IMPACT OF CYP2A6 GENETIC VARIATION ON NICOTINE METABOLISM AND SMOKING BEHAVIOURS IN LIGHT SMOKING POPULATIONS OF BLACK-AFRICAN DESCENT

By

Man Ki Ho

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

© Copyright by Man Ki Ho 2011
IMPACT OF CYP2A6 GENETIC VARIATION ON NICOTINE METABOLISM AND SMOKING BEHAVIOURS IN LIGHT SMOKING POPULATIONS OF BLACK-AFRICAN DESCENT

Man Ki Ho

Doctor of Philosophy

Graduate Department of Pharmacology and Toxicology
University of Toronto

2011

ABSTRACT

Populations of Black-African descent have slower rates of nicotine and cotinine metabolism, smoke fewer cigarettes (~10 cigarettes/day), and have higher incidences of tobacco-related illnesses compared to Caucasians. Cytochrome P450 2A6 (CYP2A6) is the main enzyme involved in the metabolism of nicotine and its proximal metabolite cotinine, as well as tobacco-specific nitrosamines. Genetic polymorphisms in CYP2A6 contribute to the large variability observed in rates of nicotine metabolism. Reduced CYP2A6 activity has been associated with fewer cigarettes smoked, higher quit rates, and lower lung cancer risk in predominantly moderate to heavy-smoking (~20–30 cigarettes/day) Caucasians. CYP2A6 genetic variants and their impact on smoking behaviours have not been well studied among individuals of Black-African descent. The main objectives herein were to identify and characterize new CYP2A6 variants that may explain the slower rates of metabolism, and determine whether CYP2A6 variation is a predictor of smoking phenotypes in this population. Furthermore, we examined whether previously validated biomarkers of tobacco exposure have limitations among individuals of
Black-African descent given their low and sporadic smoking patterns. A new CYP2A6 variant (CYP2A6*23) was found in individuals of Black-African descent recruited for a nicotine pharmacogenetic-pharmacokinetic study. CYP2A6*23 reduced activity towards nicotine and coumarin in vitro and was associated with slower rates of CYP2A6 kinetics in vivo. In a clinical trial of African-American light smokers, CYP2A6 slow metabolizers were more successful at smoking cessation compared to normal metabolizers, although no differences in cigarette consumption were found. Two common biochemical markers of tobacco smoke exposure, cotinine and exhaled carbon monoxide, were weakly correlated with self-reported cigarette consumption. These biomarkers were not substantially affected by variables previously shown to alter amount smoked and/or rates of cotinine metabolism such as gender, age, body mass index or smoking menthol cigarettes. However, CYP2A6 slow metabolizers had significantly higher cotinine without smoking more cigarettes. Identification and characterization of novel variants adds to our understanding of nicotine pharmacokinetic differences between racial/ethnic minority groups and improves accuracy of CYP2A6 genotype groupings for genetic association studies. Furthermore, better insight into the biological factors associated with smoking behaviours will aid in the development of more efficacious targeted treatments for this understudied population.
ACKNOWLEDGEMENTS

As author of this thesis, I would like to acknowledge the people who contributed their time and efforts to this work. Firstly, I would like to thank my supervisor Dr. Rachel F. Tyndale for her support, encouragement and guidance. She has provided me with an invaluable learning experience and I am grateful for her mentorship.

I would also like to thank all of my colleagues in the lab for their technical assistance, theoretical guidance, and moral support. In particular, I would like to thank Ewa Hoffmann, Qian Zhou and Zhao Bin for their contributions to my projects.

I am grateful for my committee members Drs. Albert Wong and Usoa Busto for sharing their time and knowledge, as well as providing helpful suggestions and comments for improvement. I would also like to thank Drs. Jasjit Ahluwalia and Kola Okuyemi for their collaborations.

I would like to thank my parents (Patrick and Florence Ho), my sister (Man Ying Ho), and all the wonderful friends I made over the years for their unconditional support and encouragement.

Finally I am graciously indebted to the Natural Sciences and Engineering Research Council of Canada, the Canadian Institutes of Health Research – Interdisciplinary Capacity Enhancement Scholars Program, the Canadian Tobacco Control Research Initiative and the University of Toronto for providing the financial support without which my continued success in this program would not have been possible.
TABLE OF CONTENTS
ABSTRACT .................................................................................................................................... ii
ACKNOWLEDGEMENTS ........................................................................................................... iv
TABLE OF CONTENTS ................................................................................................................ v
LIST OF PUBLICATIONS ........................................................................................................... xi
LIST OF TABLES ....................................................................................................................... xiii
LIST OF FIGURES ..................................................................................................................... xiv
LIST OF ABBREVIATIONS ....................................................................................................... xv
STATEMENT OF RESEARCH PROBLEM .............................................................................. xvi
MAIN RESEARCH OBJECTIVES ............................................................................................ xix

GENERAL INTRODUCTION
SECTION 1: SMOKING AND NICOTINE
  1.1 Prevalence ....................................................................................................................... 1
  1.2 Health consequences of smoking ................................................................................... 3
  1.3 Heritability of smoking behaviours ............................................................................... 5
  1.4 Neurobiology of tobacco addiction ............................................................................... 6
    1.4.1 Brain reward pathway ............................................................................................. 6
    1.4.2 Nicotinic acetylcholine receptors ............................................................................ 8
    1.4.3 Other targets implicated in tobacco addiction ...................................................... 10
  1.5 Nicotine: the main addictive substance in cigarettes .................................................... 11
    1.5.1 Nicotine reinforcement ......................................................................................... 11
    1.5.2 Nicotine-titration hypothesis ................................................................................. 13
    1.5.3 Other compounds in tobacco smoke with addictive properties ............................ 16
  1.6 Nicotine pharmacokinetics ............................................................................................ 17
    1.6.1 Absorption and distribution .................................................................................. 17
    1.6.2 Nicotine metabolism ............................................................................................. 17
    1.6.3 Nicotine C-oxidation and contribution by CYP2A6 ............................................. 18
      1.6.3.1 In vitro studies ................................................................................................. 20
      1.6.3.2 In vivo studies .................................................................................................. 21
    1.6.4 Other enzymes involved in nicotine metabolism .................................................. 22
    1.6.5 Pharmacological actions of nicotine metabolites ................................................. 24
  1.7 Tobacco dependence ..................................................................................................... 25
1.7.1 Acquisition of dependence .................................................................................... 25
1.7.2 Diagnostic scales for tobacco dependence ............................................................ 26
1.8 Smoking cessation ........................................................................................................ 27
1.8.1 Treatments available ............................................................................................. 27
  1.8.1.1 Pharmacotherapies ............................................................................................ 27
  1.8.1.2 Non-pharmacological interventions ................................................................. 31
1.9 Biomarkers of tobacco smoke ....................................................................................... 32
  1.9.1 Common indicators of exposure ........................................................................... 32

SECTION 2: LIGHT SMOKING
2.1 Prevalence ................................................................................................................ ..... 33
2.2 Health consequences ..................................................................................................... 35
2.3 Demographics .............................................................................................................. . 35
  2.3.1 Ethnic minorities ................................................................................................... 35
  2.3.2 Females ................................................................................................................. 36
  2.3.3 Young adults ......................................................................................................... 36
2.4 Tobacco dependence ..................................................................................................... 37
  2.4.1 Smoking characteristics ........................................................................................ 37
  2.4.2 Interventions for smoking cessation ..................................................................... 38
2.5 African-American light smokers ................................................................................... 39
  2.5.1 Demographics ....................................................................................................... 39
  2.5.2 Smoking initiation ................................................................................................. 39
  2.5.3 Cigarette consumption .......................................................................................... 40
  2.5.4 Use of menthol cigarettes ...................................................................................... 41
  2.5.5 Smoking cessation ................................................................................................ 43
  2.5.6 Health disparities .................................................................................................. 43

SECTION 3: CYP2A6
3.1 CYP2 gene family ......................................................................................................... 45
  3.1.1 Evolutionary origins of CYP2ABFGST gene cluster ............................................. 46
  3.1.2 CYP2A subfamily .................................................................................................. 47
  3.1.3 CYP2A tissue expression ...................................................................................... 48
3.1.4 CYP2A6 substrates ........................................................................................................ 48
3.1.4.1 Tobacco-specific nitrosamines............................................................................... 50

3.2 Genetic variability in CYP2A6 activity ........................................................................ 51
3.2.1 Inter-individual and inter-ethnic variability .............................................................. 51
3.2.2 Currently identified CYP2A6 variants and their functional consequences ............ 52

3.3 Other influences of CYP2A6 activity ........................................................................ 53
3.3.1 Gender/hormonal effects ......................................................................................... 53
3.3.2 Age ......................................................................................................................... 59
3.3.3 CYP2A6 inhibitors and inducers ............................................................................. 60
3.3.3.1 Pharmaceutical agents ....................................................................................... 60
3.3.3.2 Menthol .............................................................................................................. 61
3.3.3.3 Smoking/nicotine ............................................................................................ 62
3.3.3.4 Dietary ............................................................................................................. 63

3.4 Other sources of variability in nicotine clearance rates .............................................. 64

3.5 Phenotype indicators of CYP2A6 activity .................................................................. 65
3.5.1 COT/NIC ............................................................................................................... 65
3.5.2 3HC/COT ............................................................................................................. 66
3.5.3 Formation of 7-hydroxycoumarin ........................................................................ 67

3.6 Association of CYP2A6 variation with smoking behaviours ...................................... 68
3.6.1 Smoking initiation ................................................................................................. 68
3.6.2 Cigarette consumption ......................................................................................... 69
3.6.3 Smoking cessation ............................................................................................... 70
3.6.3.1 Case-control gene association studies ............................................................... 70
3.6.3.2 Clinical trials ..................................................................................................... 70
3.6.3.3 CYP2A6 inhibition to aid smoking cessation ................................................... 73
3.6.4 Lung Cancer ......................................................................................................... 74

STATEMENT OF RESEARCH HYPOTHESES ................................................................... 75

CHAPTER 1: A NOVEL CYP2A6 ALLELE, CYP2A6*23, IMPAIRS ENZYME FUNCTION IN VITRO AND IN VIVO AND DECREASES SMOKING IN A POPULATION OF BLACK-AFRICAN DESCENT

