) is especially important in light smokers as it provides an estimate of procarcinogen exposure averaged over an extended period of time (Shiffman 1989; Goniewicz et al. 2009). Like other markers of tobacco smoke exposure, urinary NNAL levels in smokers are dose-dependently associated with the risk of tobacco-related cancers (Yuan et al. 2009). In contrast to NNK, the procarcinogenic NNN can be directly measured in urine and its levels have been associated with the risk of developing esophageal cancer (Yuan et al. 2011). Since CYP2A6 plays a larger role in the metabolic activation of NNN versus NNK, we used urinary NNAL levels as a biomarker of NNK intake and exposure, whereas we used urinary NNN levels as a biomarker of residual, unmetabolized NNN.

In this study, we used plasma NMR as the endophenotype of CYP2A6 activity. NMR accounts for both inherited and non-inherited variations in CYP2A6 activity (Binnington et al. 2012), which makes it a more thorough representation of the current rate of nicotine clearance than CYP2A6 genotype. As NMR can be influenced by exposure to inhibitors or inducers we also used CYP2A6 genotype; this dual approach also confirms that genetic variation in CYP2A6 is the major influence on the smoking behavior differences observed in individuals with different NMR endophenotypes. Using both the NMR endophenotype and CYP2A6 genotype, we tested the hypothesis that light smokers and smokeless tobacco users with lower CYP2A6 activity (i.e. in the lower NMR stratum or CYP2A6 reduced metabolizers, RM) have lower tobacco consumption as measured by urinary total nicotine equivalents (TNE), a biomarker of daily nicotine
consumption. We also tested the hypothesis that individuals with lower CYP2A6 activity will have lower urinary NNAL levels, reflecting lower tobacco consumption, and higher urinary NNN levels, reflecting reduced NNN metabolism.

2.3 Material and Methods

Study Design

A detailed description of recruitment procedures, participant characteristics and tobacco related behaviors has been reported previously (Renner et al. 2013). Briefly, 234 Yupik tobacco users were recruited in local villages near Bristol Bay, Alaska. Male and female (55%) current smokers (n=141), commercial smokeless tobacco users (n=73) and iqmil users (n=20) aged between 18 and 65 were eligible for this study; these are the same subjects as previously described except that two smokers with a mixed Yupik ancestry were not included in the current genetic analysis (Binnington et al. 2012). The research protocol was approved by the Alaska Area IRB, the Bristol Bay Area Health Corporation Board, the IRB at the University of California San Francisco, the IRB at the Centers for Disease Control and Prevention and the University of Toronto Ethics Review office.

CYP2A6 genotyping

As previously described (Binnington et al. 2012), DNA was extracted from blood (n=234) using the Genelute purification kit according to the manufacturer’s instructions (Sigma-Aldrich Ltd. Oakville, Ontario, Canada). Prevalent CYP2A6 alleles with altered function (CYP2A6: *2, *4, *7, *9, *10, *12, *17 and *35) were genotyped by two-step allele specific PCR reactions according to previously reported methods (Schoedel et al. 2004; Mwenifumbo et al. 2005; Ho et al. 2009). Those individuals with one or two copies of a reduced function allele (*2, *4, *7, *9, *10, *12, *17 and *35) were classified as CYP2A6 RM (Mwenifumbo et al. 2008; Ho et al. 2009).

Nicotine metabolite and procarcinogen exposure measurements

Blood and urine samples were collected for the measurement of nicotine and its metabolites, as well as urinary total NNAL (i.e. NNAL and NNAL-glucuronide) and NNN (i.e. NNN and NNN-glucuronide) levels. Plasma COT and 3HC levels, and urinary nicotine, nicotine metabolites,
NNAL and NNN levels were quantified by liquid chromatography-tandem mass spectrometry as previously described (Dempsey et al. 2004; Jacob et al. 2008; Bernert et al. 2011).

**Statistical Analyses**

Statistical analyses were performed using the Stata statistical package version 11 (StataCorp, College Station, TX). NMR stratification was performed by a median split of plasma NMR within each tobacco product group. Participants who were in the higher NMR stratum were considered the faster CYP2A6 activity group, while those in the lower NMR stratum were considered the lower CYP2A6 activity group. The biochemical measures (nicotine, nicotine metabolites, NNN, and NNAL levels) were not normally distributed, thus pairwise comparisons were performed by non-parametric Wilcoxon’s tests. Also for this reason, regression analyses were conducted using log-transformed data.

### 2.4 Results

**Alaska Native smokers with lower CYP2A6 activity exhibited lower tobacco consumption**

Baseline demographics (e.g. age, gender, and body mass index) did not differ between plasma NMR strata or CYP2A6 genotype groups (**Table 3**). Lower tobacco consumption was observed in individuals with slower NMRs. Specifically, urinary TNE levels were significantly lower in smokers in the slower (n=71) versus faster (n=70) plasma NMR stratum (56.3 vs. 79.2 nmol/mg Cre, respectively. P=0.006, **Fig. 8** and **Table 4**). Similar results by NMR strata were observed with a 6 item TNE (i.e. nicotine, nicotine glucuronide, COT, COT glucuronide, 3HC and 3HC glucuronide), which is sometimes used as an alternative to the 9 item TNE (**Table 4**) (Benowitz et al. 2011). The TNE levels did not significantly differ between CYP2A6 genotypes (63.8 vs. 70.7 nmol/mg Cre in CYP2A6 RM and NM, respectively. **Fig. 8** and **Table 4**).

**Alaska Native commercial smokeless tobacco users with lower CYP2A6 activity exhibited lower tobacco consumption**

Consistent with observations in smokers, commercial smokeless tobacco users in the slower (n=37) versus faster (n=36) plasma NMR stratum had 54% lower TNE (64.2 vs. 99.0 nmol/mg Cre, respectively. P=0.02, **Fig. 8** and **Table 5**). Similar effects of NMR strata could be observed
with the 6 item TNE (Table 5). The TNE levels did not significantly differ between CYP2A6 genotypes (69.8 vs. 91.5 nmol/mg Cre in CYP2A6 RM and CYP2A6 NM respectively, P=0.08. Fig. 8 and Table 5).
Table 3 | Baseline demographics by plasma NMR strata and CYP2A6 genotype.

<table>
<thead>
<tr>
<th></th>
<th>Plasma NMR Strata</th>
<th>CYP2A6 genotypes</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Smoker (n=141)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slow: n=71</td>
<td>Faster: n=70</td>
<td>P</td>
<td>Reduced: n=62</td>
<td>Normal: n=79</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>35.1 (14.5)</td>
<td>37.7 (14.7)</td>
<td>0.19</td>
<td>39.0 (15.3)</td>
<td>34.3 (12.8)</td>
<td>0.13</td>
</tr>
<tr>
<td>BMI</td>
<td>28.8 (5.6)</td>
<td>27.5 (6.6)</td>
<td>0.19</td>
<td>28.7 (6.0)</td>
<td>27.8 (6.3)</td>
<td>0.31</td>
</tr>
<tr>
<td>Age of initiation</td>
<td>17.5 (6.4)</td>
<td>17.0 (4.5)</td>
<td>0.89</td>
<td>18.1 (7.2)</td>
<td>16.6 (3.7)</td>
<td>0.25</td>
</tr>
<tr>
<td>Years smoked</td>
<td>17.6 (13.1)</td>
<td>20.7 (14.3)</td>
<td>0.20</td>
<td>21.1 (14.3)</td>
<td>17.7 (13.2)</td>
<td>0.18</td>
</tr>
<tr>
<td>FTND</td>
<td>2.2 (1.9)</td>
<td>2.8 (2.1)</td>
<td>0.09</td>
<td>2.3 (1.8)</td>
<td>2.6 (2.2)</td>
<td>0.60</td>
</tr>
<tr>
<td>Commercial Smokeless</td>
<td></td>
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<tr>
<td>tobacco users (n=73)</td>
<td>Slow: n=37</td>
<td>Faster: n=36</td>
<td>P</td>
<td>Reduced: n=34</td>
<td>Normal: n=39</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>37.0 (12.5)</td>
<td>42.1 (12.3)</td>
<td>0.09</td>
<td>37.2 (12.5)</td>
<td>41.6 (12.4)</td>
<td>0.21</td>
</tr>
<tr>
<td>BMI</td>
<td>30.5 (7.7)</td>
<td>29.1 (6.4)</td>
<td>0.57</td>
<td>29.7 (8.1)</td>
<td>29.8 (5.8)</td>
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<tr>
<td>Age of initiation</td>
<td>17.9 (6.7)</td>
<td>21.5 (1.8)</td>
<td>0.57</td>
<td>17.8 (6.7)</td>
<td>21.3 (10.7)</td>
<td>0.35</td>
</tr>
<tr>
<td>Years of usage</td>
<td>19.2 (11.9)</td>
<td>20.6 (13.3)</td>
<td>0.82</td>
<td>19.4 (12.2)</td>
<td>20.3 (13.0)</td>
<td>0.99</td>
</tr>
<tr>
<td>Iqnik users (n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>42.1 (18.4)</td>
<td>43.6 (16.4)</td>
<td>0.85</td>
<td>45.0 (18.2)</td>
<td>41.1 (16.5)</td>
<td>0.62</td>
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<tr>
<td>BMI</td>
<td>29.2 (5.1)</td>
<td>28.7 (6.5)</td>
<td>0.85</td>
<td>27.5 (4.3)</td>
<td>29.9 (6.6)</td>
<td>0.38</td>
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<tr>
<td>Age of initiation</td>
<td>30.8 (18.5)</td>
<td>19.3 (13.6)</td>
<td>0.13</td>
<td>28.9 (20.8)</td>
<td>21.9 (13.1)</td>
<td>0.37</td>
</tr>
<tr>
<td>Years of usage</td>
<td>11.3 (12.8)</td>
<td>24.3 (16.9)</td>
<td>0.07</td>
<td>16.1 (17.6)</td>
<td>19.2 (15.5)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

BMI = body mass index; FTND = Fagerstrom Test for Nicotine Dependence. Statistical comparisons were made by Wilcoxon’s test.
Figure 8 | Tobacco users with slower CYP2A6 activity have lower nicotine intake. A), by plasma NMR strata or B), by CYP2A6 genotypes. The statistical comparisons were made by Wilcoxon’s test.
Table 4 | Urinary and plasma nicotine exposure biomarkers by plasma nicotine metabolite ratio (NMR) stratum and CYP2A6 genotype in smokers.

<table>
<thead>
<tr>
<th>Smokers (n=141)</th>
<th>Plasma NMR strata</th>
<th>CYP2A6 genotypes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slower, n=71</td>
<td>Faster, n=70</td>
<td>Reduced, n=62</td>
</tr>
<tr>
<td></td>
<td>Absolute values mean (SD)</td>
<td>% of urinary nicotine equivalents recovered</td>
<td>Absolute values mean (SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$P_{\text{absol.}}$</td>
</tr>
<tr>
<td>Urinary (nmol/mg Cre)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (9-items)</td>
<td>56.3 (35.3)</td>
<td>79.2 (53.6)</td>
<td>0.006</td>
</tr>
<tr>
<td>TNE (6-items)*</td>
<td>49.5 (31.7)</td>
<td>73.6 (50.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total nicotine</td>
<td>12.0 (14.2)</td>
<td>18.0 (14.6)</td>
<td>7.3 (8.0)</td>
</tr>
<tr>
<td>Free</td>
<td>8.8 (12.3)</td>
<td>12.5 (12.9)</td>
<td>5.5 (7.1)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>3.2 (3.4)</td>
<td>5.5 (4.2)</td>
<td>1.8 (1.5)</td>
</tr>
<tr>
<td>Total COT</td>
<td>17.7 (13.0)</td>
<td>31.4 (9.2)</td>
<td>19.9 (14.8)</td>
</tr>
<tr>
<td>Free</td>
<td>8.9 (8.4)</td>
<td>14.6 (6.7)</td>
<td>9.4 (8.7)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>8.8 (5.6)</td>
<td>16.8 (6.3)</td>
<td>10.5 (7.0)</td>
</tr>
<tr>
<td>Total 3HC</td>
<td>19.8 (13.3)</td>
<td>38.6 (15.4)</td>
<td>46.3 (33.2)</td>
</tr>
<tr>
<td>Free</td>
<td>17.2 (11.7)</td>
<td>33.8 (13.7)</td>
<td>39.4 (27.6)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>2.7 (2.7)</td>
<td>5.0 (4.6)</td>
<td>6.9 (6.6)</td>
</tr>
<tr>
<td>Metabolite</td>
<td>Normal Metabolizers</td>
<td>Reduced Metabolizers</td>
<td>p-value</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>0.5 (0.4)</td>
<td>0.8 (0.4)</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>Nicotine-N-Oxide</td>
<td>4.5 (4.8)</td>
<td>7.7 (6.2)</td>
<td>2.8 (2.6)</td>
</tr>
<tr>
<td>Cotinine-N-Oxide</td>
<td>1.8 (1.1)</td>
<td>3.5 (1.4)</td>
<td>2.4 (1.5)</td>
</tr>
<tr>
<td>CPD</td>
<td>6.7 (4.4)</td>
<td>8.3 (5.9)</td>
<td>0.153</td>
</tr>
</tbody>
</table>

**Plasma (ng/mL)**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normal Metabolizers</th>
<th>Reduced Metabolizers</th>
<th>p-value</th>
<th>Normal Metabolizers</th>
<th>Reduced Metabolizers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COT</td>
<td>171 (101)</td>
<td>170 (110)</td>
<td>0.795</td>
<td>177 (96)</td>
<td>165 (112)</td>
<td>0.26</td>
</tr>
<tr>
<td>3HC</td>
<td>45.9 (33.6)</td>
<td>108.4 (77.2)</td>
<td>0.001</td>
<td>58.8 (46.3)</td>
<td>91.2 (76.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>3HC/CO T</td>
<td>0.263 (0.105)</td>
<td>0.685 (0.296)</td>
<td>0.001</td>
<td>0.316 (0.175)</td>
<td>0.595 (0.330)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Compared to TNE (9-items), TNE (6-items) did not include nornicotine, nicotine-N-oxide, and cotinine-N-oxide. Gluc=glucuronide. \( P_{\%} \) = the P values from Wilcoxon’s tests when the metabolites were normalized to % of urinary total nicotine equivalents. \( P_{\text{absol}} \) = the P values from Wilcoxon’s tests when the absolute urinary recovery values were compared. Bold values indicate statistically significant differences between the groups by Wilcoxon’s test. Levels were compared between the normal metabolizers and the reduced metabolizers (individuals with at least one copy of reduced function \( CYP2A6 \) alleles) or between the faster plasma NMR stratum and the slower plasma NMR stratum (median split).
Table 5 | Urinary and plasma nicotine exposure biomarkers by plasma nicotine metabolite ratio (NMR) stratum and CYP2A6 genotype in commercial smokeless tobacco users and iqmk users.

<table>
<thead>
<tr>
<th>Commer cial smokeles s tobacco (n=73)</th>
<th>Plasma NMR strata</th>
<th>CYP2A6 genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slower, n=37</td>
<td>Faster, n=36</td>
</tr>
<tr>
<td>Absolute values (mean (SD))</td>
<td>% of urinary nicotine equivalents recovered</td>
<td>Absolute values (mean (SD))</td>
</tr>
<tr>
<td>TNE (9-Items)</td>
<td>64.2 (44.3)</td>
<td>99.0 (74.4)</td>
</tr>
<tr>
<td>TNE (6-Items)*</td>
<td>57.2 (39.1)</td>
<td>91.7 (69.4)</td>
</tr>
<tr>
<td>Total nicotine</td>
<td>10.5 (11.1)</td>
<td>15.0 (9.9)</td>
</tr>
<tr>
<td>Free</td>
<td>7.5 (9.7)</td>
<td>9.9 (8.9)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>3.0 (2.1)</td>
<td>5.1 (2.3)</td>
</tr>
<tr>
<td>Total COT</td>
<td>22.1 (15.9)</td>
<td>34.3 (8.0)</td>
</tr>
<tr>
<td>Free</td>
<td>11.7 (10.3)</td>
<td>17.7 (8.5)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>10.3 (7.2)</td>
<td>16.6 (6.0)</td>
</tr>
<tr>
<td>Total 3HC</td>
<td>24.6 (16.2)</td>
<td>39.8 (15.3)</td>
</tr>
<tr>
<td>Free</td>
<td>22.0 (14.9)</td>
<td>35.5 (14.4)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>2.7 (2.6)</td>
<td>4.5 (3.8)</td>
</tr>
<tr>
<td></td>
<td>Slower, n=10</td>
<td>Faster, n=10</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Absolute values mean (SD)</td>
<td>% of urinary nicotine equivalents recovered</td>
</tr>
<tr>
<td>Iqnik users (n=20)</td>
<td>Absolute values mean (SD)</td>
<td>Absolute values mean (SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmatic (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COT (n=20)</td>
<td>223 (116)</td>
<td>223 (148)</td>
</tr>
<tr>
<td>3HC (n=20)</td>
<td>58 (36)</td>
<td>137 (95)</td>
</tr>
<tr>
<td>3HC/COT NMR (n=20)</td>
<td>0.256 (0.092)</td>
<td>0.700 (0.275)</td>
</tr>
<tr>
<td>Urinary (nmol/mg Cre)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (9-Items)</td>
<td>117.2 (53.0)</td>
<td>139.0 (38.7)</td>
</tr>
<tr>
<td>TNE (6-Items)*</td>
<td>104.4 (47.9)</td>
<td>129.1 (37.3)</td>
</tr>
<tr>
<td>Total</td>
<td>15.3 (8.1)</td>
<td>13.6 (6.1)</td>
</tr>
<tr>
<td></td>
<td>nicotine</td>
<td>Glucuron</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>10.3 (6.8)</td>
<td>8.5 (5.2)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>5.1 (3.7)</td>
<td>5.1 (4.1)</td>
</tr>
<tr>
<td>Total COT</td>
<td>34.3 (13.0)</td>
<td>32.0 (10.6)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>16.8 (9.3)</td>
<td>14.5 (4.5)</td>
</tr>
<tr>
<td>Total 3HC</td>
<td>52.4 (38.6)</td>
<td>40.9 (16.2)</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>2.4 (4.8)</td>
<td>2.6 (3.8)</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>1.0 (0.6)</td>
<td>0.8 (0.2)</td>
</tr>
<tr>
<td>Nicotine-N-Oxide</td>
<td>7.9 (4.3)</td>
<td>6.8 (2.5)</td>
</tr>
<tr>
<td>Cotinine-N-Oxide</td>
<td>3.8 (2.1)</td>
<td>3.3 (0.9)</td>
</tr>
<tr>
<td>Chew per day</td>
<td>4.1 (1.4)</td>
<td>4.6 (2.9)</td>
</tr>
<tr>
<td>Can per week</td>
<td>1.3 (1.7)</td>
<td>1.0 (0.6)</td>
</tr>
<tr>
<td>Plasma (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COT</td>
<td>369 (198)</td>
<td>322 (201)</td>
</tr>
<tr>
<td>3HC</td>
<td>160 (108)</td>
<td>246 (230)</td>
</tr>
<tr>
<td>3HC/COT NMR</td>
<td>0.345 (0.144)</td>
<td>0.843 (0.367)</td>
</tr>
</tbody>
</table>
*Compared to TNE (9-items), TNE (6-items) did not include nornicotine, nicotine-\(N\)-oxide, and cotinine-\(N\)-oxide. Gluc=glucuronide. \(P_\%\) = the \(P\) values from Wilcoxon’s tests when the metabolites were normalized to % of urinary total nicotine equivalents. \(P_{\text{absol}}\) = the \(P\) values from Wilcoxon’s tests when the absolute urinary recovery values were compared. Bold values indicate statistically significant differences between the groups by Wilcoxon’s test. Levels were compared between the normal metabolizers and the reduced metabolizers (individuals with at least one copy of reduced function \(CYP2A6\) alleles) or between the faster plasma NMR stratum and the slower plasma NMR stratum (median split).
Alaska Native iqnik users with lower CYP2A6 activity had lower tobacco consumption

Among the small number of iqnik users, nineteen percent lower TNE levels were observed in those in the slower (n=10) compared to faster (n=10) plasma NMR stratum (117.2 vs.139.0 nmol/mg Cre respectively, P=0.023, Fig. 8 and Table 5). However, no significant difference in TNE levels was observed by CYP2A6 genotype group (Table 5).

Urinary nicotine metabolite profiles differed between CYP2A6 activity groups in smokers

Fig. 9A shows the total and proportional level of nicotine and each metabolite in smokers’ urine (n=141) by plasma NMR stratum, illustrating the dominant role of CYP2A6 in the metabolic removal of nicotine. Smokers in the slower plasma NMR stratum excreted 72% of their TNE via the CYP2A6-mediated COT pathways (i.e. COT and its metabolites, darker shades, Fig. 9A), whereas more than 85% of TNE was excreted via the COT pathway among smokers in the faster plasma NMR stratum (P<0.0001, Fig. 9A). As a result, smokers in the slower plasma NMR stratum excreted a significantly higher percentage of consumed nicotine (i.e. TNE) as free nicotine, nicotine glucuronide, nornicotine and nicotine-N-oxide (P<0.05, Table 4 and Fig. 9A) and a significantly lower percentage as 3HC and its glucuronide (P <0.001, Table 4 and Fig. 9A). Urinary nicotine metabolite profiles among smokers also differed between CYP2A6 genotype groups (Fig. 9B and Table 4).

Urinary nicotine metabolite profiles differed between CYP2A6 activity groups in commercial smokeless tobacco and iqnik users

Similar to Alaska Native smokers, commercial smokeless tobacco users and iqnik users in the slower plasma NMR stratum excreted 78% of their TNE via the COT pathway, whereas more than 85% of TNE were excreted via the COT pathway in individuals in the faster plasma NMR stratum (P<0.01, Table 5). Similar to the results observed using plasma NMR stratum, urinary nicotine metabolite profiles also differed by CYP2A6 genotype group in commercial smokeless tobacco users and iqnik users (Table 5).
Figure 9 | Total tobacco consumption, and nicotine’s metabolic profile, differed between NMR strata (A) and CYP2A6 genotypes (B) in cigarette smokers (n=141). The total size of pie charts represents the amount of total urinary nicotine equivalents (TNE). The percentage of TNE recovered as each nicotine metabolite is represented by the size.
Neither Plasma NMR strata nor CYP2A6 genotypes were associated with self-report indicators of tobacco consumption or plasma COT levels

Despite the 41% difference in tobacco consumption as indicated by TNE, Alaska Native smokers in the slower versus faster plasma NMR stratum reported smoking similar CPD (6.7 vs. 8.3, respectively, non-significant) and had similar plasma COT levels (235 vs. 213 ng/mL, respectively, non-significant, Table 4). There were also no significant differences between plasma NMR strata in the number of chews per day, the number of cans of smokeless tobacco used per week (standard Copenhagen sized cans), or the levels of plasma COT in commercial smokeless tobacco users or iqmik users (Table 5). Likewise, CYP2A6 genotype was not associated with differences in self-report indicators of tobacco consumption or plasma COT levels in smokers, commercial smokeless tobacco users or iqmik users (Table 4 and 5).

**Urinary NNAL levels trended lower in individuals with lower CYP2A6 activity**

There were 27% and 18% difference in urinary NNAL levels between NMR strata and CYP2A6 genotype groups respectively, but they were not significant (1.1 vs. 1.4 pmol/mg Cre in the slower and faster plasma NMR stratum, respectively; 1.1 vs. 1.3 pmol/mg in CYP2A6 RM and NM, respectively, Table 6). Among the commercial smokeless tobacco users, we observed 62% lower NNAL levels in the slower compared to the faster plasma NMR stratum (4.5 vs. 7.3 pmol/mg Cre, respectively, P=0.01, Table 6). A similar difference in magnitude was also observed between CYP2A6 genotypes (4.6 vs. 7.1 pmol/mg Cre in CYP2A6 RM and NM, respectively, P=0.006, Table 6). No difference in NNAL levels was observed between NMR strata or CYP2A6 genotypes among the small iqmik group (Table 6). As expected, the effects of NMR strata and CYP2A6 genotypes on NNAL levels (a measure of exposure) were eliminated when nicotine dose (TNE) was controlled for (Table 7 A and B).

**Urinary NNN levels were higher in smokers with lower CYP2A6 activity**

As total tobacco consumption (i.e. TNE) differed between NMR strata, we compared urinary NNN levels while controlling for TNE. NNN levels were higher in slower than faster NMR strata (B=0.41, β=0.17, 95%CI=0.02–0.79, P=0.04, Table 8A). A 39% difference in NNN levels between NMR strata was observed when not controlling for tobacco dose, but this did not reach
statistically significance (0.182 vs. 0.110 fmol/mg Cre, respectively, non-significant, Table 6). Significantly higher urinary NNN levels were observed in CYP2A6 RM compared to CYP2A6 NM (0.230 vs. 0.083 fmol/mg Cre, respectively, P=0.05, Table 6), which was also greater when TNE was controlled for (by CYP2A6 genotype: B=0.60, β=0.24, 95%CI=0.21-0.98, P=0.003, Table 8A). Among the commercial smokeless tobacco users, urinary NNN levels were moderately, but non-significantly, higher in those with slower compared to faster plasma NMR (0.126 vs. 0.107 fmol/mg Cre, respectively, Table 6), and the difference between the two groups increased when TNE was controlled for (by plasma NMR strata: B=0.41, β=0.18, 95%CI=-0.067-0.887, P=0.09, Table 8B). No difference in NNN levels was observed between CYP2A6 genotype groups among the commercial smokeless tobacco users (0.119 vs. 0.113 fmol/mg Cre in CYP2A6 RM and CYP2A6 NM, respectively, P=0.67; regression analysis by CYP2A6 genotype: B=-0.06, β=-0.03, 95%CI=-0.544-0.418, P=0.793, Table 6, 8B). No difference in urinary NNN levels was observed by NMR stratum or CYP2A6 genotype group among iqmik users (Table 6).
Table 6 | Urinary procarcinogen biomarker levels in smokers, commercial smokeless tobacco users, and iqnik users.

<table>
<thead>
<tr>
<th></th>
<th>Plasma NMR strata</th>
<th>CYP2A6 genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers n=141</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=141</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slower n=71 mean (SD)</td>
<td>Faster n=70 mean (SD)</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNAL (pmol/mg Cre.)</td>
<td>1.1 (0.8)</td>
<td>1.4 (1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNN (fmol/mg Cre.)</td>
<td>0.182 (0.390)</td>
<td>0.110 (0.200)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial smokeless tobacco users n=73</td>
<td></td>
<td>Reduced n=34 mean (SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNAL (pmol/mg Cre.)</td>
<td>4.5 (3.6)</td>
<td>7.3 (6.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNN (fmol/mg Cre.)</td>
<td>0.126 (0.114)</td>
<td>0.107 (0.088)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iqnik users n=20</td>
<td>Slower n=10 mean (SD)</td>
<td>Faster n=10 mean (SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNAL (pmol/mg Cre.)</td>
<td>0.8 (0.6)</td>
<td>0.7 (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNN (fmol/mg Cre.)</td>
<td>0.756 (1.95)</td>
<td>0.154 (0.195)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bold values indicate statistically significant differences between the groups by Wilcoxon’s test.