Abstract .......................................................................................................................... 77
Introduction .................................................................................................................... 78
1.1.4 Genetic association studies in African-Americans ............................................. 154
  1.1.4.1 Utility and limitations of self-reports of racial/ethnic ancestry ...................... 154
  1.1.4.2 Importance of performing studies in different racial/ethnic groups .......... 155
1.2 Origins of \textit{CYP2A6} polymorphisms............................................................................ 157
  1.2.1 Types of genetic polymorphisms ........................................................................ 157
  1.2.2 Discovery of novel \textit{CYP2A6} alleles ............................................................... 158
  1.2.3 Evolution of CYPs and their genetic variants ..................................................... 159
1.3 Comparison of \textit{CYP2A6} genetic variability between racial/ethnic groups .......... 161
1.4 Functional consequences of \textit{CYP2A6} genetic variants ..................................... 162
1.5 Other biological sources of variability in \textit{CYP2A6} activity ...................................... 163
  1.5.1 Polymorphisms in \textit{POR} .................................................................................... 163
  1.5.2 Polymorphisms in transcription factors .............................................................. 164
  1.5.3 Epigenetics .......................................................................................................... 165
  1.5.4 mRNA silencing (microRNA) ........................................................................... 166
1.6 Utility of genotype versus phenotype measures of \textit{CYP2A6} activity ..................... 167

SECTION 2: IMPACT OF \textit{CYP2A6} GENETIC VARIATION ON SMOKING BEHAVIOURS
IN AFRICAN-AMERICAN LIGHT SMOKERS
  2.1 Smoking initiation ................................................................................................... 169
    2.1.1 Factors associated with smoking initiation in African-Americans ................. 169
  2.2 Cigarette consumption ........................................................................................... 170
    2.2.1 Light smoking behaviours among African-Americans .................................... 170
    2.2.2 Comparisons between African-American light and moderate/heavy smokers ... 172
  2.3 Tobacco dependence ............................................................................................... 173
    2.3.1 Dependence in African-American light smokers ............................................. 173
    2.3.2 Models of tobacco dependence ...................................................................... 174
      2.3.2.1 Reinforcement theories ............................................................................ 174
      2.3.2.2 Classical conditioning theories .............................................................. 175
      2.3.2.3 Importance of social and cultural contexts ............................................. 176
  2.4 Smoking cessation ................................................................................................... 178
    2.4.1 Impact of \textit{CYP2A6} on smoking cessation ................................................... 179
    2.4.2 Gender differences in smoking cessation outcomes in African-Americans ...... 181
2.4.3. Improving treatment outcomes for smoking cessation .............................................. 182

2.5. Utility of biomarkers of tobacco exposure among African-Americans ...................... 184

CONCLUSIONS ......................................................................................................................... 187

REFERENCES ........................................................................................................................... 189

APPENDICES ............................................................................................................................ 221
LIST OF PUBLICATIONS

Research articles in thesis
Chapter 1:

Chapter 2:

Chapter 3:

Additional publications from doctoral work attached in the Appendices
Research articles

Review articles


Online publications


Conference presentations


Abstracts presented


LIST OF TABLES

<table>
<thead>
<tr>
<th>General introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1 A list of genes that have been implicated in tobacco addiction.</td>
<td>12</td>
</tr>
<tr>
<td>Table 3.1 List of CYP2A6 substrates</td>
<td>49</td>
</tr>
<tr>
<td>Table 3.2 Description of CYP2A6 alleles and their predicted functional impact</td>
<td>54 – 57</td>
</tr>
<tr>
<td>Table 3.3 CYP2A6 allele frequencies in various racial/ethnic populations</td>
<td>58</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1 Allele frequency of CYP2A6*23 by ethnicity</td>
<td>87</td>
</tr>
<tr>
<td>Table 2 CYP2A6*23 genotype groups and their mean adjusted 3HC/COT ratio</td>
<td>87</td>
</tr>
<tr>
<td>Table 3 Kinetic parameters of CYP2A6 wildtype and variant constructs for nicotine</td>
<td>90</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1 Factors that influence the 3HC/COT in CYP2A6*1/*1 individuals</td>
<td>105</td>
</tr>
<tr>
<td>Table 2 CYP2A6 genotypes and associated 3HC/COT ratios</td>
<td>107</td>
</tr>
<tr>
<td>Table 3 CYP2A6 allele frequencies in African-Americans in this population compared to our previous study in individuals of Black-African descent</td>
<td>108</td>
</tr>
<tr>
<td>Table 4 Logistic regression analyses of predictors of CO-verified quit rates at EOT (week 8) and follow-up (week 26)</td>
<td>116</td>
</tr>
<tr>
<td>Supplementary Table 1 Participant demographics</td>
<td>126</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1 Participant characteristics</td>
<td>133</td>
</tr>
<tr>
<td>Table 2 Variables that influences CPD, expired CO or plasma COT levels</td>
<td>136 – 137</td>
</tr>
<tr>
<td>Table 3 Correlations (r) between biomarkers and cigarette consumption by variables</td>
<td>140</td>
</tr>
<tr>
<td>Table 4 Multiple linear regression models of the predictors of CPD, expired CO and plasma COT</td>
<td>141</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>General introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1 Prevalence of current smoking by ethnicity and gender</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2 Schematic of circadian changes in plasma nicotine levels</td>
<td>14</td>
</tr>
<tr>
<td>Figure 1.3 Quantitative schematic of the various nicotine metabolism pathways</td>
<td>19</td>
</tr>
<tr>
<td>Figure 3.1 Human CYP2ABFGST gene cluster on chromosome 19q13.2.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 3.2 Quit rates by 3HC/COT quartiles in clinical trials</td>
<td>72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1 CYP2A6.23 had substantially reduced catalytic activity towards nicotine and coumarin in vitro</td>
<td>89</td>
</tr>
<tr>
<td>Figure 2 CYP2A6.17 and CYP2A6.23, but not CYP2A6.16, have reduced in vitro nicotine C-oxidation</td>
<td>90</td>
</tr>
<tr>
<td>Figure 3 CYP2A6*23 decreased the rates of nicotine metabolism in vivo, as measured by the 3HC/COT ratio</td>
<td>92</td>
</tr>
<tr>
<td>Figure 4 Individuals with the CYP2A6*23 allele trended to having a lower likelihood of being current smokers</td>
<td>92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1A CYP2A6 genotypes and their associated unadjusted 3HC/COT ratios</td>
<td>109</td>
</tr>
<tr>
<td>Figure 1B The unadjusted 3HC/COT ratio was significantly associated with CYP2A6 genotype groupings.</td>
<td>111</td>
</tr>
<tr>
<td>Figure 1C The 3HC/COT ratio adjusted by gender</td>
<td>111</td>
</tr>
<tr>
<td>Figure 2 (A-F) Association of CYP2A6 activity with smoking indices.</td>
<td>113</td>
</tr>
<tr>
<td>Figure 3 (A-F) Association of CYP2A6 activity and smoking abstinence</td>
<td>115</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1 (A-C) Histogram of self-reported CPD and biomarkers</td>
<td>135</td>
</tr>
<tr>
<td>Figure 1 (D-H) Correlations between self-reported CPD and biomarkers</td>
<td>135</td>
</tr>
<tr>
<td>Figure 2 (A-F) Relationship between CYP2A6 activity, CPD, expired CO, and plasma COT.</td>
<td>138</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3HC</td>
<td>( \text{trans-3}')-hydroxycotinine</td>
</tr>
<tr>
<td>3HC/COT</td>
<td>Ratio of ( \text{trans-3}')-hydroxycotinine to cotinine</td>
</tr>
<tr>
<td>AUC</td>
<td>Area-under-the-curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>CNVs</td>
<td>Copy number variants</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COT</td>
<td>Cotinine</td>
</tr>
<tr>
<td>CPD</td>
<td>Cigarettes per day</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Cytochrome P450 2A6</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleoside triphosphates</td>
</tr>
<tr>
<td>E.coli</td>
<td>( \text{Escherichia coli} )</td>
</tr>
<tr>
<td>EOT</td>
<td>End-of-treatment</td>
</tr>
<tr>
<td>ETS</td>
<td>Environmental tobacco smoke</td>
</tr>
<tr>
<td>FMO3</td>
<td>Flavin-containing monoxygenase 3</td>
</tr>
<tr>
<td>FTND</td>
<td>Fagerström Test for Nicotine Dependence</td>
</tr>
<tr>
<td>HE</td>
<td>Health education counseling</td>
</tr>
<tr>
<td>HNF4-( \alpha )</td>
<td>Hepatic nuclear factor 4 - ( \alpha )</td>
</tr>
<tr>
<td>hNPR*</td>
<td>Human NADPH-cytochrome P450 reductase</td>
</tr>
<tr>
<td>MI</td>
<td>Motivational interview counseling</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>nAChRs</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-butanone</td>
</tr>
<tr>
<td>NNN</td>
<td>N-nitrosonornicotine</td>
</tr>
<tr>
<td>NRT</td>
<td>Nicotine replacement therapy</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>POR*</td>
<td>NADPH-cytochrome P450 reductase</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SXR</td>
<td>Steroid/xenobiotic receptor</td>
</tr>
<tr>
<td>UGTs</td>
<td>Uridine diphosphate-glucuronosyltransferases</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>

* Note the gene name for NADPH-cytochrome P450 reductase is officially denoted \( \text{POR} \), although a number of studies have referred to it as \( \text{hNPR} \).
STATEMENT OF RESEARCH PROBLEM

Tobacco smoking remains a leading cause of preventable disease and death worldwide. While tobacco control efforts have greatly reduced the prevalence of smoking, a substantial portion (approximately 20%) of North Americans continue to smoke, and this will remain so unless additional measures are taken to reduce smoking initiation and increase cessation among current smokers (Schroeder et al.). Smoking is a complex behaviour involving multiple biological and environmental risk factors. Identifying the genetic predictors of tobacco addiction has been the focus of much research, and a great deal of progress has been made in recent years due to advancements in our knowledge of the human genome. In particular, improvements in technology have allowed for efficient screening and identification of sources of genetic variability between individuals.

Nicotine is the main psychoactive compound responsible for the highly addictive properties of tobacco smoke (Mcmorrow et al., 1983; Scherer, 1999; Benowitz, 2010). Genetic polymorphisms in CYP2A6, the main enzyme responsible for nicotine metabolism, have been shown to alter the rates of nicotine metabolism and smoking behaviours (Malaiyandi, Sellers et al., 2005). In 2004 and 2005, two large sequencing projects of several racial/ethnic minority populations identified a number of single nucleotide polymorphisms in CYP2A6, although the functional consequences of these variants were not elucidated in these studies (Solus Jf, 2004; Haberl and Anwald B, 2005). Many of these variants were found exclusively in African-Americans, a unique population with slower rates of nicotine and cotinine metabolism that suffer disproportionately from smoking-related illnesses, particularly lung cancer, despite smoking fewer cigarettes compared to Caucasians (Benowitz et al., 1999; Haiman et al., 2006). As such, unidentified sources of CYP2A6 genetic variation in this racial/ethnic group may in part explain their distinctive rates of metabolism and smoking patterns.
Tobacco control efforts and changing social norms have altered smoking patterns in Westernized countries over the past 25 years. The rising costs of cigarettes as a result of increased taxation, as well as comprehensive smoking bans, have greatly reduced smoking prevalence and consumption. The average age-adjusted cigarettes smoked daily reported by adults in the United States have declined substantially from 1980 to 2000, going from 25.1 to 16.7 in males and 21.5 to 13.2 in females (Duval et al., 2008). Non-daily and light smoking patterns are particularly prevalent among racial/ethnic minority groups. For example, among current smokers, 51.4% of African-Americans reported daily use and smoked an average of 9.3 cigarettes per day compared to 68.5% of Caucasian reporting daily use and smoking an average of 14.9 cigarettes per day (Office of Applied Studies et al., 2006).