NNAL = 4-(methylnitrosamino)-1-(3) pryridy1-1-butanol; NNN = N-Nitrosonornicotine
### Table 7 | The effect of NMR stratum or CYP2A6 genotype on urinary NNAL level was eliminated after controlling for tobacco consumption.

<table>
<thead>
<tr>
<th>Predictors of NNAL</th>
<th>NMR strata R²=0.40 P&lt;0.001</th>
<th>B</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.6</td>
<td>0.009,0.015</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>NMR (slower strata)</td>
<td>0.27</td>
<td>0.14</td>
<td>0.017,0.529</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>Age (Increasing)</td>
<td>0.01</td>
<td>0.15</td>
<td>0.001,0.018</td>
<td>0.036</td>
</tr>
<tr>
<td>A Smokers</td>
<td>CYP2A6 Genotype R²=0.39 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.55</td>
<td>0.008,0.014</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>CYP2A6 Genotype (Reduced)</td>
<td>-0.13</td>
<td>-0.07</td>
<td>-</td>
<td>0.390,0.125</td>
</tr>
<tr>
<td></td>
<td>Age (Increasing)</td>
<td>0.01</td>
<td>0.16</td>
<td>0.001,0.019</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>NMR strata R²=0.65 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>B Commercial</td>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.75</td>
<td>0.008,0.012</td>
<td>0.001</td>
</tr>
<tr>
<td>smokeless tobacco</td>
<td>NMR (slower strata)</td>
<td>-0.09</td>
<td>-0.05</td>
<td>-</td>
<td>0.352,0.167</td>
</tr>
<tr>
<td></td>
<td>Age (Increasing)</td>
<td>0.01</td>
<td>0.15</td>
<td>0.001,0.021</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>CYP2A6 Genotype R²=0.67 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.74</td>
<td>0.008,0.012</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>CYP2A6 Genotype (Reduced)</td>
<td>-0.24</td>
<td>-0.14</td>
<td>-</td>
<td>0.484,0.011</td>
</tr>
<tr>
<td></td>
<td>Age (Increasing)</td>
<td>0.01</td>
<td>0.14</td>
<td>0.000,0.020</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*B* is the unstandardized coefficient. *β* is the standardized coefficient (i.e. standardized to a variance of 1). The *B* and *β* provided refers to the variables listed in brackets beside each categorical predictor. The NNAL levels were log-transformed to obtain normal distribution.
Table 8 | NNN levels were significantly higher in smokers with slower CYP2A6 activity when tobacco consumption was controlled for.

<table>
<thead>
<tr>
<th>Predictors of NNN</th>
<th>B</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A Smokers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma NMR R²=0.30 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.53</td>
<td>0.010,0.019</td>
<td>0.001</td>
</tr>
<tr>
<td>NMR (slower strata)</td>
<td>0.41</td>
<td>0.17</td>
<td>0.023,0.791</td>
<td>0.038</td>
</tr>
<tr>
<td>Age (Increasing)</td>
<td>0.01</td>
<td>0.08</td>
<td>-0.006,0.020</td>
<td>0.297</td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R²=0.33 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.52</td>
<td>0.010,0.018</td>
<td>0.001</td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Reduced)</td>
<td>0.6</td>
<td>0.24</td>
<td>0.212,0.979</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (Increasing)</td>
<td>0.00</td>
<td>0.03</td>
<td>-0.011,0.015</td>
<td>0.730</td>
</tr>
<tr>
<td><strong>B Commercial smokeless tobacco</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma NMR R²=0.36 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.63</td>
<td>0.007,0.015</td>
<td>0.001</td>
</tr>
<tr>
<td>NMR (slower strata)</td>
<td>0.41</td>
<td>0.18</td>
<td>-0.067,0.887</td>
<td>0.091</td>
</tr>
<tr>
<td>Age (Increasing)</td>
<td>0.00</td>
<td>0.02</td>
<td>-0.021,0.017</td>
<td>0.825</td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R²=0.33 P&lt;0.001</td>
<td>B</td>
<td>β</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNE (increasing, per nmol)</td>
<td>0.01</td>
<td>0.58</td>
<td>0.007,0.014</td>
<td>0.001</td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Reduced)</td>
<td>-</td>
<td>-</td>
<td>-0.544,0.418</td>
<td>0.793</td>
</tr>
<tr>
<td>Age (Increasing)</td>
<td>-</td>
<td>-</td>
<td>-0.025,0.015</td>
<td>0.612</td>
</tr>
</tbody>
</table>

B is the unstandardized coefficient. β is the standardized coefficient (i.e. standardized to a variance of 1). The B and β provided refer to the variables listed in brackets beside each categorical predictor. The NNN levels were log-transformed to obtain a normal distribution.
2.5 Discussion

This study revealed three novel findings, each supporting the key role of CYP2A6 in influencing tobacco consumption and the risk of developing tobacco-related disease. First, Alaska Native smokers, who smoked on average less than 10 CPD (i.e. light smokers), titrated their daily tobacco consumption (i.e. TNE) according to their CYP2A6 activities similarly to previous observations among Caucasian heavier smokers (Wassenaar et al. 2011). Second, variation in CYP2A6 activity was associated with altered levels of tobacco use among commercial smokeless tobacco users and iqnik users. Third, Alaska Native smokers and smokeless tobacco users with lower CYP2A6 activities excreted lower levels of NNAL, reflecting lower NNK exposure as a result of their lower tobacco consumption and they may possess reduced NNN metabolic activation (inferred through higher residual urinary NNN levels). Together, lower tobacco and procarcinogen consumption and lower procarcinogen bioactivation would be expected to decrease the risk of tobacco-related cancers in individuals with lower CYP2A6 activities, consistent with a lower risk seen among heavy smoking Caucasians (Wassenaar et al. 2011).

In this study, the levels of urinary TNE in Alaska Native smokers were comparable to those among Caucasian heavy smokers despite smoking less than 10 CPD (6-item TNE: 61.4 nmol/mg Cre in Alaska Native smokers vs. 60-63 nmol/mg Cre in Caucasian smokers with ~20 CPD) (Le Marchand et al. 2008; Benowitz et al. 2011). This suggests that Alaska Native smokers inhaled each cigarette more deeply and/or for a longer duration, resulting in similar amounts of nicotine (and presumably procarcinogens) exposure to that observed in Caucasian heavy smokers despite smoking fewer CPD (Benowitz et al. 2012). Several studies have observed that cigarette smokers with lower CYP2A6 activities smoke fewer CPD (Schoedel et al. 2004; Wassenaar et al. 2011), but this difference in CPD has not been observed in light smoking populations such as African Americans (Ho et al. 2009), nor was it observed in this Alaska Native group. Smokers who consume comparatively fewer cigarettes typically inhale more intensely than smokers who consume more cigarettes, hence nicotine and procarcinogen intake per cigarette is inversely related to numbers of reported CPD (Benowitz et al. 2011). In the present study, smokers with lower CYP2A6 activity (i.e. slower plasma NMR stratum or CYP2A6 RM) had lower TNE levels while reporting similar daily cigarette use. This suggests that Alaska Native smokers with lower CYP2A6 activity reduced their nicotine consumption per cigarette to compensate for their slower
nicotine inactivation, presumably by inhaling smaller and/or fewer puffs, a phenomenon observed previously in heavier smokers (Strasser et al. 2007). The 41% reduction in TNE between NMR strata in Alaska Native smokers was larger than the 28% reduction in CPD observed in Caucasian heavy smokers between CYP2A6 genotypes (6), suggesting consumption differences between CYP2A6 activity groups, when measured more accurately, could be larger than those predicted by CPD (Wassenaar et al. 2011). Substantial inter-subject variation in TNE levels was observed between NMR strata and CYP2A6 genotypes in the iqmik users. This is likely due to variation in the preparation procedures (e.g. content of the basic ash and pre-mastication) and chewing behavior (chew duration and whether swallows the iqmik juice) (Renner et al. 2005). Due to these differences, biochemical markers such as TNE provide a better measure of nicotine exposure than the self-report iqmik consumption measures.

In the current study, the effect of NMR strata on urinary TNE was stronger than the effect of CYP2A6 genotype likely reflecting a closer relationship of NMR (versus CYP2A6 genotype) to the phenotype of interest, CYP2A6 activity. This is most likely because of the influence of gender, BMI, and dietary inducers/inhibitors on CYP2A6 activity (Ho et al. 2009; Al Koudsi et al. 2010). Further, there may be novel CYP2A6 genetic variants among the Alaska Native people, which were not investigated in our study (Binnington et al. 2012).

To the best of our knowledge, this is the first demonstration of tobacco consumption titration by CYP2A6 activity among smokeless tobacco users. The consumption differences between plasma NMR strata among the commercial smokeless tobacco users were slightly larger than those observed in smokers (54% versus 41% lower TNE, respectively). This may be a reflection of slower nicotine absorption from smokeless tobacco versus cigarette smoking (Benowitz 1997), which could allow for a longer period of time for metabolism to occur thereby increasing the effect of differences in the rates of nicotine metabolism.

Although urinary TNE levels differed by CYP2A6 activity group among Alaska Native smokers and commercial smokeless tobacco users, plasma COT levels did not. Plasma COT is often used as a biomarker of nicotine exposure, however it is both metabolically formed and removed by CYP2A6. Lower CYP2A6 activity might differentially reduce the relative rates of COT formation versus COT removal resulting in the accumulation of COT among slower CYP2A6
metabolizers. Thus plasma COT might over-estimate nicotine dose in individuals with lower CYP2A6 activities.

In addition to influencing tobacco consumption, genetic variation in CYP2A6 can also alter lung cancer risk (Wassenaar et al. 2011). Some earlier studies in Caucasians did not find an association between CYP2A6 and the risk for developing lung cancer (Rossini et al. 2008). However these studies did not genotype the majority of loss of function CYP2A6 variants and suffered from reduced power (Rossini et al. 2008). In recent studies, with more comprehensive genotyping, significant associations between CYP2A6 and the risk for developing lung cancer have been found (Fujieda et al. 2004; Wassenaar et al. 2011). This may be due to differences in both tobacco consumption and in procarcinogen metabolic activation by CYP2A6 genotype. Tobacco specific nitrosamines such as NNK, NNN and NNAL are all potent procarcinogens (Hecht 2003). Commercial smokeless tobacco users with lower CYP2A6 activity had lower NNAL levels, which was eliminated when controlling for TNE levels, suggesting that the lower NNAL levels are a result of lower tobacco consumption rather than differences in NNK metabolism. In fact, we observed no evidence that variation in CYP2A6 activity had any effect on NNAL levels beyond the effect of CYP2A6 on tobacco consumption: the relative amount of NNK eliminated via the NNAL pathway did not differ between CYP2A6 activity groups in our study consistent with human genetic association studies (Ter-Minassian et al. 2012).

Higher NNN levels were observed in the smokers with lower CYP2A6 activity, which was even greater after controlling for nicotine intake (i.e. TNE). We speculate that the lower CYP2A6 activity resulted in lower NNN clearance (i.e. lower bioactivation) resulting in higher levels of urinary NNN. CYP2A6 mediates the α-hydroxylation of NNN to genotoxic metabolites (Wong et al. 2005). If not detoxified, these genotoxic metabolites can covalently bind to DNA which can result in mutations. This is consistent with human transfection studies in which a higher degree of NNN induced mutagenesis was observed in the CYP2A6 transfected cells than the control cells (Kushida et al. 2000). More research is needed to understand the role of urinary NNN as a predictor of tobacco-related cancers cancer risk – to date, a dose dependent relationship has only been seen for esophageal cancer (Yuan et al. 2011).
There are limitations to our study. Our study only included Alaska Native individuals and the application of the findings to other race/ethnicities requires further investigation. Secondly, this study had a relatively small smokeless tobacco group, particularly for individuals who exclusively used iqmk.

Together our data indicate that Alaska Native light smokers and smokeless tobacco users titrate their tobacco consumption according to CYP2A6 activity. Previous failures to see evidence of titration by CYP2A6 activity among light smokers may be due to the poor sensitivity of the tobacco consumption indicators used (e.g. CPD). Furthermore, consistent with the lower tobacco consumption, individuals with lower CYP2A6 activities also had lower procarcinogen exposure (i.e. NNK and NNAL). In addition, lower CYP2A6 activity was associated with higher NNN levels suggesting reduced bioactivation. Together these findings support a reduced risk of tobacco related cancers for slow, versus fast, CYP2A6 metabolizers, in both smokers and smokeless tobacco users.
2.6 Significance to Thesis

This chapter contributes to the literature in three ways. First, it demonstrated that variation in CYP2A6 activity significantly alter tobacco consumption in light smokers. This suggests that light smokers titrate their tobacco consumption to maintain desirable nicotine levels in the body as seen with heavy smokers. This would imply that the associations between CYP2A6 gene variants and smoking related disease risk/cessation outcomes previously observed in heavy smokers are likely applicable to light smokers as well. Secondly, this study demonstrated that smokeless tobacco users titrate their tobacco consumption according to their CYP2A6 activity. About 7% of men in the United States use smokeless tobacco, yet little is known about the genetic influences on smokeless tobacco use behaviors. This is the first study to demonstrate that genetic variation can significantly influence smokeless tobacco use. Lastly, this study also provided evidence that variation in CYP2A6 activity is associated with altered carcinogen bioactivation (as indicated by the higher NNN levels in individuals with reduced CYP2A6 activity). Individuals with reduced CYP2A6 activity are at a lower risk of developing lung cancer even after adjusting for tobacco consumption. This study suggests that reduced carcinogenic TSNA activation could contribute to this association.
CHAPTER 2: THE ABILITY OF PLASMA COTININE TO PREDICT NICOTINE AND CARCINOGEN EXPOSURE IS ALTERED BY DIFFERENCES IN CYP2A6: THE INFLUENCE OF GENETICS, RACE AND SEX

Andy Z.X. Zhu, Caroline C. Renner, Dorothy K. Hatsukami, Gary E. Swan, Caryn Lerman, Neal L. Benowitz, and Rachel F. Tyndale

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GES, NLB and RFT conducted the pharmacokinetic study. CCR, DKH, NLB and RFT conducted the cross sectional study. CL, NLB and RFT conducted the clinical trial. AZZ determined the experimental questions and derived the mathematical equations. AZZ performed all analyses and modeling. AZZ and RFT wrote the manuscript.
3.1 Abstract

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

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

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

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

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

In humans, cotinine is metabolically formed from nicotine in a reaction catalyzed by CYP2A6 and is further metabolized to trans-3’-hydroxycotinine (3HC) by CYP2A6 (Nakajima et al. 1996; Nakajima et al. 1996). Cotinine is routinely used as an objective index of tobacco and tobacco-derived carcinogen exposure. The use of cotinine is particularly useful because of wide individual variability in the relationship between self-report cigarettes smoked per day and systemic exposure to tobacco and tobacco-derived carcinogen (Benowitz et al. 2011). Higher plasma cotinine levels have been associated with increased lung cancer risk (Boffetta et al. 2006), and variation in cotinine levels have recently been used as evidence that the mechanism mediating the association between genetic variation in CHRNA5-A3-B4 and lung cancer risk is almost entirely through the modulation of tobacco consumption (Munafo et al. 2012).

The systemic intake of nicotine is correlated with exposure to tobacco-derived carcinogen (Benowitz et al. 2011). When used as a biomarker of tobacco-derived carcinogen exposure, it is generally assumed that plasma cotinine levels reflect intake of nicotine, and that variability in the relationship between cotinine levels and nicotine intake is random among smokers. However, some research suggests that a particular level of cotinine does not predict the same level of tobacco exposure among different groups of smokers. For example, directional inconsistencies have been observed between plasma cotinine levels and other indicators of tobacco exposure in genetic association studies involving CYP2A6. Caucasian smokers with one or more reduced function CYP2A6 alleles (i.e. CYP2A6 reduced metabolizers, or RM) smoke fewer cigarettes per day compared to the CYP2A6 normal metabolizers (NM), consistent with titration of smoke intake to obtain desired levels of nicotine in the body (Schoedel et al. 2004; Wassenaar et al. 2011). Yet despite smoking fewer cigarettes per day, smokers with reduced CYP2A6 activity have similar plasma cotinine levels compared to the smokers with faster CYP2A6 activity (Strasser et al. 2011). Another example is found in studies of African American light smokers, where similar levels of cigarettes per day are reported between CYP2A6 genotypes (i.e. CYP2A6 RM have similar cigarettes per day compared to CYP2A6 NM), but the plasma cotinine levels are significantly higher in CYP2A6 RM compared to CYP2A6 NM (Ho et al. 2009). Furthermore, gene variants at the CYP2A6 loci (i.e. tag SNPs) that are associated with decreased lung cancer risk are paradoxically associated with increased, rather than decreased, plasma cotinine levels.
(Timofeeva et al. 2011). These observations suggest that CYP2A6 reduced activity genotype may increase plasma cotinine levels in addition to its role in reducing nicotine inactivation, tobacco consumption and nitrosamine metabolic activation (Zhu et al. 2013).

Variation in plasma cotinine levels is also observed between the sexes. The male participants of the National Health and Nutrition Examination Surveys have significantly higher plasma cotinine levels compared to the female participants even after adjusting for cigarettes per day, machine-determined nicotine delivery of cigarettes, race, age, body mass index, poverty status, and the use of either menthol or regular cigarettes (Gan et al. 2008). Since CYP2A6 activity differs between the sexes, this systematic variation in cotinine levels between the sexes could be the result of the difference in CYP2A6 activity (Al Koudsi et al. 2010).

Notable variation in cotinine levels is also observed between races. African American smokers generally have higher plasma cotinine levels compared to Caucasian smokers after adjusting for the number of cigarettes smoked per day and the machine-determined nicotine delivery in cigarettes (Wagenknecht et al. 1990), which could be partially due to the slower cotinine clearance in African Americans compared to Caucasians (Perez-Stable et al. 1998). Since the prevalence of CYP2A6 RM is high in African Americans compared to Caucasians, it is possible that the racial variation in cotinine levels originates from the difference in CYP2A6 activities between racial groups.

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

$$[COT_{\text{plasma}}] = Dose_{\text{nic}} \times \frac{f_{\text{NIC} \rightarrow \text{COT}}}{Cl_{\text{COT}}}$$

where $COT_{\text{plasma}}$ represents the steady state plasma cotinine levels, $Dose_{\text{nic}}$ is the daily nicotine intake, $f_{\text{NIC} \rightarrow \text{COT}}$ is the fraction of nicotine converted to cotinine (i.e. cotinine formation), and $Cl_{\text{COT}}$ represents systemic plasma cotinine clearance (i.e. cotinine removal) (Benowitz 1996). Hence, the steady state plasma cotinine levels are proportional to the ratio of $f_{\text{NIC} \rightarrow \text{COT}}/Cl_{\text{COT}}$ and the $Dose_{\text{nic}}$. Cotinine is both formed and removed by CYP2A6. Thus, both $f_{\text{NIC} \rightarrow \text{COT}}$ and cotinine
clearance are highly dependent on CYP2A6 activity. We hypothesize that reduced CYP2A6 activity will increase the ratio of $\frac{f_{\text{NIC}}}{{\text{COT}}} / \text{Cl}_{{\text{COT}}}$, and alter the quantitative relationship between plasma cotinine and nicotine and tobacco-derived carcinogen exposure in cigarette smokers.

In this study, we use $CYP2A6$ genotype as the primary indicator of CYP2A6 activity. $CYP2A6$ genetic variants are known to alter in vivo CYP2A6 activity as well as nicotine and cotinine clearance (Oscarson et al. 2002; Xu et al. 2002; Fukami et al. 2004; Benowitz et al. 2006; Mwenifumbo et al. 2007; Ho et al. 2008; Mwenifumbo et al. 2008). In addition to $CYP2A6$ genotype, we also use the nicotine metabolite ratio (NMR) as a secondary indicator of CYP2A6 activity (Dempsey et al. 2004). For the evaluation of nicotine intake in ad libitum smokers, we use urinary total nicotine equivalents (TNE) (Wang et al. 2011). For the evaluation of tobacco-derived carcinogen in ad libitum smokers, we used total urinary (methylnitrosamino)-1-(3) pyridyl-1-butanol (NNAL), 1-hydroxyfluorene and 1-hydroxypyrene levels (Yuan et al. 2011; StHelen et al. 2012).

### 3.3 Material and Methods

**Study design**

Data for the present analysis were taken from two studies that have been previously published (Benowitz et al. 2006; Benowitz et al. 2012).

**Study 1:** The pharmacokinetics of nicotine and cotinine were characterized in 181 non-smoking Caucasian male and female sets of twins (both monozygotic and dizygotic). Demographic variable comparisons are shown in **table 9**. A comprehensive description of the study procedures has been published previously (Swan et al. 2004; Swan et al. 2005). Briefly, participants received a simultaneous 30-minute infusion of deuterium-labeled nicotine and cotinine in the fasting condition. Blood samples were collected for the measurement of labeled nicotine and cotinine for determination of pharmacokinetic parameters and DNA was extracted for $CYP2A6$ genotyping. The cotinine and 3HC levels were measured in the 480-minute plasma sample for determination of the NMR (Benowitz et al. 2006). The nicotine and cotinine clearance were estimated as follows:
\[ Cl_{\text{NIC}} = \frac{Dose_{\text{NIC} - d^2}}{AUC_{\text{NIC} - d^2}} \]

\[ Cl_{\text{COT}} = \frac{Dose_{\text{COT} - d^4}}{AUC_{\text{COT} - d^4}} \]

Where CI is the total body clearance, NIC is nicotine, COT is cotinine, dose is the infusion dose, and AUC is the area under the plasma concentration-time curve extrapolated to infinity. \( f_{\text{NIC} \rightarrow \text{COT}} \) was estimated as follows:

\[ f_{\text{NIC} \rightarrow \text{COT}} = \frac{AUC_{\text{COT} - d^2}}{Dose_{\text{NIC} - d^2}} \times Cl_{\text{COT} - d^4} \]

**Study 2:** The relationship between plasma cotinine, nicotine intake and carcinogen exposure was studied in daily *ad libitum* smokers (table 9). In a cross sectional study of 163 Alaska Native smokers, the plasma cotinine levels urinary TNE, total NNAL and PAH metabolites were measured. A comprehensive description of the study procedures has been published elsewhere (Benowitz et al. 2012).
### Table 9 | Descriptive participant characteristics.

<table>
<thead>
<tr>
<th></th>
<th>1. Pharmacokinetic Study</th>
<th>2. Ad libitum smoking study</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>181</td>
<td>163</td>
</tr>
<tr>
<td>Male</td>
<td>29.9%</td>
<td>43.6%</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Non-smokers</td>
<td>All smokers</td>
</tr>
<tr>
<td>Race (%)</td>
<td>All Caucasians</td>
<td>All Alaska Native people</td>
</tr>
<tr>
<td>Age (years) Mean (SD)</td>
<td>37.7 (11.9)</td>
<td>36.0 (14.4)</td>
</tr>
<tr>
<td>Baseline cigarette per day Mean (SD)</td>
<td>N/A</td>
<td>7.8 (5.2)</td>
</tr>
</tbody>
</table>

**Study descriptions**
- Examines the source of variability in nicotine and cotinine pharmacokinetics
- Examines tobacco consumption behaviors in Alaska Native smokers during *ad libitum* smoking.

**Outcome measures**
- $f_{NIC\_COT}$
- Cotinine clearance
- Urinary TNE, NNAL, 1-hydroxyprene and 1-hydroxyfluorene
- Plasma cotinine levels

#### Study 1 by CYP2A6 genotype

<table>
<thead>
<tr>
<th></th>
<th>CYP2A6 Reduced metabolizers (n=33)</th>
<th>CYP2A6 Normal metabolizers (n=148)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>36 (32,40)</td>
<td>38 (36,40)</td>
</tr>
<tr>
<td>Sex (% Females)</td>
<td>70%</td>
<td>70%</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75 (70,81)</td>
<td>71 (69,74)</td>
</tr>
<tr>
<td>Race (% Caucasians)</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Study 1 by sex**

<table>
<thead>
<tr>
<th></th>
<th>Males (n=54)</th>
<th>Females (n=127)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>40 (37,44)</td>
<td>37 (34,39)</td>
</tr>
<tr>
<td>CYP2A6 genotype (% Reduced metabolizers)</td>
<td>19%</td>
<td>18%</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83 (80,86)</td>
<td>67 (65,69)</td>
</tr>
<tr>
<td>Race (% Caucasians)</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

#### Study 2 by CYP2A6 genotype

<table>
<thead>
<tr>
<th></th>
<th>CYP2A6 Reduced metabolizers (n=33)</th>
<th>CYP2A6 Normal metabolizers (n=148)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>38 (34,41)</td>
<td>35 (32,38)</td>
</tr>
<tr>
<td>Sex (% Females)</td>
<td>53%</td>
<td>47%</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75 (71,78)</td>
<td>77 (72,81)</td>
</tr>
<tr>
<td>Race (% Alaska Native people)</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Study 2 by sex**

<table>
<thead>
<tr>
<th></th>
<th>Males (n=71)</th>
<th>Females (n=92)</th>
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<td>Age</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>(Years)</td>
<td>(33.39)</td>
<td>(33.40)</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>CYP2A6 genotype</td>
<td>34%</td>
<td>54%</td>
</tr>
<tr>
<td>(% Reduced metabolizers)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72 (68,76)</td>
<td>81 (77,85)</td>
</tr>
<tr>
<td>Race (% Alaska Native people)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Smoking status</td>
<td>All current smokers</td>
<td>All current smokers</td>
</tr>
</tbody>
</table>

Values are mean (95% confidence interval) unless otherwise noted. Bold values indicate statistical significance.

**Tobacco and tobacco derived carcinogen exposure biomarkers**

To evaluate the quantitative relationship between plasma cotinine and tobacco exposure in cigarette smokers, we used urinary total nicotine equivalents (TNE) from Study 2 participants as the reference biomarker of nicotine and tobacco exposure as it is the summation of multiple nicotine metabolic pathways. TNE is the total urinary level of nicotine and 8 of its metabolites (i.e. nicotine, nicotine glucuronide, cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, nicotine-N-oxide, cotinine-N-oxide, and nornicotine). Together, the 9 analytes account for about 90% of nicotine (determined from a transdermal administered nicotine dose (Benowitz et al. 1994)), and creatinine adjusted spot urinary TNE correlates with daily tobacco consumption (Benowitz et al. 2010). TNE is not influenced by the different rates of nicotine and cotinine metabolism since it measures the nicotine metabolites generated via different metabolic pathways.