Classic models of tobacco dependence based on observations in moderate and/or heavy (i.e. pack a day) smokers of Caucasian ancestry suggest that continual maintenance of blood and brain nicotine levels are necessary for avoidance of withdrawal symptoms. As such, smoking behaviours need to occur at regular intervals over the course of the day due to the fast removal of nicotine from the body. However, these theories do not account for the phenomenon of non-daily smoking and light smoking patterns of 10 or fewer cigarettes per day. This has important implications as even smokers with low levels of consumption have difficulty quitting (Okuyemi Ks et al., 2002), and primary pharmacotherapies for smoking cessation have been based on the need for nicotine replacement or mimicking of nicotine’s effect among abstinent smokers. Nearly all of the clinical trials published to date demonstrating the efficacy of these pharmacotherapies have excluded smokers consuming 10 or fewer cigarettes per day, and it is unknown whether these medications would be efficacious among light smoking populations. Investigations into the biological factors underlying smoking behaviours among light smokers,
particularly in understudied racial/ethnic minority groups such as African-Americans, are warranted.
MAIN RESEARCH OBJECTIVES

There are gaps in our current understanding of CYP2A6 genetic variation in relation to nicotine metabolism and smoking behaviours. Much progress has been made in identifying novel CYP2A6 genetic variants in recent years, particularly among populations of Black-African descent; however, functional characterization of these variants remains incomplete. A better understanding of the genetic differences in nicotine metabolism between racial/ethnic groups may potentially explain their unique smoking patterns, as well as improve future genetic studies by reducing the heterogeneity within the wildtype group. In addition, the association of CYP2A6 genetic variation with smoking behaviours has been found predominantly in moderate to heavy smokers of Caucasian ancestry. There is a need to determine whether these findings can be extrapolated to light smoking populations in order to better understand the mechanisms of dependence and factors motivating tobacco addiction in this growing segment of smokers.

Thus, the main objectives of this thesis are to:

- Identify and determine the functional consequences of novel CYP2A6 genetic variants
- Determine whether CYP2A6 genetic variation influences cigarette consumption, smoking cessation and the utility of biomarkers of smoking in light smokers, with a focus on populations of Black-African descent
GENERAL INTRODUCTION

SECTION 1: SMOKING AND NICOTINE

1.1 Prevalence

Tobacco smoking remains a leading cause of preventable illness, disability, and death (World Health Organization, 2009). Despite the numerous negative health effects associated with cigarette smoking, and tobacco control efforts aimed to reduce its accessibility and acceptability, a substantial number of individuals continue to smoke. Adult cigarette smoking has been declining in North America, with prevalence reduced by nearly half from the mid-1960s to mid-1990s (World Health Organization, 2002). However, the rate of decline in smoking prevalence has slowed in recent years and appears to have reached a plateau, with approximately 21% of adults in North America currently reported as smokers (Health Canada, 2008; Centers for Disease Control and Prevention, 2009; Benowitz, 2010; National Cancer Institute et al., 2010).

There are considerable differences in smoking prevalence between racial/ethnic groups. Rates of smoking are highest among American Indians/Alaskan Natives (32.4%), followed by Caucasians (22.0%), African-Americans (21.3%), Hispanics (15.8%) and Asian-Americans (9.9%) (Centers for Disease Control and Prevention, 2009) (Figure 1.1). Smoking prevalence also tends to be higher among males compared to females (Figure 1.1) (Hatsukami et al., 2008; Centers for Disease Control and Prevention, 2009). According to a 2008 survey, 23.1% of men were smokers in the United States compared to 18.3% of women, with even greater differences observed among certain ethnic groups (Centers for Disease Control and Prevention, 2009). Smoking is also more prevalent among those of poorer socioeconomic status (Bobak et al., 2000) with highest rates observed in those without a high school diploma (27.5%), or with only high school equivalency (41.3%), and lowest
rates observed among those with a graduate degree (5.7%) (Centers for Disease Control and Prevention, 2009).

Figure 1.1: Prevalence of current smoking by ethnicity and gender. Current smoking is defined as having smoked at least 100 lifetime cigarettes and reporting smoking every day or some days at the time of interview. By definition Caucasians refers to individuals self-reported as non-Hispanic Whites and African-Am refers to individuals self-reported as non-Hispanic Blacks. Am-Ind refers to American-Indians, AK Natives refers to Alaskan Natives, and Asian-Am refers to Asian-Americans. Data adapted from the 2008 National Health Interview Survey (United States) (Centers for Disease Control and Prevention, 2009).
The global prevalence of smoking varies from 5% in some countries to more than 55% in others (Hatsukami, Stead et al., 2008). Of particular concern is the rising rate of tobacco consumption in developing countries such as China, India, and parts of Latin America and Africa (World Health Organization, 2002; World Health Organization, 2009). Of the 1.3 billion smokers worldwide, more than 80% live in low- to middle-income nations where smoking and its associated illnesses will add to the already existing health burdens and poor quality of life in these countries (Gajalakshmi Ck et al., 2000).

1.2 Health consequences of smoking

Tobacco smoking is responsible for an estimated 5 million deaths each year worldwide, and one out of two lifetime smokers will die from smoking-related illnesses (Doll R et al., 2004). There are over 4,000 compounds in cigarette smoke, many of which have harmful effects on health, including carbon monoxide, ammonia, hydrogen cyanide, and acrolein (World Health Organization, 2002). Numerous carcinogenic compounds are also present in tobacco smoke; of the 69 carcinogens identified, 11 are known human carcinogens and an additional seven are likely to be carcinogenic in humans (National Cancer Institute). Evidence for a causal relationship between smoking and cancer has been accumulating since the late 1930s. The 2004 Surgeon General Report concluded that smoking causes 30% of all cancer deaths, accounting for approximately 80 to 90% of deaths attributed to lung cancer and approximately 60 to 90% of deaths from upper aerodigestive cancers (Centers for Disease Control and Prevention, 1998; U.S. Department of Health and Human Services, 2004; National Cancer Institute, National Institutes of Health et al., 2010). Cigarette smoking is also the cause of numerous respiratory and cardiovascular ailments, being responsible for 75 to 90% of chronic obstructive pulmonary disease (COPD) related deaths, 20 to 25% of coronary heart disease deaths, and 18% of deaths from stroke (Centers for Disease Control
Exposure to environmental tobacco smoke (ETS) also causes lung cancer and respiratory problems in healthy non-smokers (Centers for Disease Control and Prevention, 1998). In addition, maternal smoking during pregnancy, or ETS exposure *in utero*, has numerous adverse effects on the fetus including increased risk for premature birth, low birth rates, Sudden Infant Death Syndrome, birth defects and possible long-term physical and mental effects (World Health Organization, 2002). In summary, tobacco smoking has immense economic costs to society with an estimated $168 billion spent annually on health care expenditures and loss in productivity in the United States alone (Office of National Drug Control Policy, 2004).

The risk for many smoking-related illnesses, including cancers, cardiovascular diseases and COPD has been shown to depend on dose, at least to some extent (Garfinkel et al., 1988; Us Department of Health Education and Welfare, 1990; Jiménez-Ruiz C et al., 1998; Mucha et al., 2006). Those who smoke 10 or fewer cigarettes per day (CPD) have 5.5-times the risk of developing lung cancer compared to nonsmokers, with the comparative risk increasing to approximately 14-times for those smoking 20 to 30 CPD and further to 22-times for those smoking in excess of 31 CPD (Garfinkel and Stellman, 1988; Jiménez-Ruiz C, Kunze M et al., 1998). The risk of myocardial infarction and death is greater among those with more cumulative cigarettes smoked (Us Department of Health Education and Welfare, 1984; Jiménez-Ruiz C, Kunze M et al., 1998). Similarly, reductions in ventilatory function are seen in those smoking 1 to 10 CPD, although the effect is even greater among those smoking more than 20 CPD (Us Department of Health and Human Services, 1984; Jiménez-Ruiz C, Kunze M et al., 1998).
1.3 Heritability of smoking behaviours

Tobacco smoking is a complex behaviour with various stages including initiation, experimentation, progression to regular use, cessation and relapse. While environmental factors such as familial, peer, and social/cultural influences undoubtedly affect one’s smoking behaviour, numerous studies have also demonstrated a substantial genetic contribution. Large variations in estimation of the heritability of “ever” smoking, a broad phenotype that can encompass those who have only tried cigarettes once to regular heavy smokers, have been reported in the ranges of 11 to 78% (Ho et al., 2007; Rose et al., 2009). Once smoking is initiated, the heritability for persistence to regular smoking has been estimated at 28 to 84%, for number of cigarettes smoked at 45 to 86%, for diagnosed nicotine dependence at 31 to 75%, for severity of nicotine withdrawal symptoms at 26 to 48% and for smoking cessation at 50 to 58% (Ho and Tyndale, 2007; Rose, Broms et al., 2009; Ho et al., 2010). It is notable that the heritability for the different behavioural stages of smoking (e.g. initiation vs. persistence) only partially overlap, suggesting some genes are specific to one smoking phenotype while others contribute to multiple aspects of smoking (Rose, Broms et al., 2009). Variability in these estimates likely results, at least in part, from differences in age and gender of participants. There is some evidence that the genetic influence on likelihood of smoking increases with age as one progresses from early adolescence to young adulthood (Rose, Broms et al., 2009). For example, a longitudinal study of adolescent twins found the heritability of tobacco use increased from 15% at initial assessments (ages 13 to 18) to 35% at final follow-up (ages 20 to 25) (White et al., 2003). Some studies have suggested a greater genetic influence on smoking among females although these results have not been consistently reported (Rose, Broms et al., 2009). One meta-analysis found modest differences in the heritability of smoking initiation and persistence between male versus female twins (Li et al., 2003), although more recent studies did not report such findings.
(Broms et al., 2006; Rose, Broms et al., 2009). Cohort effects and inconsistent phenotype definitions may also contribute to the wide variability observed in heritability estimates.

1.4 Neurobiology of tobacco addiction

A common feature of all drugs with addiction liability is their ability to activate the mesolimbic brain reward pathway which evolved to respond to “natural” rewards critical for species survival such as food and sex (Ross et al., 2009). Repetitive drug use alters the normal function of this circuitry, resulting in neuroadaptive changes and a new homeostatic balance that underlies persistent drug-seeking behaviour and compulsive drug use despite harmful consequences.

Nicotine is the major alkaloid in *Nicotiana* plants cultivated for tobacco products, where it likely acts as natural insecticide (Tomizawa et al., 2003). It is the main psychoactive substance in tobacco smoke that sustains addiction, and is readily absorbed into the arterial circulation via inhalation through the lungs. Through this route, nicotine rapidly enters the brain in an estimated 10 – 20 seconds; this likely contributes to the highly addictive potential of tobacco smoke and makes it the most reinforcing and dependence-producing form of nicotine administration (Hukkanen, Jacob et al., 2005). Nicotine acts upon nicotinic acetylcholine receptors (nAChRs), which are located throughout the body and centrally in the brain, to mediate a number of physiological and psychological effects.

1.4.1 Brain reward pathway

Nicotine activates dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain projecting to the nucleus accumbens (NAc) (Nestler, 2005; Changeux). This can occur directly via interactions with nAChRs on dopaminergic neurons, as well as indirectly via
inhibitory and excitatory inputs coming into the VTA from other sources (such as GABAergic and glutamatergic interneurons) (Mansvelder et al., 2002). Dopamine release via this pathway signals a pleasurable experience and is critical in mediating drug-induced reward; chemical or anatomical lesions of dopaminergic neurons in the VTA abolish nicotine self-administration in rats (Corrigall et al., 1992). Dopaminergic neurons in the VTA also project to the prefrontal cortex, amygdala, and hippocampus, regions that are important for associative learning processes such as addiction (Nestler, 2005; Changeux).

In addition to dopamine, other neurotransmitters such as γ-aminobutyric acid, glutamate, opioid peptides, serotonin, acetylcholine, norepinephrine, and endocannabinoids are also released via nicotine’s binding to presynaptic nAChRs (Wonnacott, 1997; Benowitz, 2008). These neurotransmitters contribute to the various other effects mediated by nicotine including enhancement of cognitive functions, learning and memory processes, mood modulation, anxiolytic effects, improvement of fine motor abilities, analgesia, and appetite suppression (Benowitz, 2008; Heishman et al.).

Prolonged repeated exposure to nicotine results in long-lasting alterations of neuronal function and synaptic plasticity of the brain reward circuitry (Mao et al., 2010). Classical theories of dependence suggest these neuroadaptations form the basis of tolerance and sensitization that leads to withdrawal symptoms and cravings upon smoking abstinence (Watkins et al., 2000). Chronic tolerance, where increasing doses are needed to achieve the same drug effect, has been demonstrated for the subjective, cardiovascular, and performance enhancing effects of nicotine in humans (Perkins et al., 1994). Repeated drug use can also result in sensitization, whereby enhanced response to subsequent drug exposure is observed. Increased locomotor response (Clarke et al., 1983; Benwell et al., 1992; Shim et al., 2001)
and increased dopamine release in the NAc in response to nicotine administration (Benwell and Balfour Dj, 1992; Cadoni et al., 2000; Shim, Javaid et al., 2001; Rahman et al., 2003) have been reported in animals following chronic nicotine treatment, although sensitization in humans has not been well established (Difranza et al., 2007). Upon abstinence, cravings and withdrawal symptoms emerge that often oppose the effects of nicotine such as difficulty concentrating, irritability, depressed mood, restlessness, anxiety, increased appetite and insomnia (Benowitz, 2008).

1.4.2 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors are transmembrane, ligand-gated, pentameric cation channels composed of some combination of nine $\alpha$ ($\alpha_{2} - \alpha_{10}$) and three $\beta$ ($\beta_{2} - \beta_{4}$) subunits. The number of binding sites for brain nAChRs vary depending on subunit composition with heteromeric receptors having two sites between alpha and beta subunits while homomeric receptors can have up to five sites per pentamer (Penton et al., 2009). Most brain nAChRs are located pre-synaptically where they modulate the release of neurotransmitters, although some are also located post-synaptically (Gotti et al., 2009).

Upon binding of nicotine or the endogenous ligand acetylcholine, a conformational change occurs and the channel opens to allow the permeation of sodium, potassium, and in some cases calcium ions (Picciotto et al., 2008). Activation of the receptor is quickly followed by desensitization wherein a second conformational change closes the channel and renders it non-functional (Picciotto, Addy et al., 2008). Receptors in the desensitized state have higher affinity towards ligands even though the channel no longer opens in response to agonist binding (Picciotto, Addy et al., 2008). The rates of desensitization and recovery may differ
by pre- and post-synaptic nAChRs, or by different neuronal populations (Picciotto, Addy et al., 2008).

Studies have shown that different combinations of α and β subunits result in neuronal receptors with different pharmacological and functional properties (Penton and Lester, 2009). These various nAChRs differ in their affinity for ligands, electrophysiological properties, calcium permeability and upregulation by nicotine (Govind et al., 2009). High-affinity nicotine binding sites are located in discrete regions throughout the brain, with the highest densities found in the VTA and NAc (Azam et al., 2002). Animal studies have determined that receptors with α4 and β2 subunits comprise approximately 90% of the high-affinity nicotine binding sites in the brain (Flores et al., 1992), and mouse knockout models for these subunits suggest they are critical for nicotine self-administration and nicotine-induced dopamine release (Picciotto et al., 1998; Tapper et al., 2004). There are also other high-affinity nicotine binding sites that have not been as well characterized which include α3, α5, α6, α7, β3 and β4 subunits in various combinations (Wang et al., 1996; Gerzanich et al., 1998; Kuryatov et al., 2000; Grinevich et al., 2005).

In moderate to heavy daily smokers, cigarettes are smoked regularly during the day to maintain nicotine at elevated levels in the plasma (10 to 50 ng/ml) since nicotine has a relatively short half-life at approximately one to two hours (Benowitz et al., 1994; Hukkanen, Jacob et al., 2005). It has been shown that human β2-containing nAChRs become nearly fully occupied after smoking one cigarette (Brody et al., 2006). The activation and desensitization of nAChRs from smoking over the course of the day, and resensitization of these receptors during overnight abstinence, likely alter states of neuronal activity and play an important role in the behavioural effects of nicotine.
Chronic exposure to nicotine results in a number of neuroadaptive changes, many of which are mediated through nAChRs (Gotti, Clementi et al., 2009). An increased number of high-affinity nicotine binding sites are found following long-term exposure to nicotine in post-mortem human brains of smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999), human cell lines (K-177 cells, a human embryonic kidney cell line stably expressing α4 and β2 nAChR subunits) (Buisson et al., 2001) and rodent animal models (Marks et al., 1983; Schwartz et al., 1983; Yates et al., 1995; Nguyen et al., 2003; Perry et al., 2007). Several mechanisms have been proposed for the nicotine-induced upregulation of nAChRs including decreased receptor turnover, increased assembly or maturation, increased trafficking of receptors to cell surface, or slower subunit degradation (Govind, Vezina et al., 2009). Chronic nicotine treatment may also potentially induce subunit stoichiometry changes or receptor conformational changes (Govind, Vezina et al., 2009). It is notable that nicotine acts on a variety of nAChRs of different subtype combinations, and the effect of chronic nicotine exposure can differ depending on receptor subunit, cell type and brain region (Gotti, Clementi et al., 2009).

1.4.3 Other targets implicated in tobacco addiction

In general, genes implicated in tobacco addiction can be categorized into those that influence the liability to experiment with the drug in the first place, and those that are involved in the biological processes underlying addiction once the individual has been exposed to the drug. As such, genes that are related to personality traits (such as impulsivity, risk-taking or response to stress) may predispose one to experimentation. Alternatively, genes encoding proteins in the brain reward system (such as receptors, transporters and metabolic enzymes of the neurotransmitter systems) and enzymes involved in nicotine metabolism may contribute
to the initial, immediate subjective and physiological reaction to tobacco smoke, thus
determining whether use will continue and escalate into development of dependence. A list
of genes implicated in tobacco dependence and smoking behaviours can be found in Table
1.1. These targets were hypothesized based on the current understanding of the neurobiology
underlying tobacco addiction. Genome-wide association studies without _a priori_ hypotheses
have also identified a number of new genetic loci related to smoking phenotypes. For
example, the cell adhesion molecule neurexin 1 (NRXN1) has been associated with nicotine
dependence (Bierut et al., 2007; Nussbaum et al., 2008). These novel targets provide
additional insights into the neurobiology underlying addiction, although further assessments
of their functional impact are still necessary.

1.5 Nicotine: the main addictive substance in cigarettes

Nicotine has long been implicated as the main compound responsible for the highly addictive
properties of tobacco smoke; this has been supported by several lines of evidence.

1.5.1 Nicotine reinforcement

Historically, only tobacco products containing nicotine have been used habitually by
individuals over long periods of time. Cigarettes with nicotine removed, or that have ultra-
low nicotine yield, have not been used extensively by the public, although other nicotine-
containing products such as chewing tobacco and snuff are widely used (Benowitz, 2008).
Nicotine has reinforcing properties as demonstrated by self-administration paradigms and
conditioned place preference models in a number of rodent and non-human primate species
(Le Foll et al., 2009). However, nicotine self-administration is highly dependent upon
experimental conditions (such as state of food-deprivation, exposure to drug prior to testing,
rate of drug administration, dose, handling history, or presence of environmental
Table 1.1: A list of genes that have been implicated in tobacco addiction. References in (Ho, Goldman D et al., 2010).

<table>
<thead>
<tr>
<th>Protein or enzyme (Gene name)</th>
<th>Smoking phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine D2 receptor (DRD2/ANKK1)</td>
<td>Smoking initiation, persistence, cigarette consumption, cessation</td>
</tr>
<tr>
<td>Dopamine D4 receptor (DRD4)</td>
<td>Smoking risk, time to first cigarette, craving and response to smoking cues, nicotine dependence</td>
</tr>
<tr>
<td>Dopamine transporter 1 (SLC6A3, DAT1)</td>
<td>Smoking risk</td>
</tr>
<tr>
<td>Monoamine oxidase-A (MAO-A)</td>
<td>Cigarette consumption, smoking risk, nicotine dependence</td>
</tr>
<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>Smoking risk</td>
</tr>
<tr>
<td>Dopamine β-hydroxylase (DBH)</td>
<td>Smoking risk, nicotine dependence, cessation</td>
</tr>
<tr>
<td>Catechol-O-methyltransferase (COMT)</td>
<td>Smoking risk, nicotine dependence, cessation</td>
</tr>
<tr>
<td>Serotonin transporter (SLC6A4, 5HTT, SERT)</td>
<td>Smoking risk, cigarette consumption, dependence</td>
</tr>
<tr>
<td>Tryptophan hydroxylase 1, 2 (TPH1, 2)</td>
<td>Age of smoking initiation, risk of smoking</td>
</tr>
<tr>
<td>μ1 - opioid receptor (OPRM1)</td>
<td>Smoking cessation</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptor – α4 subunit (CHRNA4)</td>
<td>Nicotine dependence</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptor – α5, α3, β4 subunits (CHRNA5, A3, B4 gene cluster on chromosome 15)</td>
<td>Nicotine dependence, cigarette consumption, lung cancer and COPD risk</td>
</tr>
<tr>
<td>Cytochrome P450 2A6 (CYP2A6)</td>
<td>Smoking risk, cigarette consumption, cessation</td>
</tr>
<tr>
<td>Cytochrome P450 2B6 (CYP2B6)</td>
<td>Smoking cessation</td>
</tr>
</tbody>
</table>
cues), with results varying by species and across laboratories (Le Foll and Goldberg, 2009). In contrast, intravenous self-administration of drugs and conditioned place preference have been readily demonstrated for other drugs including psychostimulants and opioids over a wide range of conditions (Le Foll and Goldberg, 2009). This suggests that nicotine alone may be a relatively weak reinforcer, and there is evidence showing it may also enhance the reinforcement properties of non-nicotine stimuli (Chaudhri et al., 2006). Stimuli that are initially neutral can develop reinforcing values as they are repeatedly paired with nicotine through Pavlovian associative conditioning processes (Chaudhri, Caggiula et al., 2006).