To investigate the relationship between plasma cotinine levels and tobacco-derived carcinogen exposure, we used total urinary (methylnitrosamino)-1-(3) pyridyl-1-butanol (NNAL) levels as the biomarker for tobacco specific nitrosamines exposure in study 2 participants (Hecht 2003). NNAL is a reductive metabolite of the highly carcinogenic 4-(methylnitrosamino)-1-(3) pyridyl-1-butane (NNK); prediagnostic levels are associated with subsequent lung cancer risk (Church et al. 2009).

We also investigated the relationship between plasma cotinine levels and exposure to another class of tobacco-related carcinogens which are not nicotine-derived, the polycyclic aromatic hydrocarbons (Hecht 2003). The urinary levels of two hydroxylated polycyclic aromatic hydrocarbons, 1-hydroxypyrene and 1-hydroxyfluorene, were used as biomarkers of polycyclic aromatic hydrocarbons exposure. 1-Hydroxypyrene levels have been directly associated with
cancer risk (Kamangar et al. 2005), and recent evidence suggests that 1-hydroxyfluorene is more specific to tobacco exposure than 1-hydroxypyrene (StHelen et al. 2012).

**CYP2A6 genotyping**

Prevalent CYP2A6 alleles with altered function were genotyped by two step-allele specific PCR reactions in the Pharmacogenetics laboratory at CAMH and University of Toronto as previously reported (Schoedel et al. 2004; Mwenifumbo et al. 2005; Mwenifumbo et al. 2008; Ho et al. 2009). Those individuals with one or two copies of reduced function allele (*2, *4, *7, *9, *10, *12, *17 and *35) were classified as CYP2A6 RM (Mwenifumbo et al. 2005; Mwenifumbo et al. 2008; Ho et al. 2009).

**The measurement of CYP2A6 activity**

The in vivo CYP2A6 activity was measured using NMR, which is the ratio between plasma 3HC and cotinine. NMR is widely used as an in vivo indicator of CYP2A6 activity (Dempsey et al. 2004; Lerman et al. 2006; Ho et al. 2009; Lerman et al. 2010). In the context of our data analysis, the use of NMR may be confounded with plasma cotinine since it uses plasma cotinine as the denominator. Thus, we use NMR to extend our findings with CYP2A6 genotype as it is a continuous variable and for comparisons to the literature.

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

Plasma nicotine, cotinine and 3HC levels, as well as urinary nicotine and metabolites, total NNAL, and PAH metabolites were quantified by gas/liquid chromatography-tandem mass spectrometry in the Clinical Pharmacology Laboratory at the University of California San Francisco as previously described (Jacob et al. 1991; Dempsey et al. 2004; Jacob et al. 2007; Jacob et al. 2008; Bernert et al. 2011). NMR stratification was done by a median split of plasma NMR within each study population; participants who were in the higher NMR stratum were considered the faster CYP2A6 activity group, while those in the lower NMR stratum were considered the slower CYP2A6 activity group.

**Statistical analysis**
Statistical analyses were performed using R statistical package (version 2.13, R foundation for statistical computing, http://www.R-project.org). In study 1 statistical comparisons between CYP2A6 genotypes and sex were performed using mixed-effect linear regressions. All analyses controlled for non-independence of data in twin pairs by modeling twining as a random effect (Benowitz et al. 2006). Spearman’s correlation was used to evaluate the relationship between 1) NMR and f_{NIC→COT}/Cl_{COT}; 2) NMR and cotinine to TNE ratio; 3) Plasma cotinine and urinary TNE, NNAL, 1-hydroxypyrene and 1-hydroxyflurene. Fisher’s r to z transformation was used to compare the correlation coefficients. To minimize the influence of potential confounders such as race and smoking status on cotinine pharmacokinetics, the effects of CYP2A6 genotype and sex were examined within non-smoking Caucasians in Study 1.

3.4 Results

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

Fig. 10A compares cotinine formation (f_{NIC→COT}) between CYP2A6 genotypes in non-smoking Caucasians. CYP2A6 RM (n=33) had significantly lower f_{NIC→COT} compared to CYP2A6 NM (n=148). In CYP2A6 RM, 67% of the nicotine dose was metabolized to cotinine compared to the 77% in CYP2A6 NM, a 15% relative reduction, P=0.001 (Fig. 10A). CYP2A6 RM also had lower cotinine clearance compared to the CYP2A6 NM (0.58 ml/min/kg in CYP2A6 RM versus 0.77 ml/min/kg in CYP2A6 NM, a 33% reduction, P=0.01, Fig. 10B). The ratio of the fractional conversion of nicotine (reflecting cotinine formation) to cotinine clearance (reflecting cotinine removal), f_{NIC→COT}/Cl_{COT}, was significantly higher in CYP2A6 RM compared to CYP2A6 NM (1.31 in CYP2A6 RM versus 1.12 in CYP2A6 NM, P=0.01, Fig. 10C). Of note, the resulting f_{NIC→COT}/Cl_{COT} ratio is the conversion factor, or average correction needed in plasma cotinine levels to accurately indicate the same nicotine dose (Fig. 10D). These genetic differences were consistent in both males and females (Fig. 11A-F). In agreement with the findings between CYP2A6 genotypes, statistically significant differences in f_{NIC→COT}, cotinine clearance and f_{NIC→COT}/Cl_{COT} were observed when comparing the faster versus the slower NMR strata (Fig. 11G-H). There was a significant inverse correlation between NMR (i.e. a continuous measure of
CYP2A6 activity) and \( f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}} \), suggesting the effect size is even larger when comparing the two extreme ends of CYP2A6 activity (Rho=-0.57, \( P<0.0001 \)).

**Figure 10** | Reduced CYP2A6 activity had a greater impact on cotinine removal than cotinine formation (Study 1).

This would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure. A. *CYP2A6* reduced metabolizers metabolized (n=33) a significantly lower amount of nicotine to cotinine compared to *CYP2A6* normal metabolizers (n=148). B. *CYP2A6* reduced metabolizers (n=33) had lower cotinine clearance (CL_{COT}) compared to *CYP2A6* normal metabolizers (n=148). C. The \( f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}} \) ratio was higher in *CYP2A6* reduced metabolizers (n=33) compared to *CYP2A6* normal metabolizers (n=148). Data presented as mean ± 95% confidence interval. D. The \( f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}} \) ratio is the conversion factor between plasma cotinine levels and the actual nicotine dose.
Figure 11 | The effects of CYP2A6 activity on nicotine and cotinine pharmacokinetics.  
Within the male participants (Study 1): A. CYP2A6 RM (n=10) metabolized a significantly lower amount nicotine to cotinine compared to CYP2A6 NM (n=44). B. CYP2A6 RM (n=10) had lower cotinine clearance (CL_{COT}) compared to CYP2A6 NM (n=44). C. The f_{NIC→COT}/CL_{COT} ratio was higher in CYP2A6 RM compared to CYP2A6 NM.  
Within the female participants (Study 1): D. CYP2A6 RM metabolized a significantly lower amount of nicotine to cotinine compared to CYP2A6 NM. E. CYP2A6 RM had lower cotinine clearance (CL_{COT}) compared to CYP2A6 NM. F. The f_{NIC→COT}/CL_{COT} ratio was higher in CYP2A6 RM compared to CYP2A6 NM.  
With all participants (Study 1): G. Individuals with slower NMR metabolized a significant lower amount of nicotine to cotinine compared to the individuals with faster NMR. H. Individuals with slower NMR had lower cotinine clearance (CL_{COT}) compared to the individuals with faster NMR. I. f_{NIC→COT}/CL_{COT} was higher among individuals in the slower NMR stratum compared to the individuals in the faster NMR stratum. Data presented as mean ± 95% confidence interval.
Different relationships between plasma cotinine and tobacco exposure were observed between smokers with different CYP2A6 genotype groups (Study 2)

Next, the quantitative relationship between plasma cotinine and nicotine intake in smokers was investigated. Demographic variable comparisons are shown in table 9. Independent regression lines between urinary TNE and plasma cotinine were constructed in CYP2A6 RM and NM (n=74 and n=89, respectively). As illustrated by fig. 12, the slope of the regression line in CYP2A6 RM was significantly lower compared to the slope in CYP2A6 NM (slope: 0.33 in CYP2A6 RM versus 0.42 in CYP2A6 NM, \( P=0.001 \), Fig. 12). Statistical significance is demonstrated by a significant interaction between CYP2A6 genotype and cotinine levels in the linear regression analysis presented in table 10A. A similar difference in regression line slopes was observed between the NMR strata (Fig. 13). There was a significant inverse correlation between NMR, a continuous measure of CYP2A6 activity, and the cotinine to TNE ratio (Rho=-0.46, \( P<0.0001 \)). These observations indicate that cotinine predicts nicotine intake, and therefore tobacco consumption, differently in smokers with reduced compared to normal CYP2A6 activity. As illustrated by fig. 12, a 300 ng/mL (which is equivalent to 1702 nmol/L) cotinine level was indicative of a 125 nmol nicotine equivalents exposure in CYP2A6 NM, whereas the same cotinine level was indicative of roughly 100 nmol exposure in CYP2A6 RM. Alternatively, at 100 nmol tobacco exposure, CYP2A6 RM would have 300 ng/mL plasma cotinine levels, whereas the CYP2A6 NM would have only 240 ng/mL. This is a 25% difference (60 ng/mL or 340 nmol/L) in cotinine levels between CYP2A6 genotype groups. Since reduced metabolizers include those with a range of decreased activity combined together, the 25% reflects an average difference (Fig. 14A). As illustrated by some specific genotypes, or quartiles of NMR, these differences in cotinine estimates of dose can be \( \approx 2 \) fold when comparing between the extremes of CYP2A6 activity or among different genotypes (fig. 14B and fig. 14C).
Figure 12 | Cotinine’s ability to predict tobacco exposure was different between CYP2A6 genotypes (Study 2).

The slope between urinary TNE and plasma cotinine was significantly lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, table 10A), suggesting the quantitative relationship between cotinine and tobacco exposure (i.e. TNE) differed between CYP2A6 genotypes. # indicates statistical significant difference in Spearman’s Rho compared to the CYP2A6 normal metabolizers. The numbers after the slopes are standard error. Of note, the strength of correlations between plasma cotinine and urinary TNE in the CYP2A6 RM was significantly weaker than in CYP2A6 NM (Fisher’s r to Z transformation: P<0.01).
Table 10 | Regression analyses of the predictive ability of cotinine and *CYP2A6* genotype (or sex) on tobacco and tobacco-derived carcinogen exposure (Study 2).

<table>
<thead>
<tr>
<th>Y=TNE</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>( r^2=0.86 ) ( P&lt;0.0001 )</td>
<td>B</td>
<td>( \beta )</td>
<td>95% CI</td>
<td>P</td>
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<tr>
<td>Cotinine (Increasing, per ng/mL)</td>
<td>0.42</td>
<td>0.94</td>
<td>0.38 to 0.45</td>
<td>0.001</td>
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<tr>
<td><em>CYP2A6</em> genotype (reduced)</td>
<td>17.1</td>
<td>0.19</td>
<td>2.1 to 32.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Interaction term</td>
<td>-0.16</td>
<td>-0.39</td>
<td>-0.24 to -0.08</td>
<td>0.001</td>
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</table>

<table>
<thead>
<tr>
<th>Y=NNAL</th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>( r^2=0.78 ) ( P&lt;0.0001 )</td>
<td>B</td>
<td>( \beta )</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Cotinine (Increasing, per ng/mL)</td>
<td>1.7</td>
<td>0.81</td>
<td>1.5 to 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td><em>CYP2A6</em> genotype (reduced)</td>
<td>56.2</td>
<td>0.13</td>
<td>-22.4 to 134.9</td>
<td>0.160</td>
</tr>
<tr>
<td>Interaction term</td>
<td>-0.7</td>
<td>-0.33</td>
<td>-1.1 to -0.2</td>
<td>0.003</td>
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</table>

<table>
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<th>Y=NNAL</th>
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<tbody>
<tr>
<td>( r^2=0.84 ) ( P&lt;0.0001 )</td>
<td>B</td>
<td>( \beta )</td>
<td>95% CI</td>
<td>P</td>
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<tr>
<td>TNE (increasing, per nmol)</td>
<td>3.9</td>
<td>0.83</td>
<td>3.6 to 4.2</td>
<td>0.001</td>
</tr>
<tr>
<td><em>CYP2A6</em> genotype (reduced)</td>
<td>31.2</td>
<td>0.07</td>
<td>-30.3 to 92.8</td>
<td>0.318</td>
</tr>
<tr>
<td>Interaction term</td>
<td>-0.6</td>
<td>-0.11</td>
<td>-1.5 to 0.3</td>
<td>0.21</td>
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<table>
<thead>
<tr>
<th>Y=1-hydroxyfluorene</th>
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</tr>
</thead>
<tbody>
<tr>
<td>( r^2=0.73 ) ( P&lt;0.0001 )</td>
<td>B</td>
<td>( \beta )</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Cotinine (Increasing, per ng/mL)</td>
<td>0.027</td>
<td>0.76</td>
<td>0.024 to 0.030</td>
<td>0.001</td>
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<tr>
<td><em>CYP2A6</em> genotype (reduced)</td>
<td>1.63</td>
<td>0.22</td>
<td>0.108 to 3.15</td>
<td>0.036</td>
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<td>Interaction term</td>
<td>-0.01</td>
<td>-0.314</td>
<td>-0.185 to -0.002</td>
<td>0.013</td>
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</table>

<table>
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<th>Y=1-hydroxypyrene</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>( r^2=0.38 ) ( P&lt;0.0001 )</td>
<td>B</td>
<td>( \beta )</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Cotinine (Increasing, per ng/mL)</td>
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<td>0.42</td>
<td>0.011 to 0.017</td>
<td>0.001</td>
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<td><em>CYP2A6</em> genotype (reduced)</td>
<td>0.800</td>
<td>0.12</td>
<td>-0.733 to 2.330</td>
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<tr>
<td>Interaction term</td>
<td>-0.007</td>
<td>-0.24</td>
<td>-0.016 to 0.0009</td>
<td>0.079</td>
</tr>
</tbody>
</table>
Figure 13 | Cotinine’s ability to predict tobacco exposure was different between NMR strata (Study 2).
The slope between urinary TNE and plasma cotinine was lower in CYP2A6 RM compared to that of CYP2A6 NM, suggesting cotinine over estimates tobacco exposure in CYP2A6 RM. Regression analysis was not performed due to the interrelatedness of plasma cotinine and NMR (i.e. NMR is the ratio 3HC to plasma cotinine levels). The numbers after the slopes are standard error.
Figure 14 | Cotinine to TNE ratio significantly differ between individuals with difference CYP2A6 activity.
A). The cotinine to TNE ratio (i.e. cotinine levels per nicotine intake) was significantly lower in CYP2A6 NM compared to the CYP2A6 RM. (Study 2) B). The cotinine to TNE ratio decreased with CYP2A6 genotypes with increasing activity (Study 2). As illustrated using some different CYP2A6 genotypes, containing reduced function (*9) or loss of function (*4) allele compared to the wild type individuals (*1/*1). Of note, individuals who are fully null for CYP2A6 (i.e. these with two copies of gene deletions, CYP2A6*4/*4) had unexpectedly lower COT to TNE ratio compared to the wild-type individuals (*1/*1, data not shown), suggesting the minor remaining cotinine formation pathway (likely CYP2B6 or CYP2A13) is considerably slower than the low affinity cotinine removal pathway (likely UGT2B10 glucuronidation or renal clearance). C). The cotinine to TNE ratio decreased by NMR quartiles (Study 2). Data presented as mean ± 95% confident intervals. Statistical comparisons were performed by Mann Whitney or Kruskal-Wallis tests.
Different relationships between plasma cotinine and tobacco specific nitrosamine exposures were observed in smokers with different CYP2A6 genotype groups (Study 2)

Next, we investigated the quantitative relationship between plasma cotinine and tobacco specific nitrosamines exposure in smokers. As seen with TNE, the slope of the regression line between urinary NNAL and plasma cotinine in CYP2A6 RM was significantly lower compared to the slope in CYP2A6 NM (slope: 1.279 in CYP2A6 RM versus 1.687 in CYP2A6 NM, P=0.003, fig. 15. Statistical significance demonstrated by a significant interaction term in the linear regression analysis presented in table 10B). These results demonstrated that that cotinine levels predict NNAL levels, and therefore nitrosamine exposure, differently in smokers with reduced compared to normal CYP2A6 activity. As illustrated by fig. 15, a 300 ng/mL (which is equivalent to 1702 nmol/L) cotinine level was indicative of a 510 pg/mg creatinine NNAL exposure in CYP2A6 NM, whereas the same cotinine level was indicative of roughly 375 pg/mg creatinine NNAL exposure in CYP2A6 RM. Alternatively, at 375 pg/mg creatinine NNAL exposure, CYP2A6 RM would have 300 ng/mL plasma cotinine levels, whereas the CYP2A6 NM would have only 225 ng/mL. The magnitude of the CYP2A6 genotype effect (25%) on plasma cotinine levels was similar to that observed with TNE as the exposure marker; as with the TNE biomarker this is an underestimation of effect size for some genotype or phenotype comparisons (see figure 14).

To determine whether the above observations were unique to plasma cotinine, independent regression lines between urinary NNAL and urinary TNE levels were constructed in CYP2A6 RM and NM. The slope of the regression lines did not differ between CYP2A6 genotypes (fig 16, and table 10C), indicating that as expected TNE’s relationship to tobacco specific nitrosamines exposure in smokers was independent of CYP2A6 genotype. Together this demonstrates that the impact of CYP2A6 genotype on plasma cotinine levels was specific to cotinine as an exposure biomarker.
Figure 15 | Cotinine’s ability to predict tobacco specific nitrosamines exposure (as indicated by NNAL levels) was different between CYP2A6 genotypes (Study 2). The slope between urinary NNAL levels and plasma cotinine was significantly lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, table 10). This suggested the quantitative relationship between cotinine and tobacco specific nitrosamines exposure differed between CYP2A6 genotypes. The numbers after the slopes are standard error.
Figure 16 | TNE’s ability to predict tobacco specific nitrosamines exposure is independent of CYP2A6 genotype (Study 2).
Different relationships between plasma cotinine and polycyclic aromatic hydrocarbon exposure were observed in smokers with different CYP2A6 genotype groups (Study 2)

Next, we investigated the quantitative relationship between plasma cotinine and polycyclic aromatic hydrocarbon exposure in smokers. As illustrated by fig. 17 and fig. 18, the slopes of the regression lines in CYP2A6 RM were lower compared to the slope in CYP2A6 NM (1-hydroxyfluorene slopes: 0.024 in CYP2A6 RM vs. 0.027 in CYP2A6 NM, \( P=0.013 \), fig. 18 and table 10D; 1-hydroxypyrene slopes: 0.010 in CYP2A6 RM versus 0.014 in CYP2A6 NM, \( P=0.079 \), Fig. 17 and table 10E).

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

Because CYP2A6 activity differs between the sexes, we investigated the impact of sex on cotinine formation and cotinine clearance. The \( f_{\text{NIC} \rightarrow \text{COT}} \) did not differ between the sexes (74% in males compared to 75% in females, non-significant, Fig. 19A). However males had a significantly lower cotinine clearance compared to females (0.58 versus 0.81 ml/min/kg in males and females, respectively, \( P<0.001 \), Fig. 19B). A significantly higher \( f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}} \) ratio, which is the conversion factor between plasma cotinine levels and the nicotine dose (Fig. 10D), was observed in males compared to females (1.39 versus 1.12 in males and females, respectively, \( P=0.001 \), Fig. 19C). As observed with the entire study population, similar impacts of sex on \( f_{\text{NIC} \rightarrow \text{COT}} \), cotinine clearance and \( f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}} \) could be observed when examined only within the subgroup of CYP2A6 NM (i.e. excluding the CYP2A6 RM, Fig. 20).

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

Independent regression lines between urinary TNE and plasma cotinine were constructed by sex. The slope of the regression line in males was significantly lower compared to the slope in females (0.34 in males versus 0.42 in females, \( P=0.01 \), Fig. 21 and Table 10F). The magnitude of the sex effect on cotinine levels was similar to the effect of CYP2A6 genotype. At 100 nmol tobacco exposure, there is a 25% difference in plasma cotinine levels (60 ng/mL or 340 nmol/L) between the sexes. As observed with the entire study population, similar impacts of sex could be
observed when examined within just the CYP2A6 NM (slopes: 0.36±0.02 in males versus 0.54±0.03 in females, \(P=0.001\)).

Figure 17 | Cotinine’s ability to predict polycyclic aromatic hydrocarbon exposure (as indicated by 1-hydroxypyrene) was different between CYP2A6 genotypes (Study 2). The slope between 1-hydroxypyrene levels and plasma cotinine was lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, table 10E). The numbers after the slopes are standard error.
Figure 18 | Cotinine’s ability to predict polycyclic aromatic hydrocarbon exposure (as indicated by 1-hydroxyfluorene) was different between CYP2A6 genotypes (Study 2). The slope between 1-hydroxyfluorene levels and plasma cotinine was significantly lower in CYP2A6 reduced metabolizers (n=74) compared to that of CYP2A6 normal metabolizers (n=89, supplementary table 10D). The numbers after the slopes are standard error.
Figure 19 | Males (n=54), who have reduced CYP2A6 activity compared to females (n=127), had a greater ratio of cotinine formation over cotinine removal, which would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure in male smokers (Study 1).

A). The $f_{\text{NIC} \rightarrow \text{COT}}$ did not differ between the sexes. B). Males had significantly lower $\text{CL}_{\text{COT}}$ compared to the females. C). Males had a higher $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$ compared to females indicating an over-estimation of nicotine dose for their cotinine levels. Data presented as mean ± 95% confidence interval.
Among the CYP2A6 normal metabolizers (Study 1, n=148), males, who have reduced CYP2A6 activity compared to females, had a greater ratio of cotinine formation over removal. This would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure in male smokers. **A**). The $f_{\text{NIC} \rightarrow \text{COT}}$ did not differ between the sexes. **B**). Males had significantly lower $\text{Cl}_{\text{COT}}$ compared to the females. **C**). Males had a higher $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ compared to females. Data presented as mean ± 95% confidence interval.

**Figure 20** | Among the CYP2A6 normal metabolizers (Study 1, n=148), males, who have reduced CYP2A6 activity compared to females, had a greater ratio of cotinine formation over removal. This would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure in male smokers. **A**). The $f_{\text{NIC} \rightarrow \text{COT}}$ did not differ between the sexes. **B**). Males had significantly lower $\text{Cl}_{\text{COT}}$ compared to the females. **C**). Males had a higher $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ compared to females. Data presented as mean ± 95% confidence interval.
Cotinine's ability to predict tobacco exposure was different between the sexes. The slope between urinary TNE and plasma cotinine was significantly lower in males (n=71) compared with that of females (n=92, Table 10F), suggesting cotinine overestimates tobacco exposure (i.e., TNE) in males compared with females (Study 2). The numbers after the slopes are standard error.

3.5 Discussion

Our analyses demonstrate that variation in CYP2A6 metabolic activity, such as those that exist between CYP2A6 genotypes or the sexes, may alter cotinine removal (i.e. cotinine clearance) to a different extent than cotinine formation (i.e. $f_{\text{NIC} \to \text{COT}}$). Individuals with lower CYP2A6 activities, such as CYP2A6 RM and males, have higher ratios of cotinine formation to cotinine removal (i.e. $f_{\text{NIC} \to \text{COT}} / \text{CL}_{\text{COT}}$) compared to individuals with faster CYP2A6 activity, such as CYP2A6 NM and females. Thus, the quantitative relationship between plasma cotinine and tobacco and tobacco derived carcinogen exposures in smokers varies between those with different CYP2A6 activity.
The pharmacokinetic mechanism

The greater effect of altered CYP2A6 activity on cotinine clearance compared to formation is likely related to differences in nicotine and cotinine’s hepatic metabolism. Nicotine exhibits a higher hepatic extraction ratio compared to cotinine, which is largely due to differences in their intrinsic clearances. The intrinsic clearance ($\frac{V_{\text{max}}}{K_m}$) of nicotine is $1.69 \, \mu$L/min/mg protein in human liver microsomes, which is 10 times faster than the $0.16 \, \mu$L/min/mg protein of cotinine (Benowitz et al. 1983; Nakajima et al. 1996; Nakajima et al. 1996; Hukkanen et al. 2005). For drugs like nicotine, with high extraction ratios, clearance is determined in large part by liver blood flow and is less affected by changes in liver enzymatic activity. In contrast for low extraction drugs like cotinine clearance is largely affected by changes in liver enzymatic activity. This differential impact of variation in CYP2A6 activity is consistent with previous observations on the effects of CYP2A6 induction. Estrogen induces CYP2A6 protein expression in humans (Higashi et al. 2007). During pregnancy, when estrogen levels are elevated, the clearance of nicotine increases by 60% whereas the clearance of cotinine increased by 140% (Dempsey et al. 2002). Thus, higher CYP2A6 activity during pregnancy has a greater effect on cotinine clearance than nicotine clearance.

CYP2A6, sex and race

Consistent with a higher $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ ratio predicting higher cotinine levels for the same tobacco and tobacco-derived carcinogen exposure (equation 1), we observed different quantitative relationships between cotinine and tobacco and tobacco-derived carcinogen exposure in smokers with reduced CYP2A6 activities (CYP2A6 RM and males). These observations provide a mechanistic explanation for the systematic variation in plasma cotinine levels previously observed between CYP2A6 genotypes, the sexes and possibly races (Gan et al. 2008; Ho et al. 2009; Timofeeva et al. 2011).

Our observations explain the directional inconsistencies between cotinine and other indicators of tobacco consumption in genetic association studies involving CYP2A6 (Ho et al. 2009; Timofeeva et al. 2011). Due to the higher $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ ratio, CYP2A6 RM would have higher plasma cotinine levels upon the same tobacco exposure compared to the CYP2A6 NM. This masks, or reduces, the true size of the effect of CYP2A6 genotype on tobacco consumption based
on plasma cotinine (i.e. CYP2A6 RM have higher cotinine levels with lower tobacco consumption). These results could also explain why the same CYP2A6 genetic variants have been associated with lower lung cancer risk while having paradoxically higher plasma cotinine levels (Timofeeva et al. 2011).