A number of laboratory experiments have shown that humans will self-administer intravenous nicotine (Henningfield Je, 1983; Henningfield Je, 1983; Rose et al., 2003; Harvey et al., 2004; Sofuoglu et al., 2007). However, the results of these studies need to be regarded with caution as they contain small sample sizes, and many participants were multiple-drug users who also readily administered saline in some cases (Dar et al., 2004).

1.5.2 Nicotine-titration hypothesis

Nicotine has a short elimination half-life of approximately one to two hours (Hukkanen, Jacob et al., 2005), and smokers are thought to smoke regularly over the course of the day to maintain plasma nicotine levels in the body (Figure 1.2) (Mcmorrow and Foxx Rm, 1983; Russell, 1987). This observed pattern of regular daily smoking forms the basis of a model for tobacco dependence that emphasizes avoidance of withdrawal as the driving force behind continued use (Shadel et al., 2000; Shiffman, 2009).
Figure 1.2: Schematic of circadian changes in plasma nicotine levels. This figure illustrates the changes in nicotine plasma levels in individuals smoking at regular intervals over the course of a day (approximately 20 to 30 cigarettes per day). The upper dotted line represents the threshold level of nicotine needed to produce arousal and pleasure while the lower dotted line represents the threshold level of nicotine needed to prevent withdrawal symptoms. The first cigarettes of the day have the most pleasurable effects, and as tolerance to these effects develops, subsequent cigarettes are smoked as a means to maintain nicotine levels and prevent withdrawal symptoms. Overnight abstinence allows for resensitization of nAChRs and response to subsequent nicotine intake. Modified from (Benowitz, 1992).

There are several lines of evidence supporting the nicotine-titration or nicotine-uptake-regulation hypothesis. Alterations of nicotine renal excretion rates change smoking behaviours. Urinary acidification using ammonium chloride increased renal clearance by 208%, reduced average blood nicotine concentration by 15%, and increased daily intake of nicotine from smoking by 18% (Schachter et al., 1977; Benowitz et al., 1985). In contrast,
urinary alkalization using sodium bicarbonate reduced smoking (Cherek et al., 1982). Alterations in smoking behaviours were also observed when smokers switched between cigarettes that differed in their nicotine yields (Scherer, 1999). Smoking behaviour was generally increased when nicotine yields were lowered, as indicated by increased puff volume, duration, number of puffs, and exhaled carbon monoxide (CO) levels per cigarette (Kozlowski et al.; Gust et al., 1982; Benowitz et al., 1983; Sepkovic et al., 1984; Scherer, 1999; Strasser et al., 2007). Conversely, smoking was reduced when using cigarettes enriched with nicotine (Dunn Pj et al., 1978; Fagerström, 1982; Scherer, 1999). Methoxalen is a known inhibitor of CYP2A6, the main enzyme involved in nicotine metabolism (Maenpaa et al., 1993). Methoxalen increased the bioavailability of a co-administered oral nicotine dose and together they decreased the desire to smoke, the amount smoked, and the total number of puffs taken more than oral nicotine given alone (Sellers et al., 2000).

One further line of evidence is the reduction of smoking behaviours by nicotine supplementation. Administration of nicotine prior to ad libitum smoking reduces the amount of nicotine intake, cigarettes consumed, exhaled CO levels, and latency to smoking the next cigarette (Benowitz et al., 1990; Perkins et al., 1992; Benowitz et al., 1998; Scherer, 1999). Furthermore, nicotine replacement therapy as gum, patch, inhaler or nasal spray formulation is a first-line smoking cessation aid (Stead et al., 2008). A number of studies have also tested the effects of mecamylamine, a nAChR antagonist, on smoking behaviours. Chronic treatment with mecamylamine results in an immediate, transient increase in smoking behaviours during the first few days, likely as compensatory response in an attempt to obtain the desired effects of smoking (Rose et al., 1997; Scherer, 1999; Rose, Behm et al., 2003). This is followed by a gradual reduction in smoking as the reinforcing effects of smoking are blocked and behavioural extinction occurs (Rose and Corrigall, 1997; Scherer, 1999; Rose,
Similarly, the nAChR partial agonist varenicline can be used to aid smoking cessation; it is thought to work by mimicking the effects of nicotine through its agonist effects, thereby alleviating withdrawal symptoms (Tonstad et al.; Mcneil et al., 2010). In addition, varenicline can attenuate the rewarding effects of nicotine derived from subsequent lapses of cigarette smoking by competitively inhibiting nicotine binding to nAChRs (Tonstad and Rollema; Mcneil, Piccenna L et al., 2010).

1.5.3 Other compounds in tobacco smoke with addictive properties

While nicotine appears to be the main addictive substance in tobacco smoke, there is evidence that other compounds may also be of importance. Chronic exposure to tobacco smoke reduces the activity of monoamine oxidase A and B by 30 to 40% compared to non-smokers or former smokers (Fowler et al., 1996; Fowler et al., 1996; Fowler et al., 1998; Fowler et al., 1999). These enzymes catabolize the oxidation of dopamine, serotonin and norepinephrine; increased levels of these neurotransmitters in the synapses likely contribute to the addiction liability of tobacco smoke by altering reward, mood, and anxiety processes. Acetaldehyde, a component of tobacco smoke, can form condensation products with biogenic amines to produce tetrahydroisoquinolines (e.g. salsolinol) and β-carbolines (e.g. harman) which are known inhibitors of monoamine oxidase (Fowler, Volkow Nd et al., 1996; Fowler, Volkow et al., 1996; Fowler, Volkow Nd et al., 1998; Fowler, Wang et al., 1999). Indeed, acetaldehyde has reinforcing properties in rodent behavioural models, increasing activity of dopaminergic neurons in the VTA, and potentiating the reinforcing properties of nicotine (Talhout et al., 2007; Le Foll and Goldberg, 2009). However, it remains to be validated whether these effects of acetaldehyde occur at concentrations found in the brains of human smokers (Talhout, Opperhuizen et al., 2007; Le Foll and Goldberg, 2009).
1.6 Nicotine pharmacokinetics

1.6.1 Absorption and distribution

Most cigarettes contain 10 to 14 mg of nicotine and, on average, 1 to 1.5 mg is absorbed systemically during smoking (Hukkanen, Jacob et al., 2005). During smoking, nicotine is rapidly absorbed from the small airways and alveoli due to their large surface area. Nicotine is a weak base with $pK_a$ of approximately 8.0, and absorption across membranes in the lungs is highly dependent on pH (Hukkanen, Jacob et al., 2005). Blood and brain concentrations of nicotine rise rapidly during smoking; this rapid rate of absorption allows smokers to titrate the nicotine dose as needed and underlies the highly reinforcing and dependence-producing nature of cigarette smoke.

Nicotine plasma levels sampled in smokers during the afternoon typically range from 10 to 50 ng/ml (Hukkanen, Jacob et al., 2005). Smoking a single cigarette increases venous nicotine blood concentrations by 5 to 30 ng/ml, with blood levels peaking at the end of smoking and declining rapidly over the next 20 minutes as a result of tissue distribution (Hukkanen, Jacob et al., 2005; Benowitz et al., 2009). Nicotine is 69% ionized and 31% unionized in the bloodstream, with less than 5% protein-bound (Benowitz et al., 1982). Nicotine is extensively distributed to body tissues with the highest affinity for liver, kidney, spleen, lung and brain and lowest affinity for adipose tissue (Hukkanen, Jacob et al., 2005). The plasma half-life of nicotine is approximately one to two hours (Benowitz and Jacob P 3rd, 1994), and the terminal half-life is approximately 11 hours, as measured from urinary metabolites, due to the slow release of nicotine from body tissue stores (Jacob 3rd et al., 1999).

1.6.2 Nicotine metabolism
Nicotine is eliminated via extensive hepatic metabolism; it is a high extraction drug and undergoes a significant first-pass effect with oral bioavailability estimated at approximately 20 to 45% (Benowitz et al., 1991). The various pathways by which nicotine is removed in humans are presented in Figure 1.3. The majority of nicotine (70 to 80%) undergoes C-oxidation to form cotinine along with five other primary metabolites (Benowitz and Jacob P 3rd, 1994; Hukkanen, Jacob et al., 2005). Cotinine is also metabolized to a number of compounds, with the majority undergoing oxidation to trans-3-hydroxycotinine (3HC) (Bowman et al., 1962; Neurath Gb et al., 1988). Quantitatively, 3HC and its glucuronide conjugate are the primary nicotine metabolites found in the urine of smokers, representing 40 to 60% of the nicotine dose (Byrd et al., 1992; Benowitz et al., 1994). Free cotinine and its glucuronide also represent a substantial portion of the urinary metabolites found at approximately 22 to 32%, while free and glucuronidated nicotine represent 11 to 15% of the recovered nicotine dose (Byrd, Chang et al., 1992; Benowitz, Jacob et al., 1994).

1.6.3 Nicotine C-oxidation and contribution by CYP2A6

Nicotine is converted into cotinine by two enzymatic steps. CYP2A6 metabolizes nicotine into an unstable nicotine-Δ1(5')-iminium ion intermediate that is rapidly metabolized to cotinine by aldehyde oxidase in a reaction that is not rate-limiting (Brandange et al., 1979). Cotinine is further metabolized to 3HC; this reaction is also primarily mediated through CYP2A6 (Nakajima et al., 1996). Several lines of evidence have demonstrated that CYP2A6 is the main enzyme involved in the metabolism of nicotine and cotinine.
Figure 1.3: Quantitative schematic of the various nicotine metabolism pathways. The major pathway of nicotine elimination in humans is highlighted in bold arrows. The excretion of metabolites as a percent of total urinary nicotine is listed in brackets. Adapted with modifications from (Haberl et al., 2005; Hukkanen et al., 2005; Malaiyandi et al., 2005; Nakajima et al., 2006; Mwenifumbo et al., 2008; Ho, Mwenifumbo et al., 2009).
1.6.3.1 *In vitro* studies

A predominant role of CYP2A6 in nicotine metabolism has been substantiated by studies using human liver microsomes. Levels of CYP2A6 immunoreactive protein were highly correlated with rates of nicotine C-oxidation activity ($r = 0.60$ to $0.94$), with up to 88% of the variability in activity accounted for by CYP2A6 protein levels (Nakajima et al., 1996; Messina et al., 1997; Yamazaki et al., 1999). Nicotine C-oxidation was inhibited more than 75% using a monoclonal CYP2A6 antibody (Nakajima, Yamamoto et al., 1996; Messina, Tyndale et al., 1997; Yamazaki, Inoue et al., 1999) while antibodies towards CYP2E1, CYP2B1, CYP2D6 or CYP3A2 had no such effects (Messina, Tyndale et al., 1997). Nicotine C-oxidation was also inhibited by more than 85% using coumarin (Nakajima, Yamamoto et al., 1996; Messina, Tyndale et al., 1997; Yamazaki, Inoue et al., 1999), a commonly used probe drug that is converted to 7-hydroxycoumarin selectively by CYP2A6 (Pelkonen et al., 2000). In contrast, substrates specific towards CYP1A, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 did not inhibit nicotine C-oxidation activity (Nakajima, Yamamoto et al., 1996; Messina, Tyndale et al., 1997; Yamazaki, Inoue et al., 1999).

Cotinine formation correlated well with formation of 7-hydroxycoumarin ($r = 0.83$) and *trans*-3’-hydroxycotinine ($r = 0.74$) (Nakajima, Yamamoto et al., 1996). Of the hepatic CYPs, cDNA-expressed CYP2A6 had the greatest capacity for nicotine C-oxidation, with CYP2B6 and CYP2D6 showing minor metabolic activity (Yamazaki, Inoue et al., 1999).

Similar experiments using human liver microsomes suggest the metabolism of cotinine into 3HC is exclusively mediated by CYP2A6 (Nakajima, Yamamoto T et al., 1996). Cotinine 3’-hydroxylase activity was highly correlated with levels of CYP2A6 immunoreactive protein ($r = 0.76$), but was not significantly correlated with liver content of CYP2B, CYP2C8, CYP2C9, CYP2E1, CYP3A4 and CYP4A (Nakajima, Yamamoto T et al., 1996).
Cotinine 3’-hydroxylation was nearly abolished through competition by coumarin while inhibitors selective for other CYPs showed no effect (Nakajima, Yamamoto T et al., 1996). CYP2A6 monoclonal antibodies reduced cotinine 3’-hydroxylation by 64% to 69%. Cotinine 3’-hydroxylation was significantly correlated with coumarin-7-hydroxylation (r = 0.89), and cDNA-expressed CYP2A6 mediated cotinine 3’-hydroxylation while CYP1A1, CYP1A2, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 had no detectable activity (Nakajima, Yamamoto T et al., 1996).

1.6.3.2 In vivo studies

Further support for a role of CYP2A6 in nicotine metabolism has been found from in vivo studies examining the metabolic profile in individuals who are homozygous for the CYP2A6*4 gene deletion allele and thus completely lack functioning enzyme. Cotinine was not detected in the plasma of these individuals two hours following oral nicotine intake (Kwon et al., 2001; Nakajima et al., 2001). Similar results have been observed in other studies showing greatly reduced cotinine levels, representing approximately 10% of total metabolites, in the urine of smokers completely lacking CYP2A6 enzyme (Kitagawa et al., 1999; Yang et al., 2001). Another study examined the urinary metabolite profile in nonsmokers following administration of nicotine gum. Among those with fully functioning CYP2A6, unchanged nicotine and nicotine N-glucuronide represented 25 to 30% of the excreted urinary metabolites, with cotinine and cotinine-derived metabolites consisting of 58 to 67% of the excreted urinary metabolites (Yamanaka et al., 2004). In contrast, approximately 68 to 70% of nicotine was excreted unchanged or as nicotine N-glucuronide in CYP2A6*4/*4 individuals (n = 2), with trace amounts of cotinine or cotinine N-glucuronide (2 to 3%), and no 3HC or its O-glucuronide detected (Yamanaka, Nakajima et al., 2004). Nicotine N-oxide also represented 25 to 30% of the excreted urinary metabolites in
individuals lacking CYP2A6, suggesting some re-routing of nicotine elimination via this pathway (Yamanaka, Nakajima et al., 2004). While other minor pathways may contribute to the metabolism of nicotine in the absence of CYP2A6, the rate of removal of nicotine from the body is still greatly reduced. In CYP2A6*4/*4 individuals, the mean area-under-the-curve (AUC) for nicotine was increased by 3.6-fold, the AUC of cotinine was reduced by 15-fold, and the half-life of nicotine was extended from 2 to 11 hours compared to those with fully active CYP2A6 (Nakajima et al., 2000; Nakajima et al., 2005). Individuals categorized as CYP2A6 intermediate or slow metabolizers, according to their CYP2A6 genotypes, also had significantly higher AUC values for nicotine (Xu et al., 2002; Mwenifumbo, Al Koudsi N et al., 2008) and lower AUC values for cotinine (Xu, Rao et al., 2002).

1.6.4 Other enzymes involved in nicotine metabolism

Nicotine, cotinine and 3HC undergo substantial glucuronidation whereby glucuronic acid is enzymatically attached to the substrate, making the conjugated compounds more water-soluble and readily excreted. This reaction is mediated by uridine diphosphate-glucuronosyltransferases (UGTs). Rates of nicotine and cotinine N-glucuronidation were highly correlated in vitro (r = 0.95) suggesting the same isoforms were responsible for both reactions (Nakajima et al., 2002). Earlier studies have suggested the involvement of UGT1A4, UGT1A9 and UGT2B7 (Kuehl et al., 2003); however, more recent studies have implicated UGT2B10 as the major isoform involved in the N-glucuronidation of nicotine and cotinine (Chen et al., 2007; Kaivosaari et al., 2007). UGT2B10 may also contribute to the detoxification of several tobacco-specific nitrosamines via glucuronidation (Chen et al., 2008; Chen et al., 2008). While 3HC can undergo N- and O-glucuronidation in human liver microsomes (Kuehl et al., 2003; Yamanaka et al., 2005), only 3HC O-glucuronide has been identified in the urine of smokers (Byrd et al., 1994). The rate of 3HC O-glucuronidation
was not correlated with the rates of nicotine or cotinine N-glucuronidation (Kuehl and Murphy, 2003); thus, the O-glucuronidation of 3HC is likely catalyzed by a different enzyme, and evidence suggests UGT2B7 is involved in this reaction (Yamanaka, Nakajima et al., 2005).

There is large inter-individual and inter-ethnic variability in the rates of nicotine and cotinine N-glucuronidation. In studies using human liver microsomes, the rates of nicotine N-glucuronidation varied by approximately 22-fold and the rates of cotinine N-glucuronidation varied by approximately 89-fold (Nakajima and Yokoi T, 2005). A trimodal distribution for nicotine N-glucuronidation and bimodal distribution for cotinine N-glucuronidation was found in African-Americans while an unimodal distribution for both of these reactions was found among Caucasians (Benowitz, Perez-Stable et al., 1999). This suggests there are some African-Americans with particularly slow N-glucuronidation activity towards nicotine and cotinine (Benowitz, Perez-Stable et al., 1999), and indeed, African-Americans excreted a lower proportion of nicotine and cotinine as their glucuronide conjugates compared to Caucasians (18 vs. 29% for nicotine glucuronidation, 41 vs. 62% for cotinine glucuronidation) (Berg et al., 2010). Interestingly, the distribution of 3HC O-glucuronidation was unimodal for both racial/ethnic groups (Benowitz, Perez-Stable et al., 1999).

A genetic polymorphism altering enzyme function in UGT2B10 was recently identified. The UGT2B10*2 (199 G>T, D67Y) reduced-function allele has been associated with slower rates of nicotine and cotinine N-glucuronidation in human liver microsomes (Chen, Blevins-Primeau et al., 2007; Chen, Dellinger Rw et al., 2008) and an approximately 20% decrease in the excretion of nicotine and cotinine as their glucuronide conjugate in vivo (Berg, Mason et
al., 2010; Berg et al.). Although further studies are needed to evaluate the impact of UGT2B10 genetic variability on nicotine clearance rates and smoking phenotypes, one recent study suggests individuals with UGT2B10*1/*2 have lower total excreted nicotine equivalents, an indicator of nicotine intake, compared to wildtype individuals (Berg, Von Weymarn et al.).

A portion of absorbed nicotine is also eliminated as nicotine N’-oxide; approximately 4 to 7% of absorbed nicotine is found as nicotine N’-oxide in the urine of smokers (Byrd, Chang et al., 1992; Benowitz, Jacob et al., 1994), and in individuals lacking CYP2A6, up to 31% of absorbed nicotine is excreted as this metabolite (Yamanaka, Nakajima et al., 2004). Nicotine N’-oxide is formed by flavin-containing monooxygenase 3 (FMO3); large variability in the protein and activity levels have been reported, ranging nine- and six-fold, respectively (Overby et al., 1997). A number of polymorphisms in the FMO3 gene have been identified although their functional consequences, particularly toward nicotine N’-oxidation, are unclear. Loss-of-function FMO3 variants have been identified in individuals with trimethylaminuria, an inherited disorder also known as fish odor syndrome, although such variants are likely extremely rare (Cashman et al., 2001).

1.6.5 Pharmacological actions of nicotine metabolites

Intravenous infusion of cotinine to plasma levels seen in moderate to heavy smokers did not result in any physiological effects (i.e. changes in heart rate, blood pressure, skin temperature), nor were any subjective effects reported (i.e. increased tension or anxiety, depression, anger, hostility, vigor, confusion, or fatigue) (Benowitz et al., 1983; Zevin et al., 2000). Oral administration of cotinine to achieve plasma levels that are up to 10-times higher than those observed in smokers resulted in similar physiological and subjective effects as the
placebo control (Hatsukami et al., 1997). In this study, cotinine did not affect heart rate, blood pressure, skin temperature, food intake or sleeping patterns, and changes in mood or subjective effects were not reported (Hatsukami, Grillo et al., 1997). Cotinine does not up-regulate nAChRs in the brain (Terry Jr et al., 2005) but it can evoke DA release from rat striatal slices, albeit with much lower potency compared to nicotine (EC$_{50}$ of 30 μM for cotinine versus EC$_{50}$ of 0.1 to 4.0 μM for nicotine) (Dwoskin et al., 1999). Similarly, 3HC has been infused intravenously into smokers during abstinence without any significant changes in heart rate or blood pressure (Benowitz et al., 2001). Subjective effects were not consistently reported, although no placebo control was used in this study (Benowitz and Peyton J Iii, 2001). Together, this suggests that cotinine and 3HC are unlikely to contribute to the psychoactive effects mediated by nicotine.