Similarly, during transdermal nicotine patch treatment (i.e. one week after starting treatment) steady state plasma cotinine levels in biochemically confirmed abstinent individuals (Lerman et al. 2006) were inversely related to the pre-treatment CYP2A6 activity (Fig. 22). The 25% (50 ng/mL) average differences in cotinine levels between participants with faster and slower CYP2A6 activity (NMR) was in agreement with the effect size observed in smokers in study 2. The difference double to 50% (100 ng/mL) when comparing those in the first versus fourth quartile of CYP2A6 activity. Thus for the same systemic nicotine exposure (delivered by 21mg patch for all participants), the individuals with slower CYP2A6 activity had higher steady state cotinine levels than the individuals with faster CYP2A6 activity (Fig. 22).
A nicotine patch study: abstinent individuals with slower NMR have higher steady state plasma cotinine compared to individuals with faster NMR after receiving the same 21 mg nicotine patch.

The solid black bar represents the mean cotinine levels for the faster NMR stratum (on a median split). The dashed-bar represents the mean cotinine levels for the slower NMR stratum. The analyses were done in the biochemically verified abstinent individuals only. Statistical comparisons were performed by non-parametric Kruskal-Wallis (all 4 quartiles) or Mann-Whitney tests (between the faster and slower strata). Data presented as mean ± 95% confidence interval.

**Figure 22**
Our results could explain the systematic variation in plasma cotinine levels between the sexes (Raunio et al. 2001). Due to the lower average estrogen levels, males have lower CYP2A6 protein expression and activity compared to females (Al Koudsi et al. 2010). Thus, males have higher $f_{\text{NIC}\rightarrow\text{COT}}/\text{CL}_{\text{COT}}$ ratios, which suggest that males would have higher plasma cotinine levels compared to females with similar tobacco exposure.

Finally, our observation could also explain the differences in plasma cotinine levels between Caucasians and African Americans even when controlling for tobacco exposure. About 50% of African Americans are CYP2A6 RM compared to the 20% in Caucasians (Haberl et al. 2005). Therefore, African Americans have, on average, higher $f_{\text{NIC}\rightarrow\text{COT}}/\text{CL}_{\text{COT}}$ ratios compared to Caucasians, resulting in higher plasma cotinine levels for similar tobacco exposure.

**Implications for cancer research**

Plasma cotinine is often used as an objective, non-self-report, indicator of tobacco exposure in smoking-related cancer research. However, there could be a 25% or greater variation in plasma cotinine levels following the same tobacco and tobacco-derived carcinogen exposure depending on CYP2A6 genotype or in the presence of other inducers/inhibitors of CYP2A6 activity. To put this difference in perspective, Munafo et al. reported that each risk allele in rs16969968, a single nucleotide polymorphism in the α5 nicotinic acetylcholine receptor, is associated with a 138.4 nmol/L (or 24 ng/mL) increase in plasma cotinine levels, which in turn was predicted to increase lung cancer risk by 1.3 times (Munafo et al. 2012). Our data suggest that the difference between CYP2A6 genotypes (or sex) would be approximately 340 nmol/L (or 60 ng/mL) at 100 nmol tobacco exposure (or roughly 375 pg/mg Cre NNAL). Hence, cotinine levels alone, without considering CYP2A6 activity, may not provide the most accurate information regarding tobacco and tobacco-derived carcinogen exposure in smokers, particularly when comparing groups with different frequencies of CYP2A6 genotypes (or race) or sex. Biomarkers of nicotine intake such as TNE that are less dependent on individual differences in nicotine metabolism or biomarkers of toxicants that are more directly involved in the carcinogenic process, will enhance our understanding of the contribution of variable levels of tobacco consumption on lung cancer risk.
In conclusion, the quantitative relationship between plasma cotinine level and tobacco and tobacco-derived carcinogen exposure differs among groups with different levels of CYP2A6 activity. These comparison groups include the different CYP2A6 genotype groups within a race (e.g. CYP2A6 RM versus CYP2A6 NM among Caucasians) and different races with distinct prevalence of CYP2A6 RM (e.g. Caucasians with 20% CYP2A6 RM versus African Americans with 50% CYP2A6 RM). Furthermore, groups with different estrogen levels may also have variation in cotinine levels unrelated to differences in tobacco exposure. Those include different sexes, or those with varying pregnancy and menopause statuses. Therefore cotinine levels, as a quantitative marker of tobacco and tobacco-derived carcinogen exposure, are not directly comparable between CYP2A6 genotypes, sexes, and races.
3.6 Significance to Thesis

This chapter contributes to the existing literature in two ways. First, it provided some caveats when using cotinine as a biomarker of tobacco consumption. Plasma cotinine is often used as an objective, non-self report, biomarker of tobacco consumption in smoking-related research. There are currently more than 30000 publications, including epidemiological studies, clinical trials, genetic studies and laboratory studies, which used cotinine as a biomarker of tobacco consumption and exposure to secondhand smoke. The findings of this chapter suggest that cotinine has significant limitations as a biomarker of tobacco consumption. There could be a 25% or greater variation in plasma cotinine levels following the same tobacco consumption.

Second, the findings of this chapter provided a mechanistic explanation for the inconsistent findings in the field of nicotine biomarkers. In chapter 1, we observed that higher CYP2A6 activity was paradoxically associated with higher TNE levels (suggesting higher tobacco consumption) and lower cotinine levels (suggesting lower tobacco consumption). The findings of chapter 2 provided a mechanistic explanation for this paradoxical finding. Our findings in chapter 2 suggested that the utilization of better biomarkers, such as TNE (which is less dependent on individual differences in nicotine metabolism) or NNAL (which is more directly involved in the carcinogenic process), could enhance our understanding of the contribution of tobacco consumption to disease risks.
4 CHAPTER 3: *CHRNA5-A3-B4* GENETIC VARIANTS ALTER NICOTINE INTAKE AND INTERACT WITH TOBACCO USE TO INFLUENCE BODY WEIGHT IN ALASKA NATIVE TOBACCO USERS

Andy Z. X. Zhu, Caroline C. Renner, Dorothy K. Hatsukami, Neal L. Benowitz

& Rachel F. Tyndale

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DKH, CCR, NLB and RFT designed the study. CCR recruited the participants. NLB determined the levels of tobacco consumption biomarkers. AZZ performed the DNA extraction from blood and plated the DNA for genotyping. AZZ designed the haplotype tagging block and conducted the genotyping. AZZ performed all analyses and modeling. AZZ and RFT wrote the manuscript.
4.1 Abstract

**Aims:** Gene variants in *CHRNA5-A3-B4*, which encodes for the α5, α3 and β4 nicotinic receptor subunits, are associated with altered smoking behaviors in Caucasians. Little is known about *CHRNA5-A3-B4* and its association with smoking behaviors and weight in Alaska Native people; a population with high prevalence but low levels of tobacco consumption, a greater prevalence of smokeless tobacco use, and high rates of obesity. We investigated *CHRNA5-A3-B4* genetic architecture and its association with nicotine intake and obesity in Alaska Native people.

**Design, Setting, Participants:** A cross sectional study of 400 Alaska Native individuals including 290 tobacco users of which 163 were cigarette smokers.

**Measurements:** *CHRNA5-A3-B4* genotype, body weight, and tobacco consumption biomarkers such as plasma cotinine and urinary total nicotine equivalents (TNE).

**Findings:** Alaska Native people have a unique *CHRNA5-A3-B4* genetic architecture. In 290 Alaska Native tobacco users, the ‘G’ allele of rs578776, which uniquely tagged a 30kb haplotype in *CHRNA5-A3-B4*, was prevalent (16%) and significantly associated with nicotine intake (20% higher plasma cotinine, *P*<0.001, 16% higher TNE, *P*=0.076), while rs16969968 was not associated with nicotine intake. Rs578776 acted in combination with CYP2A6, the main nicotine-metabolizing enzyme, to increase TNE by 1.8 fold compared to the low risk group (*P*<0.001). Furthermore a *CHRNB4* variant, rs2869950, was significantly associated with increased BMI (*P*<0.01) in the tobacco users even after controlling for differences in nicotine intake (*P*<0.01).

**Conclusions:** Genetic variants in *CHRNA5-A3-B4* alter nicotine intake and BMI in a population with distinct genetic structure, smoking behaviors and prevalence of obesity.
4.2 Introduction

Smoking and obesity are the greatest preventable causes of premature death. Alaska Native people experience a higher incidence rate of lung cancer than the United States national average (Centers for Disease Control and Prevention 2010), despite reporting on average lower tobacco consumption compared to Caucasian Americans (United States Public Health Service. Office of the Surgeon General. 1998) as observed in our study population of the Alaska Native smokers in the Bristol Bay region (Renner et al. 2013). More than 70% of Alaska Native adults are also considered over-weight or obese (Behavioral Risk Factor Surveillance System 2008). Although smoking and obesity appear to be distinct epidemics with different etiologies, smoking and body weight are intertwined in many ways. Here we investigate whether gene variants in the α5, α3, and β4 nicotinic acetylcholine receptor gene cluster (CHRNA5-A3-B4) are associated with nicotine intake and weight in Alaska Native people as previously observed in Caucasian smokers (Thorgeirsson et al. 2008; Freathy et al. 2011).

Nicotine is the major psychoactive component of tobacco. A number of independent genome wide association studies demonstrate a significant association between CHRNA5-A3-B4 gene variants and self-reported smoking quantity as well as with the incidence rate of lung cancer (Amos et al. 2008; Hung et al. 2008; Thorgeirsson et al. 2008; Thorgeirsson et al. 2010). Currently, most association studies are conducted in Caucasians; little is known about the haplotype structure of CHRNA5-A3-B4 and its relationship with nicotine intake in racial minority groups such as Alaska Native people.

Multiple independent studies have identified a strong association between tobacco consumption and rs16969968 and its correlated SNP rs1051730 (Thorgeirsson et al. 2008; Keskitalo et al. 2009; Saccone et al. 2009; Thorgeirsson et al. 2010). For example, the ‘AA’ genotype of rs16969968 is associated with an increase of roughly one cigarette per day (CPD), 24-100 ng/mL in cotinine, ≈15 nmol in nicotine equivalents and a 1.6 fold higher odds ratio of developing lung cancer when compared to the ‘GG/GA’ genotype group (Le Marchand et al. 2008; Keskitalo et al. 2009; Wassenaar et al. 2011; Munafo et al. 2012). In addition to rs16969968-rs1051730, other independent SNPs in CHRNA5-A3-B4 have also been reported to affect self-reported smoking
quantity after adjusting for the effect of rs16969968-rs1051730 (Chen et al. 2012). These include rs588765 and correlated SNPs as well as rs578776 and correlated SNPs (Saccone et al. 2010).

The first objective of our study was to characterize the haplotype structure of *CHRNA5-A3-B4* gene cluster in Alaska Native people and to evaluate the impact of genetic variants in *CHRNA5-A3-B4* on nicotine intake and tobacco-derived carcinogen exposure. Variation in *CHRNA5-A3-B4* and *CYP2A6*, the latter is the main nicotine-metabolizing enzyme, were previously shown in Caucasians to act in combination to alter CPD (and lung cancer risk) (Wassenaar et al. 2011). Here, we directly compared the quantitative effects of the variants in these two genes on self-report and objective biomarkers of nicotine intake.

Our second objective was to explore the association between *CHRNA5-A3-B4* gene variants and body weight. Like smoking, the prevalence of obesity is very high (37.5%) in Alaska Native people, and another 34.3% are considered overweight (Behavioral Risk Factor Surveillance System 2008). Nicotine both increases metabolic rate and suppresses the expected compensatory increase in appetite and feeding. Smokers on average weigh less than nonsmokers; and smokers typically gain 4 to 5 kg when they stop smoking (Seeley et al. 2011). This can be attributed to the activation of sympathetic nervous system peripherally and nicotine’s ability to activate α3β4 nicotinic receptors located on POMC (Pro-opiomelanocortin) neurons in the arcuate nucleus of the hypothalamus centrally (Audrain-McGovern et al. 2011; Mineur et al. 2011). Since *CHRNA5-A3-B4* encodes for the α3β4 nicotinic receptor, it is possible that *CHRNA5-A3-B4* gene variants can modulate nicotine’s ability to regulate body weight by altering α3β4 nicotinic receptor function. Previous research suggests that a nicotine intake altering *CHRNA5-A3-B4* variant, rs1051730, is associated with altered body weight (Freathy et al. 2011). Yet it is not clear whether this association is an indirect effect of rs1051730 altering nicotine intake or a direct effect of rs1051730 altering nicotine’s effect on body weight, possibly by modulating nicotinic receptor function (Freathy et al. 2011). In this study, we seek to replicate and extend the association between *CHRNA5-A3-B4* variants and body weight by looking at the impact of *CHRNA5-A3-B4* variants on body weight with, and without, controlling for nicotine intake. Together, these findings will further our understanding of the role of genetic variations in the nicotinic receptor genes in two pressing public health issues, smoking and obesity.
4.3 Material and methods

Study Design

A detailed description of recruitment procedures, participant demographics and tobacco related behaviors has been reported previously (Renner et al. 2013). Briefly, 400 Alaska Native individuals were recruited in local villages near Bristol Bay, Alaska. The total current tobacco user group (n=290) included 163 cigarette smokers, 76 commercial smokeless tobacco users, 20 iqmik (a type of smokeless tobacco used with alkaline ash) users, and 31 mixed products users. The non-tobacco user group included 82 former smokers and 28 never users. Ethics approval was obtained from Alaska Area IRB, the Bristol Bay Area Health Corporation Board and Ethics Committee, UCSF and the University of Toronto.

CHRNA5-A3-B4 Genotyping

Seventeen SNPs in the CHRNA5-A3-B4 gene cluster were genotyped using Applied Biosystem Taqman genotyping assays. The CHRNA5-A3-B4 SNPs were selected to 1) represent loci which have been previously associated with smoking behavior (e.g. rs16969968-rs1051730; rs588765, and rs7164030-rs578776) and 2) tag the region between CHRNA5 and CHRNA3 (where most of the previous significant associations with smoking behavior have been observed) based on the ‘CHB’ (Han Chinese) and ‘JPT’ (Japanese) datasets of the International Hapmap Project release #28. The position and minor allele frequency of the genotyped SNPs are listed on Table 11.
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The measurement of CYP2A6 activity

We estimated in vivo CYP2A6 activity previously using the plasma ratio of tran-3’-hydroxycotinine to cotinine (also known as nicotine metabolite ratio, NMR) (Binnington et al. 2012). Using a median split of plasma NMR within the total tobacco user group (n=290) as described previously (Zhu et al. 2013), participants who were in the higher NMR stratum were considered the faster CYP2A6 activity group, while those in the lower NMR stratum were considered the lower CYP2A6 activity group.

Plasma and urinary biomarkers

We used a panel of biomarkers to assess nicotine intake and tobacco-derived carcinogen exposure. To evaluate nicotine intake, plasma cotinine as well as the urinary sum of nicotine and 8 of its metabolites is known as total nicotine equivalents (TNE) and accounts for about 90% of an administered nicotine dose (Benowitz et al. 1994). Total urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) was used to evaluate tobacco specific nitrosamine exposure (Hecht 2003; Church et al. 2009). 1-Hydroxyfluorene was used in this study as a biomarker of tobacco related polycyclic aromatic hydrocarbons exposure (StHelen et al. 2012). Plasma cotinine and tran-3’-hydroxycotinine levels, as well as urinary TNE, total NNAL, and 1-hydroxyfluorene were quantified by gas/liquid chromatography-tandem mass spectrometry as previously described (Dempsey et al. 2004; Jacob et al. 2007; Jacob et al. 2008; Bernert et al. 2011).

Statistical Analyses

Statistical analyses were performed using ‘R’ version 2.14 (R foundation for statistical computing). The association between CHRNA5-A3-B4 gene variants and biomarkers of tobacco and tobacco derived carcinogen exposure were assessed by ‘PLINK’ (Purcell et al. 2007). Haplotyping results of CHRNA5-A3-B4 were obtained using the ‘Haploview’ software (Barrett et al. 2005). The biomarkers (plasma cotinine, urinary TNE, NNAL and 1-hydroxyfluorene levels) were normalized by log transformation before statistical analyses. With regard to nicotine intake and tobacco derived carcinogen exposure, no multiple comparison adjustments were used since we considered our analyses a replication and extension of previous findings in a new racial population. Since there were 12 independent loci, we considered P values below 0.0042 (i.e.
0.05/12) statistically significant for the assessment of the association between \textit{CHRNA5-A3-B4} and BMI.

4.4 Results

The \textit{CHRNA5-A3-B4} gene cluster structure in Alaska Native people

The minor allele frequency of the 17 genotyped SNPs in comparison to other racial groups is listed in Table 11. All SNPs were in Hardy-Weinberg equilibrium. A haplotype analysis revealed a 30 KB region of high linkage disequilibrium (LD) between \textit{CHRNA5} and \textit{CHRNA3} (i.e. a haplotype block between rs569207 and rs6495308, Fig. 23). Within this haplotype block, four haplotypes with a prevalence of more than 1% were identified (Fig. 23 Insert), the total prevalence of these four haplotypes was 99.9% in the 400 participants. The most common haplotype, with a 77.6% frequency, was designated as haplotype A. The remaining haplotypes were designated to haplotype B, C and D according to their prevalence respectively. As illustrated by the insert of fig. 23, these four haplotypes could be discerned by a few tag SNPs. For example, haplotype A was uniquely tagged by rs569207, and haplotype B was uniquely tagged by rs578776.
Figure 23 | Observed CHRNA5-A3-B4 haplotype structure in Alaska Native people. The shade and number in the squares indicate the D’ score. Insert: A haplotype analysis revealed a 30kb region of high linkage disequilibrium between CHRNA5 and CHRNA3. Within this haplotype block, four haplotypes with a prevalence of more than 1% were identified. These four haplotype could be discerned by a few tag SNPs. For example, haplotype A was uniquely tagged by rs569207 (marker #5), while haplotype B was uniquely tagged by rs578776 (marker #8)
The association between gene variants in \textit{CHRNA5-A3-B4} and nicotine intake

The associations (by additive models) between \textit{CHRNA5-A3-B4} gene variants and nicotine intake and tobacco-derived carcinogen exposure are primarily evaluated in the total tobacco users group (n=290). The associations within the smokers (n=163) and smokeless tobacco users (n=76) are also presented to illustrate the effect in these subgroups. The results are summarized in Table 12 with a few key findings outlined below.

\textbf{Rs578776:} The ‘G’ allele of rs578776, which uniquely identified haplotype B in Alaska Native people, was significantly associated with higher cotinine levels in the total tobacco users group and in the cigarette smokers (Fig. 24A and Table 12). As illustrated by fig. 24A, each ‘G’ allele of rs578776 was associated with a roughly 20\% (33 ng/mL) increase in plasma cotinine levels in the total tobacco users group and a roughly 22\% (33 ng/mL) increase in the smokers ($P<0.001$ and $P=0.008$, respectively). When evaluated by a dominant model (i.e. ‘AG’ and ‘GG’ groups combined), rs578776 was weakly associated with plasma cotinine levels in the smokeless tobacco group ($P=0.06$, Fig. 25A). In addition to cotinine, weak associations between rs578776 and urinary TNE were also observed among the total tobacco user group and among the smokers ($P=0.076$ and $P=0.05$, respectively. Fig. 24B). Rs578776 was also significantly associated with CPD among the smokers. The average CPD was 7.2 for the ‘AA’ genotype, 8.6 for the ‘AG’ genotype and 11.7 for the ‘GG’ genotype ($P=0.02$, Table 12). No significant associations between rs578776 and urinary NNAL or 1-hydroxyfluorene levels were observed (Fig. 24C and D). The associations were similar when assessed by dominant models (i.e. pooling the ‘AG’ and ‘GG’ genotypes, Table 12). Individuals with the ‘G’ allele of rs578776 exhibited a greater, although non-significant, odds ratio of being a current smoker than a former smoker (OR=1.72, $P=0.06$, n=163 and 82 respectively).

\textbf{Rs16969968:} rs16969968 is a nonsynonymous (amino acid change: D398N) variant previously associated with nicotine intake in Caucasians thought to alter $\alpha_5$ nicotinic receptor function (Bierut et al. 2008; Fowler et al. 2011). The prevalence of the ‘A’ allele of rs16969968, and the correlated rs1051730, was low (3\%) in Alaska Native people compared to Caucasians (\~{}38\% according to the Hapmap project). Similar to the Caucasian populations of the international Hapmap project, rs16969968 was in very low LD with rs578776 in Alaska Native
people ($r^2=0.15$). While rs16969968 had what appeared to be a large impact on nicotine intake (Fig. 26), this did not reach significance at least in part due to the low prevalence and power to detect the association. The ‘GA’ genotype of rs16969968, compared to the ‘GG’ genotype, was non-significantly associated with 1.51 fold (89 ng/mL) higher plasma cotinine in the total tobacco user group and 1.46 fold (73 ng/mL) higher plasma cotinine in the smokers (Fig. 26A). The ‘GA’ genotype of rs16969968 was also non-significantly associated with 16% (10 nmol/mg Cre) higher urinary TNE levels in the total tobacco user group and 38% (21 nmol/mg Cre) higher TNE levels in the smokers (Fig. 26B). Associations of similar directions and magnitudes could also be observed with urinary NNAL and 1-hydroxyflurene (Fig. 26C and Fig. 26D). The ‘A’ allele of rs16969968 was found at a greater frequency in the current smokers compared to the former smokers, although this difference did not reach statistical significance (6.1% vs. 3.7%, n=163 and 82 respectively). Of note, the association between plasma cotinine levels and rs578776 remained significant after adjusting for rs16969968 or excluding the participants with at least one ‘A’ variant allele of rs16969968 (data not shown).

Rs588765: rs588765 and correlated SNPs have previously been associated with increased risk of heavy smoking (Chen et al. 2012). In our study, no significant associations were observed between rs588765 and nicotine intake with/without adjusting for the effect of rs578776 and/or rs169699698 (Fig. 27).
The risk alleles are indicated in the parentheses. MAF= Minor allele frequency. PAH=1-hydroxyflurenene levels.
Figure 24 | The association between rs578776 with A) cotinine levels B) Urinary TNE C) Urinary NNAL D) Urinary 1-hydroxypryrene.
IQR= interquartile range. The $P$-values were obtained from additive models.
Figure 25 | The association (by dominate models) between rs578776 with A) cotinine levels B) Urinary TNE C) Urinary NNAL D) Urinary 1-hydroxypryrene.

IQR= interquartile range. The $P$-values were obtained from dominant models.
The association between rs16969968 with A) plasma cotinine levels B) Urinary TNE C) Urinary NNAL D) Urinary 1-hydroxypryrene.

IQR = interquartile range
Figure 27 | The association between rs588775 with A) cotinine levels B) Urinary TNE C) Urinary NNAL D) Urinary 1-hydroxypryrene.
IQR= interquartile range.
Combined influence of *CHRNA5-A3-B4* and *CYP2A6* activity on nicotine intake

Next, we examined the combined influence of gene variants in *CHRNA5-A3-B4* and altered CYP2A6 activity on nicotine intake. Since CYP2A6 activity can influence the quantitative relationship between nicotine intake and cotinine levels (Zhu et al. 2013), urinary TNE was used as the primary tobacco exposure biomarker. The analyses focused on the total tobacco user group (n=290) for sufficient statistical power. The effects of rs578776 and CYP2A6 activity, alone or together, on tobacco intake are shown in **fig. 28**. To assess the combined influence of *CHRNA5-A3-B4* and CYP2A6, we assigned the tobacco users into one of three risk groups. The low risk group included participants with both rs578776 AA genotype and slower CYP2A6 activity. Participants with either rs578776 AG/GG genotype or faster CYP2A6 activity were assigned to the intermediate risk group. Participants with both rs578776 AG/GG genotype and faster CYP2A6 activity were considered the high risk group. As illustrated in **fig. 28**, urinary TNE levels were significant different between the low risk group (mean=47 nmol TNE/Cre), the intermediate risk group (mean=69.2 nmol TNE/Cre) and the high risk group (84.4 nmol TNE/Cre, Kruskal-Wallis test: $P=0.0003$, **Fig 28**). Urinary TNE levels also increased with the number of risk genotypes in a linear trend (Jonckheere Trend Test: $P=2.6\times10^{-5}$). Due to the low prevalence of rs16969968, our ability to evaluate the combined effect of rs16969968 and CYP2A6 activity in this study was limited (**Fig. 29**).
The combined impact of genetic variants in *CHRNA5-A3-B4* and *CYP2A6* activity on urinary TNE levels among the total tobacco user group.

The low risk group included participants with both rs578776 AA genotype and slower CYP2A6 activity. Participants with either rs578776 AG/GG genotype or faster CYP2A6 activity were assigned to the intermediate risk group. Participants with both rs578776 AG/GG genotype and faster CYP2A6 activity were considered as the high risk group. IQR= interquartile range. The *P*-values were obtained by non-parametric comparisons (Mann-Whitney or Kruskal-Wallis).
Figure 29 | The combined impact of rs16969968 in CHRNA5-A3-B4 and CYP2A6 activity on urinary TNE levels.
The P-values were obtained by non-parametric comparisons (Mann-Whitney tests or Kruskal-Wallis tests).

The association between CHRNA5-A3-B4 gene variants and BMI

The total tobacco users had significantly lower BMI compared to the non-tobacco group (Fig. 30A, \(P<0.001\)). Among the total tobacco user group, there was a significant inverse correlation between plasma cotinine levels and BMI, suggesting nicotine dose dependently reduced body weight (Rho=-0.128, \(P=0.04\), Fig. 30B). Notably, the Y-intercept of the regression line is 30.4 kg/m\(^2\) which is the same as the average BMI of the non-tobacco user group. Similar correlation coefficients could be observed when examined in smokers (Rho=-0.117, \(P=0.15\)) or the commercial smokeless tobacco users (Rho=-0.133, \(P=0.27\)). Neither of the nicotine intake
altering SNPs, rs578776 and rs16969968, were associated with BMI in the total tobacco user group or in the smokers only (Table 12). Adjusting for cotinine levels did not improve the associations between rs578776 or rs16969968 with BMI. On the other hand rs2869550, a CHRNA4 SNP with a 36% allele frequency which was not associated with altered nicotine intake in Alaska Native people (Table 12), was associated with BMI in the total tobacco user group ($P=0.006$, Table 12). The association was even stronger after adjusting for plasma cotinine levels ($P=0.0035$, Fig. 31, Table 13). Adjusting for the nicotine intake altering SNPs, such as rs578776, did not change the strength of the association. Rs2869950 was not significantly associated with BMI in the non-tobacco user group (Fig. 31).