Studies in rats have shown that nornicotine accumulates in the brain following repeated nicotine exposure (Ghosheh et al., 2001) because it has a substantially longer half-life at approximately 8 hours (Kyerematen Ga et al., 1990; Hukkanen, Jacob et al., 2005). Receptor binding studies have shown that nornicotine can bind to nAChRs with high affinity and in vitro can evoke DA release from rat nucleus accumbens slices, although with lower potency (EC$_{50}$ = 0.48 to 3.0 μM) compared to nicotine (EC$_{50}$ = 70 nM) (Reavill et al., 1988; Green et al., 2001). Nornicotine can also maintain i.v. self-administration in rats and induce locomotor stimulation; however, these effects were weak and did not occur at pharmacologically relevant concentrations (Bardo et al., 1999; Dwoskin et al., 1999).

1.7 Tobacco dependence

1.7.1 Acquisition of dependence
The majority of smoking experimentation and initiation occurs during adolescence; approximately 80% of current smokers started smoking prior to age 18 (Giovino, 1999; Centers for Disease Control and Prevention, 2006). Even though over 50% of young adults in the United States have experimented with tobacco at least once (Centers for Disease Control and Prevention, 2002; Pergadia et al., 2006; Centers for Disease Control and Prevention, 2010), only a portion of those who have ever tried cigarettes (25-35%) will become addicted regular smokers (Centers for Disease Control and Prevention, 1998). In addition to the genetic factors that contribute to smoking initiation and progression to dependence, a number of environmental factors have been identified. Studies have demonstrated the importance of social-environmental factors such as familial and peer influences (Simons-Morton, 2002; Vink et al., 2003), parental monitoring (Simons-Morton, 2002), academic achievements (Wilkinson et al., 2007), socioeconomic status (Soteriades et al., 2003), initial reactions to the first cigarette (Pomerleau et al., 1993; Difranza et al., 2007), tobacco industry marketing and access to tobacco (e.g. local cigarette taxation rate) (Evans et al., 1995; Robinson et al., 1997). Various psychological factors and personality traits such as impulsivity, novelty-seeking and risk taking, low self-esteem, depressed mood/affect disorder, maladaptive coping skills and attitudes and beliefs about the benefits of smoking have also been identified as predictors of smoking among adolescents (Wills et al., 1995; Baker et al., 2004).

1.7.2 Diagnostic scales for tobacco dependence

A number of diagnostic scales have been created in an attempt to provide quantitative assessment of the severity of tobacco dependence. Tobacco addiction is a complex, multifaceted disorder, and the various measures each have their advantages and limitations (Piper et al., 2006). The Fagerström Test for Nicotine Dependence (FTND) (Heatherton et al.,
1991), the most widely used measure of tobacco dependence, was primarily developed as a measure of physical dependence (Piper, McCarthy et al., 2006). The FTND has utility due to its adequate test-retest reliability and acceptable predictive validity of the success of smoking cessation attempts and smoking relapse (Piper, McCarthy et al., 2006). Its brevity and ease of administration also makes it useful in clinical and epidemiological contexts. There is generally low concordance between the FTND with formal diagnostic systems such as Diagnostic Statistical Manual-IV (DSM-IV) and International Classification of Diseases-10 (ICD-10), suggesting these various measures are capturing slightly different aspects of tobacco dependence (Hughes et al., 2004). More comprehensive scales have been developed recently, such as the Nicotine Dependence Syndrome Scale (NDSS) and Wisconsin Inventory of Smoking Dependence Motives (WISDM-68), that are multidimensional and cover greater depth in the types of questions asked (Piper et al., 2004; Piper et al., 2008).

1.8 Smoking cessation

Tobacco addiction is a chronic, relapsing illness; more than 80% of moderate to heavy smokers who attempt to quit without aid relapse within a month, with only 3 to 5% maintaining long-term abstinence each year (Hughes et al., 2004). On average, four to five quit attempts are typically made before successful long-term abstinence is achieved; as such, smoking cessation is a difficult and complex process. Although a variety of methods have been developed to aid smoking cessation (e.g. pharmacotherapy and behavioural counseling), the currently available treatments are still rather limited in efficacy.

1.8.1 Treatments available

1.8.1.1 Pharmacotherapies
There are currently three types of drugs for smoking cessation approved by Health Canada. These include various formulations of nicotine replacement therapy, bupropion (Zyban®) and varenicline (Champix®) (The Canadian Lung Association, 2010).

*Nicotine replacement therapy (NRT)*

NRT is the most commonly used pharmacotherapy for smoking cessation, providing an alternative source of nicotine to reduce the intensity of nicotine withdrawal symptoms and blunt the reinforcing effects of inhaled nicotine from cigarette smoke should a lapse occur. NRT is currently offered in various formulations that are available over-the-counter including chewing gum, transdermal patch, and lozenges, while the nicotine nasal spray or oral inhaler are available with a prescription. While these preparations are buffered to alkaline pH to increase the absorption of nicotine through cell membranes, the rise in nicotine brain and blood levels is more gradual, without the sharp peaks observed from smoking that are important for its addiction liability (Benowitz et al., 1988; Hukkanen, Jacob et al., 2005). NRT provides low-level replacement of nicotine, with *ad libitum* use of these products resulting in plasma levels that are one- to two-thirds of the levels achieved from moderate to heavy cigarette smoking (Hukkanen, Jacob et al., 2005). For the chewing gum, lozenges, nasal spray and oral inhaler, nicotine absorption takes place primarily through the mucosa of the oral and nasal cavity rather than the lungs (Benowitz et al., 1987; Benowitz, Jacob P 3rd et al., 1991; Compton et al., 1997; Hukkanen, Jacob et al., 2005). The bioavailability for these products is estimated at 50 to 80% as a portion of nicotine is often swallowed and undergoes extensive first-pass hepatic metabolism (Benowitz, Jacob P 3rd et al., 1987; Benowitz, Jacob P 3rd et al., 1991; Compton, Sandborn Wj et al., 1997; Hukkanen, Jacob et al., 2005). Nicotine is also well absorbed from the skin, with its rate of release from a transdermal patch being dependent on skin permeability and the rate of diffusion of
nicotine through the polymer matrix. An estimated 68 to 100% of the amount of nicotine released from the transdermal nicotine patch is absorbed into the body (Gupta et al., 1993; Fant et al., 2000; Hukkanen, Jacob et al., 2005). This variability is likely due to some evaporation of nicotine occurring from the exposed edges of the patch once it is applied on the skin (Gupta, Benowitz et al., 1993).

Meta-analyses of randomized clinical trials have shown NRT significantly improves the likelihood of smoking cessation compared to placebo, increasing the percentage of successful quitters at 6 months from approximately 10% for the placebo treatment arm to approximately 17% for NRT treatment (Mcneil, Piccenna L et al., 2010). A large meta-analysis of 132 trials found the relative risk of abstinence for any form of NRT compared to placebo control was 1.58 (95% confidence interval 1.50 to 1.66), ranging from 1.43 for nicotine gum, 1.66 for transdermal patch, 1.90 for inhaler, 2.00 for oral lozenges, to 2.02 for nicotine nasal spray (Stead, Perera R et al., 2008). There is evidence that the 4 mg gum was more efficacious than the 2 mg gum, but the efficacy of nicotine patch did not differ by dose (Stead, Perera R et al., 2008). The adverse effects related to NRT use vary by type of formulation and include skin irritation (patch), sleep disturbances (24 hour patch), local irritation of nose or throat, sneezing and coughing (spray), and hiccups (inhaler) (Mcneil, Piccenna L et al., 2010).

_Bupropion (Zyban®)_

Bupropion (Zyban®) was originally developed as an atypical antidepressant; however, it has also proven effective in aiding smoking cessation in both depressed and non-depressed smokers (Hayford et al., 1999; Smith et al., 2003; Cox et al., 2004; Hughes et al., 2007). It has been shown to reduce cravings and relieve certain withdrawal symptoms associated with smoking abstinence such as mood disturbances, difficulty concentrating and irritability.
(Shiffman et al., 2000; Durcan et al., 2002). The neurobiological mechanism(s) by which it aids smoking cessation is not well understood; it can act as a noncompetitive antagonist towards brain nAChRs (Fryer et al., 1999; Slemmer et al., 2000), bind to striatal dopamine transporters and prevent dopamine reuptake (Learned-Coughlin et al., 2003), as well as inhibit the firing of noradrenergic neurons in the locus coeruleus at concentrations reached during smoking cessation treatment (Cooper et al., 1994). 6’-Hydroxybupropion, the primary metabolite of bupropion, is also pharmacologically active (Siu et al., 2007). Randomized clinical trials have shown the quit rates observed at 6 months from bupropion treatment are approximately 17% compared to an average of 9% in the placebo group (Mcneil, Piccenna L et al., 2010). A meta-analysis of 36 trials for bupropion found the relative risk for long-term abstinence at 6 months was 1.69 (95% confidence interval 1.53 to 1.85) as compared to placebo (Hughes et al., 2007).

Varenicline (Champix ®)

Varenicline (Champix ®) is a partial agonist for the α4β2 nAChRs (Mcneil, Piccenna L et al., 2010). Randomized clinical trials have shown that the long-term abstinence rates reported at 6 months were approximately 26% for varenicline treatment compared to approximately 11% for the placebo treatment (Cahill et al., 2008; Mcneil, Piccenna L et al., 2010). A meta-analysis of seven trials found the relative risk of long-term abstinence at 6 months for varenicline treatment was 2.33 (95% confidence interval 1.95 to 2.8) compared to placebo (Cahill, Stead Lf et al., 2008). The relative risk of long-term abstinence at 12 months for varenicline treatment was 1.5 (95% confidence interval 1.22 to 1.88) compared to bupropion treatment in three clinical trials (Cahill, Stead Lf et al., 2008). One open-label trial also found the relative risk of long-term abstinence at 12 months was 1.3 (95% confidence
interval 1.01 to 1.71) for varenicline treatment compared to nicotine patch treatment (Aubin et al., 2008).

There is evidence that concomitant uses of different types of medication may have additional benefits compared to monotherapy. Meta-analyses found that combining nicotine patch with a rapid titratable form of NRT (such as nasal spray) or bupropion was more effective than monotherapy alone (Shah et al., 2008; Stead, Perera R et al., 2008).

1.8.1.2 Non-pharmacological interventions

In addition to pharmacological options, various non-pharmacological approaches are useful in aiding smoking cessation. Counseling is provided in most clinical trials in addition to the pharmacological agents tested, and the quit rates observed in the placebo treatment arms are often reflective of the effects that counseling alone had on smoking cessation. The most common form is cognitive behavioural therapy; its overall goal is to help smokers identify and break the association between environmental and social cues that motivate their smoking, as well as to teach coping strategies for dealing with stress associated with nicotine withdrawal (Black Jh 3rd).