Figure 30 | The association between tobacco consumption and BMI. A). Non-tobacco user group have higher BMI than the tobacco users. B). BMI negatively correlated with plasma cotinine levels within the total tobacco user group, suggesting a dose dependent relationship between nicotine intake and body weight.
Rs2869550 was significantly associated with BMI in the total tobacco user group, but not in the non-tobacco user group. Similar directions of effect were observed between rs2869550 and BMI within the smokers and smokeless users. The $P$-value of the total tobacco user group was derived from an additive model after adjusting for plasma cotinine levels (Table 13). $P_{COT adj} = $ The $P$ value of rs2869950 after adjusting plasma cotinine levels.

Table 13 | The association between rs2869550 BMI (Additive model).

<table>
<thead>
<tr>
<th>Predictors of BMI</th>
<th>B</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r^2=0.055$ $P=0.002$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotinine (Increasing, per ng/mL)</td>
<td>-0.0083</td>
<td>-0.002 to -0.014</td>
<td>0.0062</td>
</tr>
<tr>
<td>rs2869550 (Per T allele)</td>
<td>2.41</td>
<td>0.799 to 4.02</td>
<td>0.0035</td>
</tr>
</tbody>
</table>


4.5 Discussion

We characterized the gene structure of CHRNA5-A3-B4 among Alaska Native people. In addition, we identified a significant association between rs578776 and nicotine intake. Secondly, we demonstrated that CHRNA5-A3-B4 gene variants, particularly rs578776, could act in combination with CYP2A6 to modify nicotine intake. Thirdly, we demonstrated that rs2869950, a CHRNA5-A3-B4 gene variant which did not alter nicotine intake, was associated with altered body weight among tobacco users.

Haplotype

The haplotype analyses of CHRNA5-A3-B4 identified both similarities and differences between Alaska Native people and Caucasians. For example, rs16969968 and rs1051730 were in high degree of linkage disequilibrium in Alaska Native people as seen in Caucasians. However, the allele frequency of rs16969968-rs1051730 variant SNPs was substantially lower (at 3%) compared to Caucasians (~38%), and was similar to that observed in the Asian populations (at 3%) of the International Hapmap project. In contrast to rs16869996-rs1051730, the LD between rs7163730 and rs578776 was weak in Alaska Native people compared to the high degree of LD previously observed in Caucasians (Chen et al. 2012).

Associations with nicotine intake

We demonstrated a significant association between rs578776 (representing haplotype B in Alaska Native people) and nicotine intake. The ‘G’ allele of rs578776 was associated with increased nicotine intake in Alaska Native people as previously seen in Asians and Caucasians (Chen et al. 2012). Of note, the allele frequency of the rs578776 ‘G’ allele was much lower in Alaska Native people than in Caucasians. In fact, the ‘G’ allele is the major allele of rs578776 in Caucasians whereas it is the minor allele of rs578776 in Alaska Native people.

Rs16969968 and its correlated SNPs generally exhibit the strongest association with nicotine intake in Caucasians and other racial groups (Chen et al. 2012; Munafo et al. 2012). In Alaska Native people, the ‘GA’ genotype of rs16869996 was associated with a roughly 73 ng/mL increase in plasma cotinine, which is within the previously reported range of 24 to 100 ng/mL (Keskitalo et al. 2009; Munafo et al. 2012). Due to the low prevalence of rs16869996 in Alaska
Native people, the difference between ‘GG’ and ‘GA’ genotypes was not significant. Overall, while the effect size of rs16969968 on nicotine intake was larger than rs578776 (Plasma cotinine in smokers: 73 vs. 33 ng/mL, respectively), the overall impact on nicotine intake is likely to be smaller than rs578776 in Alaska Native people due to the allele frequencies.

**Associations in combination with CYP2A6**

In our study, CYP2A6 and *CHRNA5-A3-B4* acted in combination to increase nicotine intake. Of note, the combined low-risk group had lower nicotine intake compared to either the ‘AA’ group of rs578776 or the slower CYP2A6 activity group alone and the combined high risk group had higher nicotine intake compared to either the ‘AG/GG’ group of rs578776 or the faster CYP2A6 activity group alone. Consistent with previous observation in Caucasians (Wassenaar et al. 2011), we observed that CYP2A6 activity had a greater influence on nicotine intake than *CHRNA5-A3-B4* gene variants, which was particularly obvious among the smokeless tobacco users. We have previously shown that these Alaska Native smokeless tobacco users with faster CYP2A6 activity have higher nicotine intake compared to the smokeless tobacco users with slower CYP2A6 activity (Zhu et al. 2013). However, in the present analyses, we did not observe any significant associations between *CHRNA5-A3-B4* gene variants and nicotine intake in the smokeless tobacco users. Thus, it is interesting that among Caucasians *CHRNA5-A3-B4* gene variants generally have a bigger effect on lung cancer risk than CYP2A6 (Wassenaar et al. 2011). This suggests that the *CHRNA5-A3-B4* gene variants may modulate lung cancer risk by mechanisms in addition to their effects on smoking behavior, such as directly altering cell proliferation and survival (Brennan et al. 2011). The prevalence of the protective *CYP2A6* and *CHRNA5-A3-B4* alleles were higher in Alaska Native people than in Caucasians, suggesting the protective *CYP2A6* and *CHRNA5-A3-B4* alleles may contribute to the low level of self-report smoking in Alaska Native people and that other genes or environmental factors may be responsible for the higher risk for lung cancer.

**Association with BMI**

The Alaska Native non-tobacco user group had, on average, higher BMI compared to the total tobacco user group. Within the tobacco users, there was a significant negative relationship between nicotine intake and BMI, supporting a negative dose response relationship between
nicotine intake and body weight (Mineur et al. 2011). The negative dose response relationship is consistent with the known effects of nicotine to increase metabolic rate and suppress appetite (Mineur et al. 2011). Genetic variants in *CHRNA5-A3-B4* have been associated with BMI (Freathy et al. 2011), however, it was not clear whether this genetic association was mediated indirectly by the effect of *CHRNA5-A3-B4* genetic variants on nicotine intake or directly by altering nicotine’s effect on the nicotinic receptor pharmacodynamic target. In this study rs578776, which altered nicotine intake, was not directly associated with BMI, suggesting the effect of rs578776 on nicotine intake was not strong enough to alter BMI. Interestingly, there was a direct role of another *CHRNA5-A3-B4* genetic variants in modulating BMI. We demonstrated rs2869550, a prevalent β4 SNP which was not associated with nicotine intake, could alter nicotine’s ability to modulate body weight even after controlling for plasma cotinine. A possible explanation is that rs2869550 results in altered β4 nicotinic receptor subunit function, reducing nicotine’s ability to suppress eating.

**Conclusion**

Together, we found an association between *CHRNA5-A3-B4* gene variants and nicotine intake in Alaska Native people. This is novel since the gene structure and SNP prevalence in Alaska Native people differ from Caucasians, and the level and type of nicotine intake also differ. We observed that rs578776, which uniquely tags haplotype B, was significantly associated with nicotine intake. Due to the high prevalence of rs578776 in Alaska Native people, it may play a more important role in governing nicotine intake than rs16969968. We also demonstrated that variation in pharmacokinetic (i.e. CYP2A6) and pharmacodynamic (i.e. nicotinic receptors) genes could act in combination to increase nicotine intake. Lastly, we provided evidence that there is a dose response relationship between nicotine intake and body weight, and genetic variants in the *CHRNA5-A3-B4* can modulate body weight in smokers directly without altering nicotine intake. A limitation of the current study with respect to generalizability is that the findings may not be representative of the entire Alaska Native population as we only recruited from one region in southwest Alaska. Since most of our participants were Yupik, Alaska Native people from other regions with different ethnic backgrounds could have different *CHRNA5-A3-B4* gene structures. In summary, our data indicate that genetic variation in *CHRNA5-A3-B4* can modulate both nicotine intake and body weight in tobacco users. These findings provide
important insights about the contribution of *CHRNA5-A3-B4* to variation in both tobacco and obesity, the two most prevalent causes of preventable death and disease.
4.6 Significance to Thesis

This chapter extends the findings of previous chapters and contributes to the existing literature in three ways. First, the findings of this chapter demonstrated that gene variants in nicotine pharmacodynamic genes, such as *CHRNA5-A3-B4*, significantly alter tobacco consumption in light smokers. This chapter also presented the combined effects of *CHRNA5-A3-B4* variants and *CYP2A6* variants on tobacco consumption. Of note *CYP2A6* variants had a similar, if not bigger, effect on tobacco consumption compared to *CHRNA5-A3-B4* variants. This suggests that the higher lung cancer risk associated with *CHRNA5-A3-B4* variants, compared to *CYP2A6* variants, may not be exclusively mediated by altered tobacco consumption. Alterations in cell signaling pathways may also contribute to the higher lung cancer risk associated with *CHRNA5-A3-B4* variants.

Secondly, this study also highlights the importance of race specific genetic association studies. In Caucasian heavy smokers, rs16969968 and rs578776 have previously been associated with altered tobacco consumption. This chapter demonstrated that rs16969968 has little importance in governing tobacco consumption in Alaska Native light smokers largely due to the low allele frequency. In contrast, rs578776, which had a smaller influence on tobacco consumption in Caucasians compared to rs16969968, played an important role in governing tobacco consumption in Alaska Native smokers.

Lastly, the finding that gene variants in *CHRNA5-A3-B4* could significantly alter nicotine’s ability to alter bodyweight is both novel and has some important clinical implications. *CHRNA5-A3-B4* variants have previously been associated with BMI, but the mechanism was unclear. This study demonstrated that *CHRNA5-A3-B4* could influence BMI beyond the influence on altering tobacco consumption. Our findings, in combination with previous animal experiments, suggest that the α3β4 nAChR could be an important drug target for the treatment of obesity (Mineur et al. 2011).
5 GENERAL DISCUSSION

The specific contributions of the thesis findings to the literature were previously discussed in Chapters 2, 3 and 4. The broad implications of the overall thesis findings to nicotine and tobacco research will be the focus of this general discussion section, along with proposals for future research directions.

5.1 Strengths and Limitations of Tobacco Consumption Biomarkers

The strengths and limitations of tobacco consumption biomarkers are summarized in Table 14. This section provides some recommendations about biomarker selection in smoking studies.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Best use</th>
<th>Limitations</th>
<th>Specificity toward tobacco</th>
<th>Half-life</th>
<th>For evaluating tobacco consumption</th>
<th>For evaluating smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Monoxide</td>
<td>Verifying smoking status in subjects treated with nicotine replacement therapy</td>
<td>Short half-life; low specificity</td>
<td>Low</td>
<td>Short</td>
<td>Poor in infrequent smokers; Questionable in frequent smokers</td>
<td>Poor in infrequent smokers; Good in frequent smokers</td>
</tr>
<tr>
<td>Cotinine</td>
<td>Evaluating tobacco consumption when urinary samples are not available; Verifying smoking status</td>
<td>Variation in cotinine clearance; ELISA method may over estimate.</td>
<td>High</td>
<td>Long</td>
<td>Reasonable in both infrequent and frequent smokers</td>
<td>Good in both infrequent and frequent smokers</td>
</tr>
<tr>
<td>TNE</td>
<td>Evaluating tobacco consumption</td>
<td>Expansive; Limited Access; Creatinine adjustment</td>
<td>High</td>
<td>Long</td>
<td>Good in both infrequent and frequent smokers</td>
<td>Unknown, likely good</td>
</tr>
<tr>
<td>NNAL</td>
<td>Evaluating TSNA exposure</td>
<td>Variation in the metabolism of NNK to NNAL</td>
<td>High</td>
<td>Long</td>
<td>Good in both infrequent and frequent smokers</td>
<td>Good in frequent smokers; Unknown in infrequent smokers</td>
</tr>
<tr>
<td>Plasma COT+3HC</td>
<td>Evaluating tobacco consumption</td>
<td>Unknown</td>
<td>High</td>
<td>Long</td>
<td>Unknown, likely good</td>
<td>Unknown, likely good</td>
</tr>
</tbody>
</table>

Table 14 | A summary of the limitations of common tobacco biomarkers
5.1.1 Strengths and Limitations of Self-Report CPD and Self-Report Chews per Day

Smoking is a complex behavior and there is a lot of variability in daily tobacco consumption between smokers (Centers for Disease Control and Prevention 2008). In clinical and epidemiological studies, self-report CPD is often used as an indicator of daily tobacco consumption. However, as illustrated by a series of analyses presented in Chapter 1, self-report CPD gives a biased and inaccurate estimation of tobacco consumption, particularly in light/infrequent smokers (less than 10 CPD). **Fig. 32** illustrates two major weaknesses of CPD. First, the amount of nicotine extracted per cigarette varies among smokers. Among smokers who consumed less than 15 CPD, there is a significant negative correlation between self-report CPD and the amount of nicotine intake per cigarette (**Fig. 32**). This suggests that the smokers who smoked fewer CPD extracted more nicotine per cigarette than the smokers who consumed more CPD. Among the smokers who smoked more than 15 CPD, the amount of nicotine intake per cigarette was less variable (**Fig. 32**). This finding suggests that CPD may still have some utility as an indicator of tobacco consumption in heavy smokers. The second major weakness of using CPD as an indicator of tobacco consumption is number bias. As illustrated by **Fig. 32**, smokers were more likely to report their CPD in the multiples of 5 cigarettes (i.e. most smokers reported 5, 10, 15, 20, 25 or 30 CPD). This number bias limits the ability of CPD to capture the fine differences in tobacco consumption and could make CPD an ordinal variable rather than a ratio variable during statistical analyses.
Figure 32 | The relationship between self-report CPD and nicotine intake per cigarette (calculated as TNE divided by CPD).
This graph is compiled based on data from Chapter 1. It illustrates two major weaknesses of self-report CPD as an indicator of tobacco consumption: 1) variation in nicotine intake per cigarette. 2) substantial number bias toward 5, 10, 15, 20 and 30. TNE=Total nicotine equivalents.

Self-report chews per day and cans of smokeless tobacco consumed per week are often used as a measurement of smokeless tobacco consumption. Chews per day and cans of smokeless tobacco consumed per week show considerable variability in the amount of nicotine intake per chew or per can (Fig. 33). There is a significant negative correlation between self-report chews per day and the amount of nicotine intake per chew (Fig. 33). This suggests that the smokeless tobacco users who consumed fewer chews per day extracted more nicotine per chew, hence chews per day may not give an accurate estimation of tobacco consumption. Furthermore, Alaska Native smokeless tobacco users reported consuming 1.6 cans of commercial smokeless tobacco per week in comparison to the 3.6 cans per week reported by Caucasian smokeless tobacco users in the United States (Mushtaq et al. 2012). This suggests that Alaska Native smokeless tobacco users are ‘light’ smokeless tobacco users as being on average light smokers. However, despite consuming only 40% of the smokeless tobacco as Caucasians, Alaska Native smokeless tobacco
users exhibit similar cotinine levels as the Caucasian smokeless tobacco users in the United States (223 ng/mL in Alaska Natives vs. 200 to 260 ng/mL in Caucasians) (Gritz et al. 1981; SRNT Subcommittee on Biochemical Verification 2002; Mushtaq et al. 2012). This suggests that using self-report cans of commercial smokeless tobacco per week as an indicator of smokeless tobacco consumption underestimate the nicotine exposure in Alaska Native individuals.

![Graph](image)

**Figure 33 | The relationship between self-report chews per day and nicotine intake per chew (calculated as TNE divided by chews per day) in Alaska Native commercial smokeless tobacco users.**

This graph is compiled from data in Chapter 1. Like with CPD, there is a significant negative correlation between chews per day and the amount of nicotine intake per chew. However, unlike CPD, there is no apparent number bias in chews per day. TNE=Total nicotine equivalents.

Overall, the existing data suggest that self-report gives an inaccurate estimation of tobacco consumption in both smokers and smokeless tobacco users. The use of objective biomarkers would capture more of the quantitative aspects of tobacco consumption and would improve our understanding of the dose response relationships between tobacco consumption and tobacco related diseases.
5.1.2 Strengths and Limitations of CO and Cotinine as Biomarkers of Tobacco Consumption and Smoking Status

Due to the inaccuracy of self-report tobacco consumption, biomarkers are used as objective indicators of tobacco consumption, secondhand smoke exposure, and smoking status. CO and cotinine are the most widely used tobacco consumption biomarkers. Here we discuss their strengths and limitations.

CO is a by-product of tobacco combustion and can be readily measured in smokers at a low cost (SRNT Subcommittee on Biochemical Verification 2002; Jatlow et al. 2008). CO is a reasonable tobacco consumption biomarker in heavy smokers but has significant limitations in light/infrequent smokers due to its half-life and specificity. In humans, CO has a relatively short half-life (1 to 4 hours depending on the level of physical activity) and can come from sources other than tobacco exposure (SRNT Subcommittee on Biochemical Verification 2002). Detectable levels of CO can be obtained from environmental sources (such as the exhaust from burning fossil fuel) as well as from endogenous formation of CO via the metabolism of heme (SRNT Subcommittee on Biochemical Verification 2002). As a result, CO’s signal to noise ratio in light/infrequent smokers is small as they do not smoke frequently enough to generate CO levels above background levels. This is supported by the observation that non-smokers living in major urban centers have exhaled CO levels of up to 7 ppm (Jones et al. 2006; Scherer 2006), yet 42% of African American light/infrequent smokers have exhaled CO levels below 10 ppm (Ho et al. 2009). The short half-life and low specificity also limit CO’s ability to verify smoking status in light/infrequent smokers. Together, these data suggest that CO is not a useful exposure index of tobacco consumption in light/infrequent smokers.

Cotinine is the major metabolite of nicotine and can be detected in various biological fluids (e.g. plasma, urine and saliva) in smokers. Cotinine has good specificity toward tobacco and correlates with nicotine intake (Rickert et al. 1981; Benowitz et al. 1983). Cotinine has a long half-life (12-16 hours), which increases it utility in light/infrequent smokers. However, as illustrated by Chapter 2, steady state plasma cotinine levels can give a biased estimation of tobacco consumption; differing CYP2A6 activity can result in more than a 25% bias in cotinine levels. In addition, some very commonly used immunoassays overestimate cotinine concentrations due to cross-reactivity with other nicotine metabolites (Schepers et al. 1988;
Anderson et al. 1991; Zuccaro et al. 1997). This makes comparisons between studies with different analytical methods very difficult.

Despite its shortcomings as a biomarker of tobacco consumption, cotinine is very useful for verifying smoking status. In heavy smokers, cotinine has 96%-97% sensitivity and 99-100% specificity for verifying smoking status (SRNT Subcommittee on Biochemical Verification 2002). Cotinine is also a better indicator of smoking status in light/infrequent smokers due to its long half-life (Ho et al. 2009). For an initial cotinine level of 200 ng/ml (a level typically observed in light/infrequent smokers), it would take four half-lives (roughly 3 days) for the cotinine level to decline below the 14 ng/ml cutoff. Therefore, cotinine levels below 14 ng/mL are indicative of smoking abstinence for at least 2 or 3 days. In agreement with this hypothesis, only 3.1% of ad libitum African American light/infrequent smokers had cotinine levels below the 14 ng/mL cutoff, suggesting cotinine has good specificity in light/infrequent smokers (Benowitz et al. 2009). Overall, cotinine is better than CO and self-report measures for verifying smoking status in both heavy and light/infrequent smokers.

### 5.1.3 Limitations of TNE as a Biomarker of Tobacco Consumption

While we argued that TNE is a better biomarker of tobacco consumption compared to self-report CPD, CO and cotinine in the thesis chapters, TNE also has some limitations. The first limitation is the cost and accessibility. There are significant analytical costs associated with the measurement of nicotine and 5-8 of its metabolites. Some of the metabolites exist in relatively low concentrations, warranting the use of LC/MS systems. This requirement for LC/MS analysis can limit the utility of TNE (due to the high cost). The second limitation of spot urinary TNE is the need for creatinine adjustment. Although TNE measurement in a 24 hour-urine sample correlates highly with tobacco consumption, spot urinary TNE is generally used for convenience with creatinine adjustments to correct for variable dilution among spot samples. Traditionally, this approach has been used in populations without much diversity and has worked well. However, creatinine adjustments may introduce significant bias in studies with multiple demographic groups. In an analysis of more than 20000 individuals, age, sex, race, and body mass index were all significant predictors of urinary creatinine levels (Barr et al. 2005). African Americans had 5-10% higher urinary creatinine levels compared with Caucasians and males had ~20% higher urinary creatinine levels compared with females. Therefore, urinary creatinine
levels (and perhaps creatinine adjusted TNE) are best compared among individuals within similar demographic groups. However, it is worth noting that, in a predominantly Caucasian study which administered known doses of nicotine daily for 5 days, the creatinine adjusted urinary TNE predicted the nicotine dose better than plasma cotinine (creatinine adjusted TNE r=0.8, plasma cotinine r=0.75) (Benowitz et al. 2010). In the same study, creatinine adjusted TNE also predicted the nicotine dose better than unadjusted TNE (Benowitz et al. 2010). These results suggest that the bias introduced by creatinine adjustments are smaller than the bias introduced by variable dilution of the urine. Even with the creatinine bias, TNE was a better biomarker than cotinine. Analytically, the systematic differences in creatinine can be partially corrected using a multiple regression analysis with urinary creatinine added as an independent variable. This approach allows urinary TNE concentration to be appropriately adjusted for creatinine, and statistical inference of other predictors in the model is independent of creatinine levels (Barr et al. 2005).

5.1.4 Sum of Plasma COT and 3HC as A Biomarker of Tobacco Consumption

The current gold standard for measuring nicotine dose is the molar sum of all nicotine metabolites in a 24 hour-urine collection (i.e. TNE in 24 hour urine) (Benowitz et al. 2010; Wang et al. 2011). However, due to the practical difficulties of collecting a 24 hour-urine sample, plasma measurements may be a more practical approach. Additionally, plasma volumes are relatively constant between people. Therefore, there is no need for dilution adjustments, which is commonly conducted on urine samples by adjusting creatinine. Nicotine, cotinine and 3HC can be readily measured in the plasma (Benowitz et al. 1993; Benowitz et al. 2001; Hukkanen et al. 2005). In a regular smoker, plasma nicotine concentrations are generally lower than 10 ng/mL and the sum of cotinine and 3HC is higher than 300 ng/mL (Zhu et al. 2013). Plasma nicotine levels also tend to fluctuate (reflecting peak to trough variation) in smokers during the day due to its short half-life and frequent administration. Therefore, we propose to use the plasma sum of cotinine and 3HC (the addition of 3HC compensates for the effect of CYP2A6 activity on cotinine clearance) as a measurement of nicotine dose in smokers. The plasma sum of cotinine and 3HC has limited influenced by variation in CYP2A6 activity and is completely independent of variation in urinary creatinine levels. In studies that evaluated the ability of
various biomarkers to estimate nicotine dose, the sum of plasma cotinine and plasma 3HC correlated with nicotine dose better than plasma cotinine alone (Malaiyandi et al. 2006; Benowitz et al. 2010). This correlation was not significantly influenced by the time of sampling (Benowitz et al. 2010). These observations suggest that the sum of plasma cotinine and 3HC may have some utility as a tobacco consumption biomarker. However, some validation research, such as the characterization of the role of variation in rates of glucuronidation, is required before plasma cotinine plus 3HC can be recommended as a biomarker of tobacco consumption.

5.1.5 Limitations of NNAL: Variability in the Metabolism

Urinary total NNAL is often used as an indicator of TSNA exposure in tobacco users. Urinary total NNAL is associated with lung cancer risk in smokers and leukoplakia risk in smokeless tobacco users (Kresty et al. 1996; Yuan et al. 2011). Like other biomarkers of tobacco consumption, urinary total NNAL has its limitations (Maser et al. 1996; Maser 1998). It is known that urinary total NNAL levels increase with CPD, but with considerable variability (Joseph et al. 2005; Lubin et al. 2007). Although some of this variability can be explained by the variation in puff volume and depth of inhalation, individual variability in NNK metabolism (NNAL is a metabolite of NNK) could also play a role. It has been demonstrated that freshly isolated human non-tumor lung tissue exhibited a wide range of variability in the metabolism of NNK to NNAL, which was likely due to genetic variation (Smith et al. 1999). Gene variants in HSD11B1 (encoding for 11-β-hydroxysteroid dehydrogenase type 1, which converts NNK to R-NNAL) and AKR1C4 (encoding for an aldo-keto-reductase, which converts NNK to S-NNAL) were significantly associated with urinary total NNAL levels (Ter-Minassian et al. 2012). Thus, genetic variation in NNK metabolism could alter NNAL levels, decreasing the ability of NNAL to reflect NNK exposure. Diet and xenobiotic exposure may also influence urinary total NNAL. For example, watercress consumption inhibits CYP1A2 mediated NNK α-hydroxylation pathway, thereby promoting the conversion of NNK to NNAL (Hecht et al. 1995). In contrast, smokers who were exposed to indole-3-carbinol had lower urinary total NNAL levels, which was likely due to the induction of CYP1A2 which reduces NNK levels and subsequent the metabolism to NNAL (Taioli et al. 1997). Taken together, these data suggest that NNAL levels are altered by genetic variation in NNK metabolism, and may not always reflect the entirety of TSNA exposure.
Ultimately, the selection of tobacco biomarkers should be determined by the study objectives. Table 14 summarized the strengths and limitations of common tobacco biomarkers and gave biomarker recommendations. Based on the findings of this thesis, TNE is recommended for evaluating tobacco consumption in both frequent and infrequent smokers, while cotinine is recommended for verifying smoking status in both frequent and infrequent smokers. A proper selection of tobacco biomarkers will likely enhance our understanding of smoking behaviors and the risk associated with tobacco consumption.

5.2 What Did the Biomarkers Tell Us About Smoking Behaviors in Light Smokers and Smokeless Tobacco Users?

5.2.1 Light Smokers Had Similar Total Nicotine Intake to that in Heavy Smokers

Previous epidemiological studies in light smokers commonly did not include any biomarker measurements and relied on self-report tobacco consumption measures. One of the unique aspects of the publications in this thesis was the inclusion of multiple biomarker measurements. The biomarker levels suggested that, despite reporting fewer CPD, light smokers had very similar total nicotine intake to those in heavy smokers. A TNE level of approximately 60 nmol/mg Cre was observed in Alaska Native light smokers. This was comparable to the 63 nmol/mg Cre observed in Caucasian heavy smokers (Le Marchand et al. 2008; Benowitz et al. 2011). Similarly, the TNE level observed in African American light smokers was approximately 58 nmol/mg Cre (Zhu et al., unpublished observations), which suggests that the observation within Alaska Native light smokers may be generalizable to other populations of light smokers as well. Together, the biomarker data suggest that, although classified as light smokers according to self-report CPD, these Alaska Native and African American “light” smokers had very similar nicotine intake compared to Caucasian heavy smokers. Therefore, classifying smokers according to self-report CPD may not generate an accurate picture of their tobacco consumption and tobacco related disease risk.

The similar total nicotine intake observed in light and heavy smokers suggests that light smokers extract a greater amount of nicotine per cigarette than heavy smokers (perhaps by either inhaling more deeply or smoking a greater proportion of the cigarette). Hence, they would be described
more appropriately as “infrequent” smokers rather than “light” smokers. There are a few possible explanations for the distinct smoking behavior observed in these light and infrequent smokers.

**Figure 34 | Nicotine intake in light smokers vs. heavy smokers.**

**A).** Alaska Native and African American light smokers had similar nicotine intake (as indicated by TNE) as found in Caucasian heavy smokers. **B).** Alaska Native and African American light smokers had lower daily cigarette consumption compared to Caucasian heavy smokers. **C).** Alaska Native and African American light smokers extract more nicotine per cigarette than Caucasian heavy smokers do. The Caucasian tobacco consumption data were obtained from Benowitz et al., 2011 (Benowitz et al. 2011). The African American tobacco consumption data were from unpublished observation from Zhu et al. The study was described by Zhu et al., 2012. TNE=Total nicotine equivalents.

The first possibility is that light/infrequent smokers have fewer smoking opportunities than heavy smokers do. It is known that smokers with fewer smoking opportunities, such as those who work in buildings with an indoor smoking ban, tend to take more puffs per cigarette to extract more nicotine (Chapman et al. 1997). Thus, they compensate for the fewer smoking opportunities by increasing the amount of nicotine intake per cigarette. In agreement, we observed that the Alaska Native smokers who cannot smoke inside of their homes tend to extract more nicotine per cigarette (TNE=11.1 nmol/mg Cre/cig.) than smokers who can smoke inside of their homes (TNE=6.8 nmol/mg Cre/cig.) (Data compiled from Zhu et al. 2013)(Zhu et al. 2013).
Together, these observations suggest that the low CPD and high nicotine intake per cigarette in light/infrequent smokers could be a result of fewer smoking opportunities. However, it is unlikely that this possibility entirely accounts for the higher nicotine intake per cigarette in Alaska Native light/infrequent smokers since many participants were recruited from fishing villages and likely work outdoors for the fishing industry.

Another possible explanation for the high nicotine intake per cigarette is that the economic status of light/infrequent smokers dictates their cigarette consumption. Many of the light/infrequent smoking populations, such as Alaska Native peoples and African Americans experience substantial socioeconomic disparities. For example, Alaska Native peoples and African Americans commonly have lower educational attainment and lower median household income compared to Caucasians in the United States (U.S. Bureau of the Census 2004; U.S. Bureau of the Census 2005; Institute of Social and Economic Research University of Alaska Anchorage 2009). Since socioeconomic status is an important determinant of smoking behaviors (Novotny et al. 1988; Townsend et al. 1994; Graham et al. 1999), it is possible that the low CPD and high nicotine intake per cigarette observed in light/infrequent smokers is due to their inability to afford more cigarettes in comparison to Caucasian smokers. Unfortunately, this hypothesis could not be directly tested in our Alaska Native study as income levels were not collected in the baseline survey. However, in a study of African American light/infrequent smokers that was described by the manuscript attached within Appendix B, the amount of nicotine intake per cigarette did not significantly differ between different household income levels (Zhu et al., unpublished observation). Furthermore, light/infrequent smoking is also prevalent in Asian Americans, who have higher than average household income in the United States (U.S. Bureau of the Census 2004; U.S. Bureau of the Census 2005). Therefore, lower economic status cannot fully account for the high nicotine intake per cigarette observed in light/infrequent smoking populations. Ultimately, a large study (such as the southern community cohort study (Signorello et al. 2005)) that compares nicotine intake per cigarette between light and heavy smokers (i.e. less than 10 vs. more than 10 CPD) could give a precise estimation of the contribution of economic status to the distinct smoking behaviors in light/infrequent smokers.

Cultural and societal influences may be another possible explanation for the high nicotine intake per cigarette observed in light/infrequent smokers. For example, Asians living in China and
Japan tend to consume more CPD than Asians living in the United States, as smoking is more socially accepted and even encouraged for males in China and Japan (Giovino et al. 2012). Likewise, individuals of African descent who were born in the Caribbean or Africa are less likely to be ever or current smokers than those born in the United States (Taylor et al. 1997; King et al. 2000; Bennett et al. 2008). Furthermore, strong identification with the African American culture, including growing up in predominantly African American communities, is a strong predictor of being a current smoker (Klonoff et al. 1994; Klonoff et al. 1996; Klonoff et al. 1999). It is possible that growing up, initiating smoking and progressing to be a smoker in a light smoking environment can have a substantial influence on one’s smoking behaviors making him/her more likely to smoke fewer cigarettes but extract a higher amount of nicotine per cigarette. Therefore, cultural influences may play an important role on smoking behaviors than biological differences between light and heavy smoking populations (Shadel et al. 2000).

Lastly, there are genetic factors that may enable light/infrequent smokers to extract more nicotine per cigarette. As described in the introduction, light/infrequent smokers eliminate nicotine at similar rates compared to heavy smokers and show similar cardiovascular responses to cigarette smoking (Shiffman et al. 1990; Brauer et al. 1996). Thus, variations in nicotine metabolism or nAChRs are unlikely to explain the difference in nicotine intake per cigarette observed between light and heavy smokers. An alternative possible explanation is genetic variation in the sensitivity of aversive chemosensory receptors to nicotine during smoking. Nicotine activates the chemosensory TRPA1 channel in the respiratory epithelium and causes irritation and in some cases a strong burning sensation (Thuerauf et al. 2006; Talavera et al. 2009). Genetic variants in TRPA1 may alter the response of the TRPA1 channel toward nicotine, allowing some individuals to intake high levels of nicotine without experiencing as much irritation and burning sensations. Interestingly, a non-synonymous SNP located in exon 1 of TRPA1 gene, rs13268757, is found at a higher allele frequency in African Americans compared to Caucasians (Uhl et al. 2011). If rs13268757 results in lower functional activation of TRPA1 channel by nicotine, it is possible that this would allow a greater proportion of African American smokers to extract more nicotine per cigarette without experiencing as many aversive effects. In agreement with the idea that variation in nicotine’s chemosensory response can alter nicotine intake per cigarette, African American light/infrequent smokers prefer to smoke cigarettes containing menthol, which is an antagonist of TRPA1 channel (King et al. 2000; Landrine et al. 2005; Allen et al. 2007; Talavera.
et al. 2009). It is possible that menthol blocks the irritations associated with nicotine inhalation and promotes greater nicotine intake per cigarette in African American light/infrequent smokers. However, it is worth noting that light smoking can also be prevalent in populations who do not use menthol cigarettes. Currently, studies have yielded inconsistent results when comparing the amount of nicotine intake in menthol smokers and non-menthol smokers (Clark et al. 1996; Heck 2009; Muscat et al. 2009). This could be due to the limitations of the biomarker measurements used. Thus, further studies investigating the association between TRPA1 gene variants and tobacco consumption are needed to clarify the role of TRPA1 channel and menthol in determining nicotine intake in smokers.

5.2.1.1 Smokeless Tobacco Users Are Exposed to High Levels of TSNA: An Undesirable Harm Reduction Approach

Our biomarker data revealed that smokeless tobacco users are exposed to high levels of carcinogenic TSNA (Chapter 2), which suggests that smokeless tobacco has minimal utility as a harm reduction strategy in smokers in comparison to smoking cessation. In recent years, the idea that smokeless tobacco products could be used as a harm reduction approach has been actively debated. The benefits of smokeless tobacco products are largely supported by data from Sweden where a significant reduction in tobacco-related mortality and morbidity has been attributed to the extensive replacement of cigarette smoking with oral snus (a kind of smokeless tobacco) (Vainio et al. 2003; Hatsukami et al. 2004). The use of smokeless tobacco products has two major benefits. First, the lack of combustion eliminated users’ exposure to toxic combustion products from cigarettes such as carbon monoxide, acrolein and PAHs (Hatsukami et al. 2004). The low exposure to toxic combustion products could reduce smokeless tobacco users’ risk of developing pulmonary diseases such as COPD, emphysema and lung cancer. Second, data from several Swedish cohorts suggested the use of oral snus could promote smoking cessation (Tillgren et al. 1996; Lindstrom et al. 2002; Lindstrom et al. 2002; Rodu et al. 2003), which would further reduce the risk of smoking related diseases. However, it is important to note that the smokeless tobacco products are cured differently in Sweden compared to those found in North America. This different production process results in very different TSNA levels (Hecht 2002). The levels of TSNA in popular American smokeless tobacco brands are 40 to 60 µg/g, which is 15 to 20 times higher than the 2.8 µg/g found in popular Swedish brands (cigarette
tobacco has 5-10 µg/g (Connolly et al. 1986; Brunnemann et al. 2002; Stepanov et al. 2012). In agreement with this idea, we observed that urinary NNAL level, a marker of TSNA exposure, was 4 times higher in smokeless tobacco users (who used North American brands of smokeless tobacco) compared with cigarette smokers (see Chapter 1 and Fig. 35). Higher NNAL levels, a biomarker of TSNA exposure, have been associated with a higher risk of developing a number of smokeless tobacco related cancers including pancreatic, oral and esophageal cancers (Luo et al. 2007; Boffetta et al. 2008). Therefore, on the basis of NNAL levels alone, smokeless tobacco users in North American may not have a substantially lower risk for tobacco related cancers compared to smokers.

![Figure 35](image-url)

**Figure 35** | **Smokeless tobacco users are exposed to higher levels of NNAL than smokers.**

A) Smokeless tobacco users are exposed to higher levels of TSNA than smokers. B) After normalizing for nicotine intake (i.e. total nicotine equivalents), smokeless tobacco users are exposed to higher levels of TSNA than smokers.
Overall, our results suggest that the use of smokeless tobacco products as harm reduction alternatives to smoking in North America would require additional proof of concept studies. Although smokeless tobacco use can have significant public health benefits as the lack of combustion eliminates the generation of secondhand smoke, it can also act as a gateway product for adolescents to cigarette smoking and increase tobacco consumption by allowing tobacco use in environments where cigarette smoking would normally be restricted.

5.2.1.2 Iqmik Delivers High Levels of Nicotine and Low Levels of TSNA

In agreement with data from Caucasian commercial smokeless tobacco users (Wennmalm et al. 1991; Hecht et al. 2007), Alaska Native commercial smokeless tobacco users have similar levels of nicotine intake as Alaska Native smokers. In contrast, the nicotine intake (TNE level) in iqmik users was strikingly higher compared to the nicotine intake in smokers. Likewise, a previous study found that pregnant iqmik users had higher cotinine levels compared to pregnant smokers (Hurt et al. 2005). Since iqmik and commercial smokeless tobacco have similar nicotine content and both have reported similar chews per day (Benowitz et al. 2012), the higher nicotine intake observed in the iqmik users is likely the result of greater nicotine absorption. As discussed in the introduction, a basic chemical environment promotes nicotine absorption. Iqmik has a highly basic pH (pH=~11) whereas commercial smokeless tobacco’s pH is in the range of 5-8 (Renner et al. 2005). At iqmik’s pH, about 99.9% of nicotine is unionized which can be rapidly absorbed by the buccal mucosa, resulting in high nicotine intake. Interestingly, iqmik users had lower TNSA exposure compared to smokers and commercial smokeless tobacco users (Benowitz et al. 2012). This was consistent with the lower TSNA levels observed with this product.

5.2.2 Future Research: Modifications to Existing Tobacco Dependence Scales

The FTND is the most commonly used scale to assess tobacco dependence in nicotine and tobacco research. The FTND was developed in Caucasian heavy smokers; it measures the level of physical dependence to nicotine and the willingness to obtain nicotine to relieve or avoid withdrawal symptoms (Piper et al. 2008). One of the most importance characteristics of the FTND is its strong predictive ability of smoking cessation in Caucasian heavy smokers (Baker et
Light/infrequent smokers generally score very low (~3) on the FTND scale (Cox et al. 2012; Renner et al. 2013), which would suggest that they are not as dependent to nicotine and should have a higher rate of smoking cessation. However, clinical trial data suggest that light/infrequent smokers have just as much difficulty quitting as heavy smokers (Ahluwalia et al. 2002; Ahluwalia et al. 2006; Cox et al. 2012). When recounting their most recent quit attempts, light/infrequent smokers did not have longer abstinence periods before relapsing compared to heavy smokers (Choi et al. 2004). Furthermore, the unaided cessation rates observed in light/infrequent smokers were very similar to the unaided cessation rates observed in heavy smokers (Ahluwalia et al. 2002; Ahluwalia et al. 2006; Cox et al. 2012). These data suggest that the light/infrequent smokers are still highly dependent on nicotine and current tobacco dependence scales may not accurately reflect the level of nicotine dependence in light smokers.

A major issue with dependence scales in light/infrequent smokers is that the scales are heavily weighted toward self-report CPD. As we have shown previously, self-report CPD does not accurately reflect nicotine intake in light/infrequent smokers. A previous study has suggested a rescaling of the self-report CPD can better capture the level of tobacco consumption in light/infrequent smokers (Mwenifumbo et al. 2011). However, the rescaled FTND was no more predictive of smoking cessation in light/infrequent smokers than the original FTND (Fig. 36A&B). It is possible that these scales can still capture some other aspects of nicotine dependence rather than predicting smoking cessation. Here we propose modifications to the FTND to incorporate biomarker data. Table 15 provides an example of the type of modifications that could be made to the FTND to better predict smoking cessation. Preliminary analyses of two clinical trials in light/infrequent smokers suggested that the modified FTND with biomarker data was more predictive of smoking cessation outcomes than the original FTND (Fig. 36C&D, P<0.0001, Zhu et al., unpublished observations). The precise weighting of the points will need to be determined to maximize predictability.
Table 15 | An example of potential modifications to the FTND to incorporate biomarker data.
The point system represents broad estimations rather than precise regression classifications.
*This question replaced “How many cigarettes per day do you smoke?” TNE can also be used for this question. <25nmol/mg Cre = 0; 26-50nmol/mg Cre = 1; 51-75nmol/mg Cre = 2; >76nmol/mg Cre = 3. **This question replaced “Do you smoke if you are so ill that you are in bed most of the day?” and “Do you smoke more frequently during the first hours after waking than you do during the rest of the day?”.

<table>
<thead>
<tr>
<th>How soon after you wake up do you smoke your first cigarette?</th>
<th>Points</th>
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<tbody>
<tr>
<td>Within 5 min</td>
<td>3</td>
</tr>
<tr>
<td>6-30 min</td>
<td>2</td>
</tr>
<tr>
<td>31-60 min</td>
<td>1</td>
</tr>
<tr>
<td>After 60 min</td>
<td>0</td>
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<table>
<thead>
<tr>
<th>Do you find it difficult to refrain from smoking in places where it is forbidden—e.g., in church, at the library, in cinema</th>
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<tbody>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
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<table>
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<tr>
<th>Which cigarette would you hate most to give up?</th>
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<tbody>
<tr>
<td>The first one in the morning</td>
<td>1</td>
</tr>
<tr>
<td>All others</td>
<td>0</td>
</tr>
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</table>

*What is the baseline plasma cotinine level (in ng/mL by LC/MS or HPLC method)? | |
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<tr>
<td>≤150</td>
<td>0</td>
</tr>
<tr>
<td>151-250</td>
<td>1</td>
</tr>
<tr>
<td>251-350</td>
<td>2</td>
</tr>
<tr>
<td>≥351</td>
<td>3</td>
</tr>
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**Do you smoke mentholated cigarettes? | |
<table>
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<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 36 | Modified FTND with biomarker data strongly predicted smoking cessation.

A). The ability of original FTND to predict smoking cessation in African American light/infrequent smokers. Note that all the light/infrequent smokers will score zero for the question “How many cigarettes per day do you smoke?” in the traditional FTND. B). The ability of the rescaled FTND to predict smoking cessation in African American light smokers (Mwenifumbo et al. 2011). C). The ability of the modified FTND with plasma cotinine biomarker data to predict smoking cessation in African American light/infrequent smokers. D). The ability of the modified FTND with TNE biomarker data to predict smoking cessation in African American light/infrequent smokers. Low vs. High FTND was classified using a median split. The Odds ratios (OR) used the low FTND group as the reference group. For example, a 0.4 OR indicated that the high FTND group had 0.4 times the odds of quitting compared to the low FTND group.
5.3 Genetics of Nicotine Dependence in Light Smokers and Smokeless Tobacco Users

5.3.1 CYP2A6

5.3.1.1 Predicting Nicotine Clearance Using CYP2A6 Genotype and NMR Phenotype

In Chapter 1 and 3, the association between CYP2A6 genotype and tobacco consumption was weaker than the association of NMR and tobacco consumption. Currently, both CYP2A6 genotype and phenotypic (using NMR) measurements, are used to estimate in vivo nicotine clearance. In this section, we discuss the limitations of using CYP2A6 genotype, or using NMR, to estimate CYP2A6 activity and nicotine clearance.

There are a number of limitations of using CYP2A6 genotype to estimate in vivo nicotine clearance. First, many reduced function CYP2A6 alleles are rare (i.e. allele frequency ≤1.0%). Since the combined frequency of all these rare alleles is significant, selectively genotyping could reduce the statistical power in many racial/ethnic populations. Therefore, the comprehensive genotyping of these alleles requires significant time and cost. Secondly, it is often hard to accurately characterize the functional impact of rare alleles on nicotine clearance due to the limitations of the in vitro expression systems and the low number of individuals with these variants. This problem is not unique to CYP2A6; similar challenges have been reported with other xenobiotic metabolizing enzymes such as CYP2D6 (Kirchheiner 2008). The third limitation is that CYP2A6 genotype does not account for the influence of non-genetic factors on nicotine clearance (as described in the introduction). For example, the known effects of CYP2A6 genotype account for a small amount of the total variance in nicotine metabolism (Al Koudsi et al. 2010), suggesting that environmental factors (as well as unidentified genetic variants) also contribute to the variation in nicotine clearance.

The limitations of using CYP2A6 genotype to predict nicotine clearance are generally solved by using NMR, as NMR can account for both genetic and environmental variability in nicotine metabolism. However, it is important to note that NMR has some limitations as well. First, NMR is technically a measure of cotinine clearance (i.e. the ratio of 3HC/COT). Although the NMR generally correlates highly with rates of nicotine clearance (Dempsey et al. 2004; Benowitz et al.
2006; Johnstone et al. 2006; Malaiyandi et al. 2006; Malaiyandi et al. 2006), it is possible that some CYP2A6 variants could alter the clearance of cotinine differently from the clearance of nicotine (and other substrates). Thus, it remains undetermined whether the NMR is a good indicator of the metabolic clearance of other CYP2A6 substrates such as tegafur and letrozole. Secondly, the steady state 3HC levels are determined by both the rate of 3HC formation and the rate of 3HC clearance. Thus, slow rates of 3HC clearance could increase plasma 3HC levels for a given cotinine level, resulting in higher NMRs compared to those with normal rates of 3HC clearance. This effect of variable 3HC clearance on NMR is not related to nicotine clearance or CYP2A6 activity and could reduce the clinical utility of NMR. Our recent analysis demonstrates that variation in 3HC glucuronidation, including that caused by UGT2B17 gene deletions, did not significantly alter NMR, and is therefore unlikely to affect the ability of NMR to predict nicotine clearance in smoking studies (Zhu et al. 2013). This study provides further support that NMR is a reliable indicator of CYP2A6 mediated nicotine clearance (Zhu et al. 2013) (Manuscript attached in Appendix D). Lastly, while NMR can be readily measured in smokers during ad libitum smoking in regular smokers, it is not possible to measure NMR without drug (nicotine or cotinine) administration in former or never smokers. Likewise, it cannot provide information about the pharmacogenetic role of CYP2A6 during tegafur and letrozole treatment in non-smokers.

Ultimately, the choice of CYP2A6 genotype or NMR is determined by the study population, the availability of biological specimens, and the study objectives. NMR should be used in studies that want to capture transient changes in nicotine clearance, such as from temporary exposure to inducers or inhibitors. In contrast, CYP2A6 genotype should be used to predict long-term nicotine clearance as it is not influenced by the transient effects of CYP2A6 inducers and inhibitors.

5.3.1.2 The Association between CYP2A6 Gene Variants and Tobacco Consumption in Light Smokers and Smokeless Tobacco Users

This thesis also demonstrated that light/infrequent smokers titrate their nicotine intake according to their CYP2A6 activity. Previously, a number of studies have demonstrated that heavy smokers with slower CYP2A6 activity smoked fewer CPD (Schoedel et al. 2004; Wassenaar et al. 2011). However, this difference in CPD by CYP2A6 activity groups had not been observed in light
smoking populations such as African Americans (Ho et al. 2009). In this thesis, we demonstrated that the lack of association between CPD and CYP2A6 activity in light/infrequent smokers was due to their highly variable nicotine intake per cigarette. When TNE was used as a biomarker of tobacco consumption, we observed a 41% reduction in tobacco consumption (by TNE, but not with CPD) in individuals with slower CYP2A6 activity. This suggests that light/infrequent smokers with slower CYP2A6 activity reduced their nicotine intake per cigarette to compensate for the slower nicotine inactivation, possibly by taking smaller puff volumes as previously observed (Strasser et al. 2007).

This thesis also demonstrates, for the first time, that smokeless tobacco users titrate their tobacco consumption according to their rates of nicotine metabolism. Interestingly, the magnitude of CYP2A6’s effect on tobacco consumption was larger in smokeless tobacco users when compared with tobacco smokers (54% reduction in TNE in smokeless tobacco users with slower CYP2A6 activity; 41% reduction in TNE in smokers with slower CYP2A6 activity). This may be a reflection of the slower nicotine absorption from smokeless tobacco, which could allow for a longer period of time for metabolism to occur thereby increasing the effect of differences in the rate of nicotine metabolism. Overall, the data presented in this thesis suggest that genetic variation in CYP2A6 influences tobacco consumption in light/infrequent smokers and smokeless tobacco users as previously observed in heavy smokers. Therefore, previously reported CYP2A6 genetic findings in heavy smokers were extended here to light/infrequent smokers and smokeless tobacco users.

5.3.1.3 CYP2A6’s Role in Determining Tobacco Related Disease Risks

The findings of this thesis illustrate the dual roles (tobacco consumption and carcinogen activation) of CYP2A6 in influencing tobacco related carcinogen exposure. First, CYP2A6 genetic variants alter carcinogen exposure as the result of an alteration in tobacco consumption. In Chapter 1, we observed that the tobacco users with slower CYP2A6 activity had lower carcinogenic NNAL levels compared to the tobacco users with faster CYP2A6 activity, which was due to differences in tobacco consumption. These data suggest that the lower tobacco consumption observed in individuals with slower CYP2A6 activity reduces their risk of tobacco related diseases.
The second mechanism by which variation in \textit{CYP2A6} can modify tobacco related disease risk is through differential carcinogen metabolism. In Chapter 1, we observed higher NNN levels in smokers with slower CYP2A6 activity even after controlling for TNE. This suggests that NNN clearance was lower in individuals with slower CYP2A6 activity. Mechanistically, CYP2A6 is known to mediate the 5’-hydroxylation of NNN to genotoxic metabolites (Wong et al. 2005). Transfecting human CYP2A6 into a bacteria system resulted in a higher degree of mutagenesis upon NNN exposure (Kushida et al. 2000). Thus, we speculate that the slower CYP2A6 activity results in lower NNN 5’-hydroxylation, and more NNN was excreted in urine unmetabolized. Since bioactivation is required for NNN to exert its genotoxic effects, the reduced NNN bioactivation will reduce genotoxic metabolite formation and the risk of tobacco related cancers in individuals with slower CYP2A6 activity. This is supported by the human epidemiological evidence that \textit{CYP2A6} slow metabolizers exhibit a lower odds ratio of lung cancer, even when controlling for quantities smoked (Wassenaar et al. 2011). Overall, our findings indicate that individuals with slower CYP2A6 activity have lower tobacco consumption and lower carcinogen bioactivation compared to individuals with faster CYP2A6 activity. This could lead to lower carcinogenic reactive intermediate exposure and reduced tobacco related cancer risk (Wassenaar et al. 2011).

\subsection*{5.3.1.4 CYP2A6’s Role in Determining Smoking Cessation Outcomes}

A number of clinical trials and epidemiological studies strongly suggest that genetic variants in xenobiotic metabolizing enzymes, such as \textit{CYP2A6}, can significantly affect smoking cessation outcomes in heavy smokers (Lerman et al. 2006; Patterson et al. 2008). Due to the similar total nicotine intake between heavy and light smokers, these \textit{CYP2A6} pharmacogenetic findings are likely applicable to light/infrequent smokers as well. Smokers with slower CYP2A6 activity respond better to transdermal nicotine patch therapy than smokers with faster CYP2A6 activity (Lerman et al. 2006; Lerman et al. 2010). This finding suggests that smokers with slower CYP2A6 activity should be treated with nicotine patches (perhaps for an extended duration). In comparison, CYP2A6 activity does not alter the treatment response to bupropion (Patterson et al. 2008). Therefore smokers with faster CYP2A6 activity should be treated with bupropion rather than nicotine replacement therapy. Stratifications by additional genes may further improve the smoking cessation outcomes in smokers with faster CYP2A6 activity. Bupropion is metabolized
to hydroxybupropion by a genetically polymorphic enzyme CYP2B6. Animal studies suggest that hydroxybupropion is pharmacologically active. We demonstrated, in a real-world outpatient clinical trial, that higher hydroxybupropion levels, but not bupropion levels, increased smoking-cessation rates (Zhu et al. 2012). Genetic variation in CYP2B6 was identified as a significant source of variation in hydroxybupropion levels (Zhu et al. 2012; Benowitz et al. 2013). The findings indicate that personalized/stratified bupropion therapy based on hydroxybupropion levels or CYP2B6 genotype may substantially improve bupropion’s therapeutic efficacy (Manuscripts attached in Appendix B). Ultimately, we would recommend testing CYP2B6 genotypes in individuals with faster CYP2A6 activity, and treat individuals with slower CYP2B6 activity with varenicline (another non-nicotine smoking cessation treatment) and individuals with faster CYP2B6 activity with bupropion. This could further improve smoking cessation outcomes.

5.3.2 CHRNA5-A3-B4

5.3.2.1 The Association between CHRNA5-A3-B4 and Tobacco Consumption in Light Smokers

In Chapter 3, we presented novel findings with regards to CHRNA5-A3-B4 variants and nicotine intake in light/infrequent smokers. The quantitative aspects of our CHRNA5-A3-B4 findings have been discussed in the discussion section of Chapter 3. Here we focus on the functional role of CHRNA5-A3-B4 gene variants and the implications of our findings.

5.3.2.2 The Functional Consequences of CHRNA5-A3-B4 Variants

Despite extensive evidence for the association between CHRNA5-A3-B4 variants and smoking behaviors, there is very little known about the molecular impact of these variants on nAChR function. Most of the research has focused on the α5 subunit, which mediates nicotine’s aversive effects in the medial habenula (Fowler et al. 2011). It has been hypothesized that the CHRNA5-A3-B4 variants associated with higher nicotine intake reduce the function of α5-containing nAChR, resulting in a deficiency of α5 mediated aversive signaling particularly at high nicotine doses. This increases the ratio of nicotine reward to the aversive effects of nicotine at high nicotine doses. Rs16969968 is a nonsynonymous variant in exon 5 of CHRNA5 which results in an amino acid change of aspartic acid to asparagine at codon 398. The ‘A’ allele of rs16969969 has a 38% allele frequency in Caucasians, but is rare in African Americans and Asians.
(International Hapmap Project). The variant ‘A’ allele of rs16969968 (encoding for asparagine) is associated with higher nicotine intake in many racial populations including Alaska Native peoples (Saccone et al. 2009; Wassenaar et al. 2011; Chen et al. 2012; Zhu et al. 2013). Bioinformatically, rs16969968 was predicted to be benign based on the chemical effects of the amino acid substitution and the evolutionary conservation of the surrounding amino acid sequence (genetics.bwh.harvard.edu/). However, in vitro functional characterization suggests that this variant can alter α5 receptor function both in terms of receptor permeability and receptor desensitization. For instance, when human α5 nAChR subunits with either the wild-type (398D, aspartic acid) or the variant (398N, asparagine) allele were expressed in Xenopus oocytes, the 398N allele resulted in lower Ca^{2+} permeability in response to 10 µM acetylcholine when α4 and β2 were used as concatemers, but not when the α5 subunit was combined with α3 (Kuryatov et al. 2011). These observations were replicated in HEK293T cells co-expressing human α5, α4 and β2 subunits (Bierut et al. 2008). In addition to modulating nAChR permeability, rs16969968 can also influence the desensitization of α5 containing nAChR. The variant allele (398N) desensitized much faster after exposure to 3 µM acetylcholine than the wild-type allele when α4 and β2 were used as concatemers in Xenopus oocytes (Kuryatov et al. 2011). Again, this was not observed when the α3 subunits were expressed as concatemers.

Together, the lower Ca^{2+} permeability and faster desensitization suggested that the variant ‘A’ allele (398N) of rs16969968 may increase nicotine intake by reducing (α4β2)2α5 nAChR function, resulting in lower nicotine associated aversive effects. This hypothesis suggested that a positive allosteric (i.e. increase (α4β2)2α5 function) modulator at (α4β2)2α5 function may have some utility in reducing nicotine consumption and possibly promoting smoking cessation.

In comparison to rs16969968, less is known about the functional consequences of rs578776 which was significantly associated with tobacco consumption in Chapter 3. Rs578776 is not in linkage disequilibrium with rs16969968 in Caucasians (Chen et al. 2012). This lack of linkage disequilibrium is also observed in Alaska Native individuals (Chapter 3). Hence, it is unlikely that the association between rs578776 and nicotine intake is mediated by the aspartic acid to asparagine amino acid substitution of rs16969968. There is some evidence that rs578776 is associated with decreased CHRNA5 mRNA expression. In Caucasians, rs578776 is in linkage disequilibrium with rs6495308 (International Hapmap Project). The ‘C’ allele of rs6495308 (which is in linkage disequilibrium with the risk ‘A’ allele of rs578776) was significantly
associated with a 40% reduction in *CHRNA5* mRNA expression in postmortem brain (Wang et al. 2009; Saccone et al. 2010; Chen et al. 2012). This finding suggests that the ‘A’ allele of rs578776 may be associated with lower *CHRNA5* expression, which would result in lower aversive effects in response to nicotine and enhanced nicotine intake.

Due to the limited number of functional studies, it is difficult to interpret the neurobiology underlying the associations between genetic variation in *CHRNA5-A3-B4* and tobacco consumption. Future studies in the *CHRNA5-A3-B4* field should focus on identifying and functionally characterizing the causal variants (which are responsible for the altered function) that are in linkage with the tag SNPs. For example, it is unlikely that intronic variants like rs6495308 and rs578776 directly alter *CHRNA5* mRNA transcription or stability. Thus, sequencing individuals with the variant allele of rs6495308 or rs578776 may lead to the identification of novel promoter variants that are responsible for the altered *CHRNA5* mRNA levels. These findings could be followed up with bioinformatic binding site simulation, *in vitro* expression studies as well as *ex vivo* studies. This information will enhance our understanding of the molecular mechanism of nicotine addiction, allowing for the optimization and development of effective smoking cessation treatments.

5.3.2.3 Implications: Is the Association Between *CHRNA5-A3-B4* Variants and Lung Cancer Directly Mediated by Altered Tobacco Consumption?

As described in the introduction, the mechanism responsible for the association between *CHRNA5-A3-B4* and lung cancer risk has been debated since the initial genome-wide association studies were conducted (Hung et al. 2008; Thorgeirsson et al. 2008; Consortium 2010; Liu et al. 2010; Thorgeirsson et al. 2010). While some publications argue the association between *CHRNA5-A3-B4* gene variants and lung cancer risk is exclusively the result of altered tobacco consumption, others believe that *CHRNA5-A3-B4* can directly contribute to lung carcinogenesis by altering cell migration and survival.

The strongest evidence suggesting that the association between *CHRNA5-A3-B4* gene variants and lung cancer risk is exclusively mediated by tobacco consumption is from the association between *CHRNA5-A3-B4* gene variants and plasma cotinine levels. Munafò et al. reported that
each risk allele of rs16969968 was associated with a 24 ng/mL increase in plasma cotinine levels, which completely accounted for the 1.3 times higher lung cancer risk associated with rs16969968 (Munafo et al. 2012). However, as we have demonstrated in Chapter 2, cotinine levels often give a biased picture of tobacco consumption. There could be a 25% variation in plasma cotinine levels following the same tobacco and tobacco-derived carcinogen exposure depending on CYP2A6 activity. Therefore, it is hard to interpret cotinine data without considering CYP2A6 activity. Studies using TNE (which is less dependent on individual differences in nicotine metabolism) or NNAL (which is more directly involved in the carcinogenic process) will likely enhance our understanding of the contribution of tobacco consumption to the association CHRNA5-A3-B4 and lung cancer risk.

The effect size comparisons of Chapter 3 provide some indirect evidence that the association between CHRNA5-A3-B4 and lung cancer risk is not entirely mediated by tobacco consumption. For example, variation in CYP2A6 activity has a bigger effect on tobacco consumption than either rs16969968 or rs578776 in Alaska Native tobacco users, which is consistent with previous observations in Caucasian heavy smokers (Wassenaar et al. 2011). Slower CYP2A6 activity results in a 40% reduction in tobacco consumption, whereas CHRNA5-A3-B4 variants rs16969968 and rs578776 result in 30% and 20% reduction in tobacco consumption respectively. This is interesting because these CHRNA5-A3-B4 variants have a bigger impact on lung cancer risk than CYP2A6 (Wassenaar et al. 2011). This suggests the possibility that CHRNA5-A3-B4 variants have additional effects on lung carcinogenesis beyond altering tobacco consumption. This hypothesis is supported by data from never smokers. In a large study of 3500 lung cancer cases and 3300 controls in China, the effect of CHRNA5-A3-B4 variants on lung cancer were very similar among never and current smokers (OR=1.44 in never smokers and 1.42 in current smokers)(Wu et al. 2009). Similar effects of CHRNA5-A3-B4 on lung cancer risk among the never smokers and current smokers were also observed in the Japanese population (Shiraishi et al. 2009). Together, these data suggested that CHRNA5-A3-B4 variants could play a direct role in lung carcinogenesis since they also affect lung cancer risk in never smokers who do not alter their nitrosamine exposure by altering tobacco consumption.

CHRNA5-A3-B4 variants could directly contribute to lung carcinogenesis by two mechanisms. Firstly, α5 and α3 containing nAChRs, such as α3α5β2 heteropentamers, are highly expressed at
the wound edge of the bronchial mucosa injured by inhaling toxic substances and play an important role during wound repair (Grando 2006; Tournier et al. 2006; Schuller 2009). Polymorphisms resulting in low cell motility may lead to more persistent mucosal damage and inflammation, which could make mucosal cells more susceptible to precursor lesions. Secondly, \textit{CHRNA5-A3-B4} variants may modulate cancer-cell survival, invasion and metastasis. For example, \textit{CHRNA3} gene is systematically hypermethylated and down-regulated in lung cancers, and overexpression of \textit{CHRNA3} in lung cancer cells results in apoptosis, suggesting that lower \textit{CHRNA3} function provides cancer cells with a survival advantage (Lam et al. 2007; Paliwal et al. 2010). Currently, it is unclear how \textit{CHRNA5-A3-B4} variants affect these processes at the molecular level. Nevertheless, these data suggest that there is a biological role for nAChRs in lung carcinogenesis, and it is likely that the association between \textit{CHRNA5-A3-B4} variants and lung cancer is mediated by both tobacco consumption and altered cell survival and signaling in the lungs.

\subsection*{5.3.2.4 Implications for Smoking Cessation}

In contrast to the consistent associations between \textit{CHRNA5-A3-B4} variants and tobacco consumption, the pharmacogenetic impact of \textit{CHRNA5-A3-B4} variants on smoking cessation have not been consistently reported. A number of genome-wide association studies have attempted to determine the genetic and pharmacogenetic contribution to smoking cessation (Uhl et al. 2007; Uhl et al. 2008; Uhl et al. 2010). However, none of those studies observed significant associations between \textit{CHRNA5-A3-B4} variants and smoking cessation. In agreement with the genome-wide association studies, candidate gene studies in German and Korean populations also revealed no association between \textit{CHRNA5-A3-B4} gene variants and smoking cessation (Breitling et al. 2010; Li et al. 2010). Recently, more focused candidate gene studies (with more comprehensive \textit{CHRNA5-A3-B4} SNP genotyping) reported a few associations between \textit{CHRNA5-A3-B4} variants and smoking cessation (Munafo et al. 2011; Chen et al. 2012; Bergen et al. 2013). However, it is not clear whether the influence of \textit{CHRNA5-A3-B4} gene variants on smoking cessation is independent of the type of pharmacological treatments (i.e. cessation in general, or just a particular pharmacological therapy)(Munafo et al. 2011; Chen et al. 2012; Bergen et al. 2013). Furthermore, one important caveat when comparing these pharmacogenetic smoking cessation studies is that \textit{CHRNA5-A3-B4} risk alleles alter tobacco consumption and
FTND in smokers, but not all studies accounted for this. Higher CPD or FTND has been associated with increased difficulty in quitting (Dale et al. 2001; Berrettini et al. 2007). Thus, the association between CHRNA5-A3-B4 and smoking cessation may be mediated by altered dependence rather than direct interactions with smoking cessation pharmacotherapies.

We recently investigated the association of CHRNA5-A3-B4 variants with smoking cessation in light/infrequent smokers (manuscript attached in Appendix E). In two independent smoking cessation trials of 1295 African American light/infrequent smokers, we observed that rs16969698 occurred at a low allele frequency and was not associated with smoking cessation, suggesting that it has low pharmacogenetic importance in African American light/infrequent smokers. In contrast to the lack of effect with rs16969698, we observed a consistent association between the ‘A’ allele of rs2036527 and lower abstinence in active pharmacological treatment after controlling for differences in CPD and FTND, but not with the participants who received the placebo treatment. Since a significant association between rs2036527 and smoking behaviors has only been reported in African Americans, it is still untested whether this finding can be applied to other ethnic populations of smokers. Overall, the pharmacogenetic role of CHRNA5-A3-B4 variants and smoking cessation remains inconsistent and larger clinical trials are needed to clarify the associations.

5.4 Conclusions

Our findings contribute significantly to the existing literature on the genetic influence of nicotine dependence in light/infrequent smokers and smokeless tobacco users. Significant associations between genetic variants and smoking behaviors had not previously been demonstrated in light/infrequent smokers. Our data suggest that the lack of association was likely due to the limitations of the tobacco consumption biomarkers that were used.

Using improved biomarkers of tobacco consumption, we demonstrated that variants in both pharmacokinetic and pharmacodynamic genes can significantly alter tobacco consumption in light/infrequent smokers and smokeless tobacco users. Specifically, we illustrated that light/infrequent smokers and smokeless tobacco users with lower CYP2A6 activity consume less tobacco; they also have lower urinary NNAL levels and reduced NNN metabolism. Furthermore, we showed that genetic variants in CHRNA5-A3-B4 alter tobacco consumption and tobacco-
derived carcinogen exposure in light/infrequent smokers. Overall, both CYP2A6 and CHRNA5-A3-B4 genetic variants alter tobacco consumption in light/infrequent smokers as previously seen in heavy smokers.

The findings of this thesis provided mechanistic insight about smoking behaviors in light/infrequent smokers and smokeless tobacco users, which may enhance smoking cessation. Modifications to the existing scales of nicotine dependence to incorporate biomarker data will likely improve our ability to predict smoking cessation treatment outcomes in light/infrequent smokers. Future studies should focus on exploring individualized smoking cessation treatment strategies based on genetics and biomarkers in light/infrequent smokers.


Iwahashi, K., C. Waga, et al. (2004). "Whole deletion of CYP2A6 gene (CYP2A6AST;4C) and smoking behavior." Neuropsychobiology 49(2): 101-104.


(tranylcypromine), and its nonamine analog, cyclopropylbenzene." Drug Metab Dispos 29(3): 217-222.


nitrosamine exposure and lower tobacco-specific nitrosamine bioactivation."


Zhu, A. Z., C. C. Renner, et al. (2013). "The ability of plasma cotinine to predict nicotine and
carcinogen exposure is altered by differences in CYP2A6: the influence of genetics, race,

not alter the nicotine metabolite ratio or nicotine intake." PLoS One 8(8): e70938.

7 APPENDICES

OBJECTIVES: Alaska Native (AN) people have a high prevalence of tobacco use and associated morbidity and mortality when compared with the general USA population. Variations in the CYP2A6 and CYP2B6 genes, encoding enzymes responsible for nicotine metabolic inactivation and procarcinogen activation, have not been characterized in AN and may contribute toward the increased risk. METHODS: AN people (n=400) residing in the Bristol Bay region of South Western Alaska were recruited for a cross-sectional study on tobacco use. They were genotyped for CYP2A6*1X2A, *1X2B, *1B, *2, *4, *7, *9, *10, *12, *17, *35 and CYP2B6*4, *6, *9 and provided plasma and urine samples for the measurement of nicotine and metabolites. RESULTS: CYP2A6 and CYP2B6 variant frequencies among the AN Yupik people (n=361) were significantly different from those in other ethnicities. Nicotine metabolism [as measured by the plasma and urinary ratio of metabolites trans-3'-hydroxycotinine to cotinine (3HC/COT)] was significantly associated with CYP2A6 (P<0.001), but not CYP2B6 genotype (P=0.95) when controlling for known covariates. It was noteworthy that the plasma 3HC/COT ratios were high in the entire Yupik people, and among the Yupik CYP2A6 wild-type participants, they were substantially higher than those in previously characterized racial/ethnic groups (P<0.001 vs. Caucasians and African Americans). CONCLUSION: Yupik AN people have a unique CYP2A6 genetic profile that associated strongly with in-vivo nicotine metabolism. More rapid CYP2A6-mediated nicotine and nitrosamine metabolism in the Yupik people may modulate the risk of tobacco-related diseases.
Appendix B:

Full text:


Bupropion is indicated to promote smoking cessation. Animal studies suggest that the pharmacologic activity of bupropion can be mediated by its major metabolite, hydroxybupropion. We measured plasma bupropion and its metabolite levels in a double-blind, placebo controlled, randomized smoking-cessation trial. Among the treatment-adherent individuals, higher hydroxybupropion concentrations (per mug/ml) resulted in better smoking-cessation outcomes (week 3, 7, and 26 odds ratio (OR) = 2.82, 2.96, and 2.37, respectively, P = 0.005-0.040); this was not observed with bupropion levels (OR = 1.00-1.03, P = 0.59-0.90). Genetic variation in CYP2B6, the enzyme that metabolizes bupropion to hydroxybupropion, was identified as a significant source of variability in hydroxybupropion formation. Our data indicate that hydroxybupropion contributes to the pharmacologic effects of bupropion for smoking cessation, and that variability in response to bupropion treatment is related to variability in CYP2B6-mediated hydroxybupropion formation. These findings suggest that dosing of bupropion to achieve a hydroxybupropion level of 0.7 mug/ml or increasing bupropion dose for CYP2B6 slow metabolizers could improve bupropion's cessation outcomes.
Appendix C:

Full text:


BACKGROUND: Bupropion, an antidepressant and smoking cessation medication, is metabolized to hydroxybupropion (HB), an active metabolite, primarily by CYP2B6.

OBJECTIVES: To compare plasma concentrations of bupropion and metabolites at steady state in healthy volunteers with and without CYP2B6 genetic variants.

METHODS: In a genotype-guided study of 42 healthy individuals, we measured the plasma and urine concentrations of bupropion and its metabolites, HB, threohydrobupropion, and erythrohydrobupropion after 7 days of sustained-release bupropion dosing.

RESULTS: CYP2B6*6 and *18 gene variants were associated with ~33% reduced concentrations of HB, with no effects on concentrations of bupropion or other metabolites. We could account for 50% of the variation in HB concentrations in a model including genotype and sex.

CONCLUSION: As HB is active and its steady-state concentrations are more than 10 times higher than bupropion, CYP2B6 variants are likely to affect pharmacological activity. Because of the large individual variation within the genotype group, the use of therapeutic drug monitoring for dose optimization may be necessary.
Appendix D:


BACKGROUND: CYP2A6 metabolizes nicotine to its primary metabolite cotinine and also mediates the metabolism of cotinine to trans-3'-hydroxycotinine (3HC). The ratio of 3HC to cotinine (the "nicotine metabolite ratio", NMR) is an in vivo marker for the rate of CYP2A6 mediated nicotine metabolism, and total nicotine clearance, and has been associated with differences in numerous smoking behaviors. The clearance of 3HC, which affects the NMR, occurs via renal excretion and metabolism by UGT2B17, and possibly UGT2B10, to 3HC-glucuronide. We investigated whether slower 3HC glucuronidation alters NMR, altering its ability to predict CYP2A6 activity and reducing its clinical utility. METHODS: Plasma NMR, three urinary NMRs, three urinary 3HC glucuronidation phenotypes and total nicotine equivalents were examined in 540 African American smokers. The UGT2B17 gene deletion and UGT2B10*2 were genotyped. RESULTS: The UGT2B17 gene deletion, but not UGT2B10*2 genotype, was associated with slower 3HC glucuronidation (indicated by three 3HC-glucuronidation phenotypes), indicating its role in this glucuronidation pathway. However, neither lower rates of 3HC glucuronidation, nor the presence of a UGT2B17 and UGT2B10 reduced function allele, altered plasma or urinary NMRs or levels of smoking. CONCLUSIONS: Variation in 3HC glucuronidation activity, including these caused by UGT2B17 gene deletions, did not significantly alter NMR and is therefore unlikely to affect the clinical utility of NMR in smoking behavior and cessation studies. This study demonstrates that NMR is not altered by differences in the rate of 3HC glucuronidation, providing further support that NMR is a reliable indicator of CYP2A6 mediated nicotine metabolism.
Appendix E:

Association of CHRNA5-A3-B4 SNP rs2036527 with smoking cessation therapy response in African American smokers.
ABSTRACT (150/150)

Robust associations between *CHRNA5-A3-B4* variants and smoking behaviors exist, however the association with smoking abstinence is less understood, particularly among African Americans. In 1295 African Americans enrolled in two clinical trials, we investigated the association between *CHRNA5-A3-B4* and smoking abstinence. A consistent association between rs2036527[A] and lower abstinence during active pharmacotherapy was observed. Rs2056527[A] was associated with lower abstinence with nicotine gum (during-treatment: OR=0.31&P<0.001; end of treatment (EOT): OR=0.51&P=0.02), bupropion (during-treatment: OR=0.54&P=0.05; EOT: OR=0.59&P=0.08) and both together (during-treatment: OR=0.42&P<0.001; EOT: OR=0.55&P=0.004). Additionally, rs588765[T] was associated with abstinence with gum during treatment (OR=2.31&P<0.01). Rs16969968 occurred at a low frequency and was not consistently associated with abstinence. *CHRNA5-A3-B4* variants were not associated with tobacco consumption and adjustments for smoking behaviors did not alter the associations between rs2036527 and smoking abstinence. Together, our data suggest that *CHRNA5-A3-B4* variants are not associated with baseline smoking, but influence smoking abstinence during active pharmacotherapy in African Americans.
INTRODUCTION

Tobacco smoking is the largest preventable cause of premature death in the United States. There are currently 45 millions smokers in the U.S, and only 3% of them are able to quit smoking each year (1). Twin studies have estimated that the heritability of smoking cessation is roughly 50%, suggesting that genetic factors play an important role in determining smoking cessation outcomes (2). A number of genes have been significantly associated with smoking cessation outcomes in Caucasians (3-5). However, relatively few studies have focused on African American smokers despite their comparatively higher risks of smoking-related morbidity and mortality (6).

Nicotine exerts its pharmacologic effects by acting on nicotinic cholinergic receptors in the brain. In Caucasians, multiple independent SNPs (single-nucleotide polymorphisms) in the CHRNA5-A3-B4 gene cluster, which encodes for the α5, α3 and β4 subunits of the nicotinic acetylcholine receptor (nAChR), have been associated with smoking quantity (7-11) and smoking cessation outcomes (12-16). These loci included rs16969968 and correlated SNPs (sometimes referred as ‘Bin A’ or ‘Locus 1’), rs588765 and correlated SNPs (sometimes referred as ‘Bin B’ or ‘Locus 3’), and rs578776 and correlated SNPs (sometimes referred as ‘Bin C’ or ‘Locus 2’) (11, 17). However, it is not clear whether the influence of CHRNA5-A3-B4 gene variants on smoking cessation is a general effect on smoking cessation (i.e. would alter cessation in placebo treatment), is independent of the specific type of pharmacological treatment (i.e. would alter all active treatments) or alters cessation for specific pharmacological treatments (i.e. nicotine patch). One study suggested that the association between CHRNA5-A3-B4 variants (rs16969968 [Bin A] and rs680244 [Bin B]) and smoking cessation is primarily observed among smokers treated with placebo, and was not observed among those who received active pharmacological therapy (15). In contrast, another study reported significant associations
between *CHRNA5-A3-B4* variant (rs1051730 which is in high linkage disequilibrium with rs16969968) and smoking cessation outcomes in smokers who were receiving nicotine replacement therapy with little effect observed in the placebo arm (13). A recent study also observed a treatment by genotype interaction on smoking cessation outcomes (i.e. the genotype effects are in opposite directions for placebo vs. nicotine replacement therapy) (18).

In African Americans, the associations between *CHRNA5-A3-B4* gene variants and smoking behaviors are not as well understood. While some studies reported positive associations between rs16969968 and smoking behaviors in African American smokers (11), other studies were not able to replicate this finding (19, 20). The low allele frequency of rs16969968 (0% to 8% in African Americans compared to 38% to 40% in Caucasians) could contribute to this discrepancy. However, a very large genome-wide meta-analysis in African American smokers (*N*=32,389) identified a significant association between rs2036527 within *CHRNA5-A3-B4* and self-reported cigarettes per day (CPD) but no significant associations between rs16969968 or rs1051730 and CPD (19). We are not aware of any studies that have investigated the association between *CHRNA5-A3-B4* gene variants and smoking cessation in African American smokers.

Here we used two placebo controlled clinical trials to compare the influence of *CHRNA5-A3-B4* variants on smoking cessation outcomes in African Americans treated with placebo and active pharmacological treatments. We hypothesized that there would be a significant association between *CHRNA5-A3-B4* gene variants and smoking cessation outcomes in the active pharmacological treatment arms as previously reported by Munafò and colleagues (13). Since rs16969968 occurs at a relatively low allele frequency in African Americans, we focused initially on rs2036527, which has previously been significantly associated with CPD and lung cancer risk in African American (19, 21); we then extended the investigation to rs16969968, rs588765 and rs578776.
RESULTS:

Descriptive data of the participants

Among the 1295 participants (6, 22), 1143 DNA samples were extracted from blood and genotyped (609 in Study 1 and 534 in Study 2). All four of the genotyped SNPs were in Hardy-Weinberg equilibrium (all $P>0.05$, Supplementary Table S1). Rs16969968 (‘Bin A’), rs588765 (‘Bin B’), and rs578776 (‘Bin C’) were selected to represent three different haplotype bins which were previously associated with smoking behaviors (11). Rs2036527 was selected for its role in influencing smoking behaviors in African Americans (19). Rs2036527 had a minor allele frequency of 21.3%. Rs16969968 had a minor allele frequency of 5.7%. Rs588765 had a minor allele frequency of 31.7%. Rs578776 had a minor allele frequency of 47.9%. These four SNPs were in low linkage disequilibrium (all $r^2=0.02-0.39$) suggesting that they were independent of each other (Supplementary Table S1). None of the four genotyped SNPs were significantly associated with baseline demographics, CPD, % smoking mentholated cigarettes, baseline plasma cotinine levels (except with rs578776 in study 2 but not study 1), baseline total nicotine equivalents levels, levels of nicotine dependence or treatment group assignment (Table 1 & Supplementary Table S2).

Associations of CHRNA5-A3-B5 variants with smoking abstinence

Fig. 1 summarizes the association between CHRNA5-A3-B4 variants and smoking abstinence rates among the participants who received the active nicotine gum treatment (Fig. 1A), the active bupropion treatment (Fig. 1B), any active pharmacological treatment (Fig. 1C), or the placebo treatment (Fig.1D). For additive models please see Supplementary Fig. S1). Some notable associations are highlighted below.

Association between rs2036527 and smoking abstinence
The ‘A’ allele of rs2036527 was significantly associated with lower abstinence rates in participants who received active pharmacological treatments.

*Nicotine gum.* Among the participants who received the nicotine gum treatment, rs2036527 was significantly associated with lower abstinence rates during treatment and weakly at end of treatment (during treatment OR=0.31, 95%CI=0.15-0.62, P<0.001; end of treatment OR=0.51, 95%CI=0.29-0.90, P=0.019. Fig.1A). Adjusting for age, sex, baseline CPD, menthol status and type of counseling sessions did not meaningfully alter the odds ratio between rs2036527 and smoking abstinence (adjusted during treatment OR=0.29, 95%CI=0.14-0.60, P<0.001; adjusted end of treatment OR=0.48, 95%CI=0.27-0.87, P=0.02. Fig.2A). The number needed to treat (NTT) were 6.3 and 14.6 for the GG and the GA/AA group respectively during the nicotine gum treatment. At the end of the nicotine gum treatment, NTT was 13.5 for the GG group (nicotine gum treatment had no benefit in the GA/AA group).

*Bupropion.* As found with nicotine gum, the ‘A’ allele of rs2036527 trended toward a significant association with lower smoking abstinence rates during treatment and at end of treatment among the participants who received active bupropion treatment (during treatment OR=0.54, 95%CI=0.29-1.01, P=0.054; end of treatment OR=0.59, 95%CI=0.33-1.07, P=0.08. Fig.1B). Adjusting for age, sex, baseline CPD, and menthol status did not meaningfully alter the odds ratios between rs2036527 and smoking abstinence (adjusted during treatment OR=0.56, 95%CI=0.30-1.06, P=0.07; adjusted end of treatment: OR=0.61, 95%CI=0.33-1.13, P=0.12. Fig.2B). During the bupropion treatment, the NTT was 13.6 for the GG group (bupropion treatment had no benefit in the GA/AA group). At the end of the bupropion treatment, NTT were 6.2 and 8.8 for the GG and the GA/AA group, respectively.

*Combined analyses.* In agreement with our primary hypothesis, the ‘A’ allele of rs2036527 was significantly associated with lower smoking abstinence rates during treatment.
and at end of treatment among the participants who received any active pharmacological treatments (during treatment OR=0.42, 95% CI=0.27-0.67, \( P<0.001 \); end of treatment OR=0.55, 95% CI=0.36-0.82, \( P=0.004 \). Fig. 1C). Adjusting for age, sex, baseline CPD, menthol status, type of counseling sessions and type of pharmacological treatment did not meaningfully alter the association between rs2036527 and smoking abstinence (Adjusted during treatment OR=0.42, 95% CI=0.27-0.67, \( P<0.001 \); adjusted end of treatment OR=0.55, 95% CI=0.36-0.83, \( P=0.005 \). Fig. 2C).

No statistically significant association between rs2036527 and smoking abstinence was observed at any time-point in the placebo arm (Fig. 1D) even after adjusting for age, sex, baseline CPD, menthol status, and type of counseling sessions (Fig. 2C).

**The association of rs588765 and smoking abstinence**

In addition to rs2036527, rs588765 was significantly associated with smoking abstinence, but only during treatment.

*Nicotine gum.* The ‘T’ allele of rs588765 was significantly associated with smoking abstinence during treatment among the participants who received nicotine gum (OR=2.31, 95% CI=1.25-4.29, \( P=0.008 \). Fig. 1A). Adjusting for age, sex, baseline CPD, menthol status and type of counseling sessions did not alter the association between rs588765 and smoking abstinence (adjusted OR=2.39, 95% CI=1.27-4.49, \( P=0.007 \). Fig. 3A).

*Bupropion.* We did not observe any consistent association between rs588765 and smoking abstinence in the bupropion treatment group (Fig. 3B).

*Combined analyses.* The ‘T’ allele of rs588765 was significantly associated with smoking abstinence during treatment among the participants who received active pharmacological treatments (OR=1.85, 95% CI=1.21-2.84, \( P=0.004 \). Fig. 1C). Adjusting for age, sex, baseline
CPD, menthol status, type of counseling sessions and type of pharmacological treatment did not meaningfully alter the association between rs588765 and smoking abstinence (adjusted during treatment OR=1.81, 95%CI=1.17-2.80, \( P=0.007 \), Fig.3C).

Among the participants who received the placebo treatment (Fig.1D), the ‘T’ allele of rs588765 was significantly associated with abstinence during treatment (OR=0.49, 95%CI=0.30-0.81; \( P=0.005 \), Fig.1D). There was also a significant treatment (combined active vs. combined placebo) by rs588765 genotype interaction during treatment (OR=3.75, 95%CI=1.96-7.19; \( P<0.001 \)). Adjusting for age, sex, baseline CPD, menthol status, and type of counseling sessions did not meaningfully alter the odds ratio between rs588765 and smoking abstinence during treatment (adjusted OR=0.47, 95%CI=0.24-0.88, \( P=0.003 \), Fig.3C).

**The association of rs16969968 and smoking abstinence**

Fig. 4 summarized the association between smoking abstinence and rs16969968 with and without baseline demographic adjustments. In these two clinical trials of African American smokers, we did not observe any consistent association between rs16969968 and smoking abstinence (Fig. 4).

**DISCUSSION**

We reported novel findings of an association between a \( \text{CHRNA5-A3-B4} \) gene variant and smoking abstinence in African Americans. We identified a consistent association between the ‘A’ allele of rs2036527 and lower rates of smoking abstinence during active pharmacotherapy treatment in African American light smokers.

**\( \text{CHRNA5-A3-B4} \) and smoking behaviors**

Gene variants in \( \text{CHRNA5-A3-B4} \), particularly rs16969968 and correlated SNPs, have
been consistently associated with heaviness of smoking and the levels of nicotine dependence in Caucasians (9-11, 23). In contrast to a recent meta-analysis in African American smokers (19), we did not observe any significant association between CHRNA5-A3-B4 variants, particularly rs2036527, and baseline smoking behaviors, including self-reported CPD, plasma cotinine levels or urinary total nicotine equivalents. This was unexpected since our study, although smaller in size compared to the meta-analysis, had objective biomarker data. Since it is well known that self-reported CPD does not account for the depth of inhalation and is a relatively weak marker of tobacco exposure (24), we expected the accuracy of biomarker data to compensate for the smaller sample size (N=1142). One reason for the lack of association between tobacco exposure and rs2036527 in our study may be that the previous meta-analyses included a substantial number of former smokers whereas our studies included only current smokers (19). It was previously observed that the influence of CHRNA5-A3-B4 gene variants on self-reported CPD is stronger in the former smokers compared to the current smokers (see Table S7 of Amos et al. study (8)). It is also possible that the lower number of cigarettes smoked per day by light smokers in the current study (mean=7.7) in contrast to the previous meta-analysis (mean ranged from 11.5-15.7) mitigated the expression of the underlying biological processes caused by the CHRNA5-A3-B4 gene variants on smoking level. However, it is important to note that CPD is a poor marker of tobacco consumption, susceptible to reporting and recalling biases and insensitive to smoking topography. The average cotinine levels, which is an more objective marker of tobacco consumption, in our study (mean=240 ng/mL, standard deviation=138) were comparable to previously observed in Caucasian and American African heavy smokers (mean≈170 ng/mL) (24). Previous studies in the CHRNA5-A3-B4 field have suggested objective biomarkers are more sensitive than CPD (7). We have previously shown that CHRNA5-A3-B4 variants are significantly associated with cotinine levels in as few as 163 smokers (25). Therefore, we believe that the accuracy of biomarkers (versus self-report CPD)
compensates for the smaller size of our study.

**CHRNA5-A3-B4 and smoking abstinence**

In Caucasians, a number of studies have examined the association between CHRNA5-A3-B4 gene variants and clinical trial outcomes for quitting smoking. These studies have varied in whether the predominant effect of CHRNA5-A3-B4 gene variants was in the placebo arm, a specific treatment arm, or in all active treatment arms more generally (13, 15, 18). Our observations regarding rs2036527 and smoking abstinence in African American smokers are in agreement with a previous report by Munafò and colleagues in Caucasians with rs1051730 (13). Both studies reported significant associations between CHRNA5-A3-B4 gene variants and abstinence 1) primarily in the active pharmacological therapy groups, with little effect observable within the placebo arm, and 2) only during treatment (no association remains at follow up, after active treatment had stopped). Of note, in Caucasians rs2036527 is in high linkage disequilibrium with rs1051730 ($r^2=0.90$). Therefore, rs2036527 may be a better pharmacogenetic marker for smoking cessation than rs16969968/rs1051730 as it predicts similar relapse risks in both Caucasians and African Americans.

The α5 nAChR subunit mediates an inhibitory effect of nicotine on brain reward systems (26). Mice with lower α5 nAChR function exhibited increased nicotine intake (26). Since rs2036527 was previously associated with increased nicotine intake in African Americans (19), it is likely that rs2036527 (or SNPs in high linkage disequilibrium) results in lower α5 nAChR function. Both nicotine gum and bupropion could have inhibitory effects on nicotine reward since both of them can bind to the nAChR (27, 28). The inhibitory effects of nicotine gum and bupropion may be weaker in individuals with rs2036527 (due to their lower α5 function), reducing the efficacy of these treatments.
Our findings with rs588765 and smoking abstinence in African American smokers are very similar to previous findings in Caucasian smokers (18). Both studies observed that the ‘T’ allele of rs588765 was associated with lower abstinence in the placebo group and higher abstinence when receiving nicotine replacement therapy (with significant interactions). However, the association between rs588765 and smoking abstinence was still observed at 6-month follow up in Caucasians, but not in African American smokers. This could be partially due to the relatively low smoking abstinence rates in African American smokers at 6-month follow up compared with the Caucasian smokers.

We did not observe any consistent association between rs16969968 and smoking abstinence in this study. It is also worth noting that the direction of the association between rs16969968 and smoking abstinence in African Americans was in the opposite direction compared with in Caucasians (18). The ‘A’ allele of rs16969968 was associated lower abstinence with nicotine gum treatment and higher abstinence with placebo treatment in our study of African American smokers. In comparison, the ‘A’ allele of rs16969968 was associated with higher abstinence with nicotine replacement therapy and lower abstinence with placebo treatment in Caucasians (18). It is possible that different linkage disequilibrium/haplotype structures between African Americans and Caucasians contributes to this discrepancy. Overall, our smoking abstinence data and the relatively low prevalence of rs16969968 in African American would suggest that rs16969968 has very little pharmacogenetic importance in African American smokers.

In contrast to the study published by Chen and colleagues (15), we did not observe any long lasting association between CHRNA5-A3-B4 gene variants and smoking abstinence rates in participants who received the placebo treatment. This discrepancy was unlikely to be the results of limited power since the current present investigation had a much bigger placebo group compared to the study by Chen and colleagues (566 in our combined studies versus 132 in the
Chen et al., study (15)). It is possible the effects of the ‘tag’ SNPs are different among different ethnic groups due to the distinct haplotype structures.

Interestingly, adjusting for CPD had little influence on the association between the CHRNA5-A3-B4 gene variants and abstinence in our study and in a number of previous studies which evaluated the association between CHRNA5-A3-B4 gene variants and smoking abstinence (13, 18). This suggests that the influence of CHRNA5-A3-B4 gene variants on smoking abstinence is not related to their modest effect size on altering tobacco consumption. In addition, the impact of rs2036527 remained unaltered when we controlled for CYP2A6 and/or CYP2B6 genotype, suggesting the impact of rs2036527 on cessation was independent of variation in nicotine or bupropion metabolism.

Lastly, our findings suggested that roughly 60% of African Americans (i.e. individuals with rs2036527[G] allele) would benefit greatly from active smoking cessation treatments, whereas the 40% with the rs2036527[A] allele may not. This demonstrates the potential value of pharmacogenetic approaches in improving smoking cessation outcomes among African American smokers (29, 30).

**Strength and Limitations**

A major strength of our study is that the smoking status (and baseline level of smoking) was biochemically verified. Our selection of African American light smokers can be viewed as both a strength and limitation. African Americans smokers experience disproportionately higher risks of smoking-related morbidity and mortality compared to Caucasians (31). However, very little is known about the efficacy of standard smoking cessation treatments in this population, particularly among light smokers who make up more than 50% of the African American smokers. However, the inclusion of light smokers may limit the generalizability of our findings to other groups. In addition, the placebo arm in both trials had relatively low smoking abstinence rates,
the low number of abstinence events could limit the statistical power to evaluate these associations. The low number of abstinent individuals also limited our ability to evaluate the effects of multiple SNPs in the same model. Another limitation was that we did not have reliable (biochemically verified) time of relapse data in this study, which could provide some time course information about the effect of rs2036527 on smoking cessation.

Population stratification is an important issue in genetic association studies. A limitation of this study was that we did not have ancestry informative markers to statistically control for population stratification. However, as improving smoking cessation treatments for Africa Americans is our goal, we believe that the limitations due to admixture are balanced against the need to improve smoking cessation in this population. Eliminating individuals who are modestly admixed, but would still self-identify as African Americans, could greatly reduce the clinical applicability of our findings.

Overall, our findings indicate that gene variants in CHRNA5-A3-B4 affect smoking abstinence in African Americans during the pharmacological treatment phase, but not during follow-up, even after adjusting for baseline smoking behaviors. The association between rs2036527 and smoking abstinence was observed with both nicotine gum and bupropion making it difficult to use for treatment personalization. Further studies should focus on understanding the mechanism(s) underlying this association in order to optimize the efficacy of smoking cessation treatments.

MATERIALS AND METHODS:

Study descriptions

We evaluated the association between the CHRNA5-A3-B4 variants and smoking cessation outcomes in two independent smoking cessation trials. Both trials were conducted in
African American light smokers (≤10 CPD) at the same community health center and the enrollment criteria were similar. These trials were focused on light smokers because 1) more than 50% of African American smokers are light smokers, and 2) little is known about the pharmacology and pharmacogenetics of smoking cessation treatments in this population.

**Nicotine gum study (Study 1):** This study was a randomized double-blind, placebo-controlled trial to evaluate the efficacy of nicotine gum (2 mg) in African American light smokers (6). Eligible participants self-identified as “African American” or “Black”, were at least 18 years old, had smoked 10 or fewer CPD for at least 6 months prior to enrollment, and smoked on at least 25 of last 30 days. This study consisted of four treatment arms (N=~190 each): 1) nicotine gum with health education (HE) counseling; 2) nicotine gum with motivational interviewing (MI) counseling; 3) placebo with HE and 4) placebo with MI. The nicotine gum treatment lasted 8 weeks, and six counseling sessions were provided to each participant. The participants were followed for a total of 26 weeks (6 months).

**Bupropion study (Study 2):** This study was a randomized double-blind, placebo-controlled trial to evaluate the efficacy of bupropion (300 mg per day, total N=540) in African American light smokers (22). Eligibility criteria were similar to Study 1 and included exclusion based on contraindication of bupropion use. Bupropion use lasted 7 weeks and HE counseling was similar to Study 1. The participants were followed for a total of 26 weeks (6 months). Both studies were approved by the University of Kansas Human Subject Committee, the University of Toronto Ethics Review Office, and the University of California San Francisco Human Research Protection Program.

Both studies assessed age, sex, menthol use, baseline CPD, baseline plasma cotinine, Fagerstrom Test for Nicotine Dependence (FTND). Study 2 also assessed urinary total nicotine equivalents, which is the total urinary level of nicotine and 8 of its metabolites. The analytical
procedures were described previously (32). Together, the 9 analytes account for about 90% of nicotine dose (33), and creatinine adjusted spot urinary TNE correlates with daily tobacco consumption (34).

**Smoking abstinence**

Biochemically verified abstinence was assessed during treatment (Week 1 for Study 1 and week 3 for the Study 2), at end of treatment (Week 8 for Study 1 and week 7 for Study 2) and at 6-month follow-up (6 month for both Study 1 and Study 2). In Study 1, abstinence was verified by exhaled CO levels of lower than 10 ppm. In Study 2, abstinence was verified by having salivary cotinine levels less than 15 ng/ml. All statistical analyses were performed on an intent-to-treatment basis, and subjects lost during follow-up were considered smokers.

**Genotyping**

*CHRNA5-A3-B4* SNPs were genotyped using ABI Viia 7 real time PCR machine (Applied Biosystems, Foster City, CA). The genotyping reaction was performed with 5 µL TaqMan GTXpress master mix and 5 µL of water containing 10 ng of DNA and 0.25 µL of 40x Taqman SNP genotyping assay (see Supplementary Table S1 for the specific probes used for each SNP). The allele discrimination data were analyzed by Viia 7 software version 1.2. All genotyping results included in our analyses had call quality values above 0.985.

**Statistical Analyses**

The comparisons of baseline demographic variables were performed by Mann Whitney tests or Chi² tests. Evaluation of the association between the dichotomized SNPs (rs2036527[GG=0 and GA/AA=1]; rs16969968[GG=0 and AG/AA=1]; rs588765[CC=0 and CT/TT=1]; and rs578776[AA=0 and AG/GG=1],) and smoking abstinence were conducted using logistic regression. Modeling the influence of the variants as ‘additive’ resulted in very similar
statistical results compared to modeling the influence of the variants as dichotomized valuable (results shown in Supplementary Fig.S1). Logistic regressions were used to evaluate the association between rs2036527 or rs588765 and smoking abstinence after adjusting for age, sex, baseline CPD, menthol status, the type of counseling sessions (Study 1) and other CHRNA5-A3-B4 SNPs. Adjusting for other CHRNA5-A3-B4 variants (such as rs16969968) did not alter the associations between rs2036527 and rs588765 with smoking abstinence (Supplementary Fig. S2, S3&S4). The P-values were not adjusted for multiple comparisons. Because there were 4 independent SNPs, we considered P-values below 0.0125 (i.e. 0.05/4) to be statistically significant. The number needed to treat represents the average number of patients needed to treat with the active versus placebo pharmacotherapy to prevent one patient from smoking relapse. Statistical analyses were performed using Stata 12 (StataCorp, College Station, TX).

**STUDY HIGHLIGHTS (145/150)**

**What is the current knowledge of the topic?**

Smoking is a major cause of preventable death globally. Smoking cessation at any age has substantial health benefits.

**What question did this study address?**

While the association between variants in CHRNA5-A3-B4 (a nicotinic receptor gene cluster) and smoking has been explored previously, the association between CHRNA5-A3-B4 variants and smoking cessation is poorly understood, particularly among African Americans.

**What this study adds to our knowledge?**
In African American smokers, this study demonstrated that a common variant in *CHRNA5-A3-B4* is associated with significantly lower smoking cessation rates during active pharmacological treatment.

**How this might change clinical pharmacology and therapeutics?**

This study suggests that roughly 60% of African Americans would benefit greatly from active smoking cessation treatments, whereas the other 40% may not. This illustrates the potential value of pharmacogenetic approaches in improving smoking cessation outcomes among African American smokers.
Acknowledgements

We acknowledge the support of NIH grant CA091912 to fund this study, National Center for Minority Health Disparities grant 1P60MD003422 (JSA), the Endowed Chair in Addiction for the Department of Psychiatry (RFT), CIHR grant MOP86471 (RFT), Ontario Graduate Scholarship (AZZ), CAMH and the CAMH foundation (RFT), NIH PGRN grants DA020830 (RFT, NB), DA017441 (SPD) and DA012353 (NB), the Canada Foundation for Innovation (#20289 and #16014) and the Ontario Ministry of Research and Innovation (RFT). The authors would like to thank Kelly Li for her technical assistance.

Conflict of Interest

NLB serves as a consultant to several pharmaceutical companies that market smoking cessation medications and has been a paid expert witness in litigation against tobacco companies. SPD is a scientific advisor to Genophen. RFT has participated in one-day advisory meetings for Novartis and McNeil.


Urine menthol as a biomarker of mentholated cigarette smoking. *Cancer 
Table 1. *CHRNA5-A3-B4* variants are not significantly associated with baseline smoking behaviors

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>Study 2</th>
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<td>GG</td>
<td>GA</td>
<td>AA</td>
<td><em>P</em>-values</td>
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<td><strong>n</strong></td>
<td>372</td>
<td>210</td>
<td>26</td>
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<tr>
<td>Baseline cigarettes per day</td>
<td>7.4 (7.1-7.7)</td>
<td>7.7 (7.3-8.2)</td>
<td>7.4 (6.2-8.6)</td>
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<td>Plasma Cotinine (ng/mL)</td>
<td>238 (223-254)</td>
<td>257 (236-279)</td>
<td>262 (193-330)</td>
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<tr>
<td>Urinary total nicotine equivalent (nmol/ mg Cre)</td>
<td>N/A</td>
<td>62.7 (54.2-71.1)</td>
<td>50.7 (45.5-55.9)</td>
<td>60.5 (43.3-77.6)</td>
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<td>FTND</td>
<td>2.8 (2.7-3.0)</td>
<td>3.0 (2.7-3.2)</td>
<td>3.5 (2.8-4.3)</td>
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<tr>
<td><strong>n</strong></td>
<td>544</td>
<td>62</td>
<td>3</td>
<td>0.84</td>
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<tr>
<td>Baseline cigarettes per day</td>
<td>7.6 (7.3-7.8)</td>
<td>7.3 (6.4-8.2)</td>
<td>7.7 (1.4-13.9)</td>
<td>0.84</td>
</tr>
<tr>
<td>Plasma Cotinine (ng/mL)</td>
<td>245 (232-258)</td>
<td>253 (210-297)</td>
<td>230 (135-325)</td>
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<td>Urinary total nicotine equivalent (nmol/ mg Cre)</td>
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<td>59 (53-65)</td>
<td>51 (41-62)</td>
<td>68 (-19.4-155)</td>
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<td>FTND</td>
<td>2.92 (2.77-3.07)</td>
<td>2.94 (2.45-3.41)</td>
<td>4.0 (1.5-6.5)</td>
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<td><strong>n</strong></td>
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<td>252</td>
<td>63</td>
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<tr>
<td>Baseline cigarettes per day</td>
<td>7.3 (6.9-7.6)</td>
<td>7.8 (7.4-8.2)</td>
<td>7.63 (6.8-8.5)</td>
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<tr>
<td>Plasma Cotinine (ng/mL)</td>
<td>251 (232-269)</td>
<td>242 (223-262)</td>
<td>243 (201-286)</td>
<td>0.68</td>
</tr>
<tr>
<td>Urinary total nicotine equivalent (nmol/ mg Cre)</td>
<td>N/A</td>
<td>61 (51-71)</td>
<td>57 (50-63)</td>
<td>55 (47-64)</td>
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<tr>
<td>FTND</td>
<td>2.9 (2.7-3.1)</td>
<td>3.0 (2.8-3.2)</td>
<td>2.9 (2.4-3.3)</td>
<td>0.68</td>
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<tr>
<td><strong>n</strong></td>
<td>173</td>
<td>303</td>
<td>133</td>
<td>0.35</td>
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<tr>
<td>Baseline cigarettes per day</td>
<td>7.3 (6.8-7.8)</td>
<td>7.5 (7.1-7.9)</td>
<td>7.9 (7.3-8.4)</td>
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<td>Plasma Cotinine (ng/mL)</td>
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<td>240 (223-257)</td>
<td>263 (236-291)</td>
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<td>Urinary total nicotine equivalent (nmol/ mg Cre)</td>
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<td>3.2 (2.8-3.5)</td>
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</tr>
</tbody>
</table>

Data presented as Mean (95% Confident Interval).
The urinary total nicotine equivalents were only available for a subset of individuals in Study 2.
FTND=Fagerstrom Test for Nicotine Dependence

Figure legends

Figure 1. The associations (dominant model) between CHRNA5-A3-B5 variants and smoking abstinence in African Americans smokers. A) Among the participants who received active nicotine gum treatment (Study 1). B) Among the participants who received active bupropion treatment (Study 2). C) Among the participants who received active pharmacological treatments (combined analysis). D) Among the participants who received placebo (combined analysis).

Figure 2. The ‘A’ allele of rs2036527 was associated with lower smoking cessation rates in African American smokers receiving nicotine gum treatment and bupropion. OR_{unadj.} = unadjusted odds ratio of quitting for the GA&AA genotype group compared to the GG genotype. OR_{adj.} = odds ratio of quitting for the GA&AA genotype group compared to the GG genotype after adjusting for age, sex, baseline CPD, menthol status and type of counseling session. A significant genotype by treatment interaction was observed during treatment ($P<0.01$).

Figure 3. The ‘T’ allele of rs588765 was associated with smoking abstinence in African American smokers receiving nicotine gum treatment and bupropion. OR_{unadj.} = unadjusted odds ratio of quitting for the CT&TT genotype group compared to the CC genotype. OR_{adj.} = odds ratio of quitting for the CT&TT genotype group compared to the CC genotype after adjusting for age, sex, baseline CPD, menthol status and type of counseling sessions.

Figure 4. Rs16969968 was not consistently associated with smoking abstinence in African American smokers receiving nicotine gum treatment and bupropion. OR_{unadj.} = unadjusted odds ratio of quitting for the GA&AA genotype group compared to the GG genotype. OR_{adj.} = odds ratio of quitting for the GA&AA genotype group compared to the GG genotype after adjusting for age, sex, baseline CPD, menthol status and type of counseling sessions.
Figure 2  A. Study 1 Nicotine Gum: rs2036527

B. Study 2 Bupropion: rs2036527

C. Combined: rs2036527
Figure 3

A. Study 1 Nicotine Gum: rs588765

B. Study 2 Bupropion: rs588765

C. Combined: rs588765
Figure 4

A. Study 1 Nicotine Gum: rs16969968

B. Study 2 Bupropion: rs16969968

C. Combined: rs16969968
Appendix F:

Full text:


OBJECTIVE: Nicotine, the main addictive ingredient in tobacco, is metabolically inactivated to cotinine primarily by the hepatic enzyme CYP2A6. Considerable genetic variation in the CYP2A6 gene results in large variation in the rates of nicotine metabolism, which in turn alters smoking behaviours (e.g. amount of cigarettes smoked, risk for dependence and success in smoking cessation). The aim of this study was to identify and characterize novel variants in CYP2A6. MATERIALS AND METHODS: The CYP2A6 gene from African American phenotypically slow nicotine metabolizers was sequenced and seven novel variants were identified [CYP2A6*39 (V68M), CYP2A6*40 (I149M), CYP2A6*41 (R265Q), CYP2A6*42 (I268T), CYP2A6*43 (T303I), CYP2A6*44 (E390K), CYP2A6*44 (L462P)]. Variants were introduced into a bi-cistronic cDNA expression construct containing CYP2A6 and P450 oxidoreductase and assessed for protein expression, enzymatic activity and stability as evaluated using western blotting and nicotine metabolism. Genotyping assays were developed and allelic frequencies were assessed in 534 African Americans. RESULTS: The variants showed significantly lower protein expression (P<0.001) when compared with the wild-type as well as reduced metabolism of nicotine to cotinine when controlling for cDNA expression using P450 oxidoreductase (P<0.001). The variants also showed reduced stability at 37 degrees C. Allelic frequencies ranged from 0.1 to 0.6% with a collective genotype frequency of 3.2%; the impact in vitro correlated significantly with in-vivo activity (R=0.40-0.48, P<0.05). Together, those with a novel variant had significantly lower nicotine metabolism in vivo than those without genetic variants (P<0.01). CONCLUSION: Here, we identified a number of novel variants with reduced/loss of CYP2A6 activity, increasing our understanding of CYP2A6 genetic variability.