Cognitive behavioural therapies

Current guidelines for smoking cessation treatment in the United States recommend a combination of counseling and pharmacotherapy as this was more effective than either alone (odds ratio = 1.4, 95% confidence interval 1.2 – 1.6) (Fiore et al., 2008; Hajek P et al., 2009). One widely used form of cognitive behavioural counseling is motivational interviewing; it is designed to help people explore and resolve uncertainties about their behaviour to help them change their behaviour and encourage their self-efficacy to quit. One meta-analysis showed
motivational interviewing increases the likelihood of abstinence (relative risk 1.27, 95% CI 1.13 – 1.42) compared to brief advice (Lai et al., 2010). Other types of cognitive behavioural counseling methods include health education, which provides information such as the addictive nature of nicotine, health consequences of smoking and benefits of quitting, and advice on developing concrete strategies to quit (Ahluwalia et al., 2006).

1.9 Biomarkers of tobacco smoke

The number of cigarettes smoked per day is often used as a proxy measure for toxin exposure, level of addiction, or level of disease risk; however, self-report of cigarette usage has a number of limitations. The way in which cigarettes are smoked can vary widely between individuals; for example, differences exist in the number of puffs taken per cigarette, puff interval and frequency, puff volume, depth of inhalation, duration of inhalation and the blocking of filter vents (Scherer, 1999). As such, objective indicators of tobacco smoke exposure are necessary. Biochemical validation of tobacco exposure is of great utility in verifying smoking abstinence in clinical trials or recruitment into research studies given the potential reporting bias of self-reported cigarette consumption. Biomarkers of cigarette consumption are also important in epidemiological studies for assessing and quantifying the dose-related risk of tobacco-related illnesses.

1.9.1 Common indicators of exposure

Two commonly used biomarkers of tobacco smoke exposure are cotinine and exhaled CO. Cotinine is detected in a number of biological fluids; it has a long half-life (13 to 19 hours) and is highly specific to nicotine exposure, although not necessarily cigarette smoke as individuals consuming alternative sources of tobacco or nicotine replacement therapy also have detectable cotinine (Benowitz and Jacob P 3rd, 1994; Benowitz et al., 2002). Exhaled
CO is a byproduct of tobacco combustion and can be readily measured, although it has a short half-life (1 to 4 hours) and is not specific to tobacco smoke due to contributions from environmental sources (such as vehicle exhaust) and endogenous formation from heme catabolism (Benowitz, Peyton J Iii et al., 2002). Non-smokers living in urban environments typically have exhaled CO levels of 3 to 5 ppm, and may be as high as 7 ppm in some cases (Jones et al., 2006; Scherer, 2006). Endogenous CO formation is estimated to result in approximately 0.7% carboxyhemoglobin levels in blood (Coburn et al., 1965), which corresponds to approximately 0.3 ppm of exhaled CO (Scherer, 2006). A number of other biomarkers have been proposed, although these also have advantages and limitations. Tobacco-specific alkaloids anabasine and anatabine have high tobacco specificity, long half-lives and are not present in NRTs (Benowitz, Peyton J Iii et al., 2002). However, these metabolites are found at less than 5% of nicotine or cotinine levels detected in the urine of smokers, and cost-effective methods for their detection remain to be developed (Benowitz, Peyton J Iii et al., 2002). Relatively simple and affordable assays are available to detect thiocyanate, a combustion product of hydrogen cyanide; however, it is limited by its lack of specificity due to its presence in dietary sources (Benowitz, Peyton J Iii et al., 2002).

SECTION 2: LIGHT SMOKING

2.1 Prevalence

The models developed to understand tobacco dependence were based primarily on observations in moderate to heavy smokers, and there is a paucity of research on tobacco use and the manifestation of tobacco dependence among non-daily users or light smokers. National surveys of tobacco use in the United States did not include questions for non-daily smoking until 1992 (Shiffman, 2009). Among current adult smokers in the United States,
recent estimates of non-daily smoking ranges from 20% to over 30% (Hassmiller et al., 2003; Office of Applied Studies and Substance Abuse and Mental Health Services Administration, 2006). An additional 25 to 30% of current adult smokers report smoking 10 or fewer CPD (Kandel et al., 2000; Trinidad et al., 2009). Similar prevalence has been reported by the Canadian Tobacco Use Monitoring Survey, with 20 to 25% of current adult smokers reporting non-daily use and 33% of current adult smokers consuming 10 or fewer CPD (Health Canada, 2008). Together, this suggests a substantial proportion of the smoking population in North America use non-daily or exhibit light smoking patterns (i.e. 10 or fewer CPD). Non-daily smoking is also highly prevalent in other parts of the world; at least two-thirds of the smokers in developing countries such as Mexico, Ecuador and Guatemala are non-daily users (World Health Organization, 2007).

Epidemiological surveys from recent years suggest that although overall smoking prevalence has been declining in North America (Health Canada, 2008; Centers for Disease Control and Prevention, 2009; Benowitz, 2010; National Cancer Institute, National Institutes of Health et al., 2010), the prevalence of non-daily smoking has been on the rise. In the United States, the percentage of current adult smokers reporting non-daily use increased from 16% in 1998 to 22% in 2009 (Wortley et al., 2003; Centers for Disease Control and Prevention, 2010). Similarly, the percentage of current adult smokers reporting non-daily use increased from 17% in 1998 to 25% in 2008 in Canada (Health Canada, 2008). The reported number of cigarettes smoked per day has also been declining among daily smokers. The average CPD smoked by adults in the United States decreased considerably from 25.1 in males and 21.5 in females in 1980, to 16.7 in males and 13.2 in females by 2000 (Duval, Jacobs Jr et al., 2008). Similar trends have been reported in Canada with the average CPD among adults declining from 20.6 in 1985 to 14.9 in 2008 (Health Canada, 2008). These changes are likely the result
of immense tobacco control efforts limiting smoking via bans, taxation, and denormalization of the behaviour.

2.2 Health consequences

There is no safe level of smoking, and even light smokers are at a significantly elevated risk for developing smoking-related illnesses. Light smokers have an increased risk of cancer, cardiovascular disease, and total mortality compared to never smokers. In a Norwegian population, smoking one to four CPD significantly increased the risk of mortality from ischemic heart disease by approximately 3-times, from lung cancer in females by approximately 5-times, and from any cause by approximately 1.5 times compared to never smokers (Bjartveit et al., 2005). Light smokers consuming between three to nine CPD also have approximately 2.1 times higher risk of myocardial infarction compared to non-smokers (Prescott et al., 2002). In addition, light smokers have a higher risk for gastrointestinal cancers and higher incidence of a number of other illnesses including respiratory ailments, cataracts, and compromised reproductive health (Schane et al., 2010).

2.3 Demographics

2.3.1 Ethnic minorities

Ethnic minorities currently constitute approximately 25% of the American population and this proportion is expected to grow to approximately 50% by 2050 (Okuyemi Ks, Harris Kj et al., 2002). Smoking prevalence and levels of cigarette consumption vary widely among racial/ethnic minority groups in the United States. While approximately 40% of Caucasian current smokers reported non-daily use or smoking 10 or fewer CPD, as much as 67% of African-Americans, 72% of Asian-Americans, and 76% of Hispanic-Americans reported smoking at these levels (Trinidad, Perez-Stable et al., 2009). Similar findings have been
found in earlier surveys, suggesting ethnic differences in cigarette consumption have existed since at least the early 1990s (Husten et al., 1998; Hassmiller, Warner Ke et al., 2003; Trinidad, Perez-Stable et al., 2009).

2.3.2 Females

Females generally smoke fewer CPD compared to males (Okuyemi et al., 2001; Health Canada, 2008; Tong et al., 2009; Trinidad, Perez-Stable et al., 2009). The factors motivating smoking behaviours appear to differ between the genders, with females being more likely to smoke to relieve stress and negative affect (Mermelstein, 1999; Perkins et al., 1999; Piper et al., 2001). They are also more responsive to the sensory component of cigarettes and to environmental cues to smoke (e.g. under certain social situations) (Mermelstein, 1999; Perkins, Donny E et al., 1999; Piper, Welsch et al., 2001). In addition, pregnancy can also greatly influence smoking behaviours. Among females who continue to smoke during pregnancy, an estimated 25 to 50% reported a significant reduction in cigarette consumption (Floyd et al., 1993).

2.3.3 Young adults

The majority of smokers begin experimentation prior to age 18, and only a portion of those who ever try cigarettes will progress to regular smoking. It takes approximately two years to establish stable smoking patterns following initial experimentation (Difranza et al., 2005). In a national survey of American high school students in Grades 9 to 12, 19.5% of respondents reported current smoking, defined as having smoked cigarettes on at least one day during the past 30 days prior to survey. Only 11.2% of respondents reported ever smoking daily, and just 7.8% of current smokers consumed more than 10 CPD on the days that they did smoke (Centers for Disease Control and Prevention, 2010). A large proportion of adolescent current
smokers (50.8%) had tried to quit in the 12 months preceding the survey (Centers for Disease Control and Prevention, 2010). Thus, light smoking among adolescents and young adults likely represents a transitory phase that smokers progress through prior to either establishing a regular smoking pattern, reducing their consumption levels, or quitting (Okuyemi Ks, Harris Kj et al., 2002). However, while light smoking appears to be a transitory phase in some cases, there are groups of individuals who maintain this low level of consumption throughout their smoking career. It is not yet clear why some progress to heavier daily smoking patterns while others maintain low levels of consumption.

2.4 Tobacco dependence

2.4.1 Smoking characteristics

Light smoking behaviours challenge traditional theories of tobacco addiction, as smoking at regular intervals over the course of the day was believed to be necessary in order to maintain sufficient nicotine levels in the body and avoid the development of withdrawal symptoms. The majority of Caucasian smokers consume a pack of cigarettes or more daily, although approximately 5% of total current smokers are considered to be light smokers who smoke five or fewer CPD on at least four days of the week. A limited number of studies have examined tobacco dependence among light smokers of Caucasian ancestry and found that they do not appear to exhibit characteristic features of tobacco dependence in contrast to Caucasian moderate to heavy smokers (Shiffman, 1989; Shiffman et al., 1995). Caucasian light smokers do not report withdrawal symptoms during smoking abstinence, have less cravings for cigarettes and fewer urges to smoke between cigarettes, are less likely to smoke even when ill or in places where it is forbidden, and have a longer latency to first cigarette in the morning (Shiffman, 1989; Shiffman et al., 2006). However, these Caucasian light smokers inhale cigarette smoke with similar smoking topography, show similar
cardiovascular responses to cigarettes, absorb equal amounts of nicotine and appear to eliminate nicotine at similar rates as heavier smokers (Shiffman et al., 1990; Brauer et al., 1996). It is unlikely these Caucasian light smokers are smoking to maintain plasma nicotine levels due to the long time interval between each cigarette smoked (Shiffman et al., 1992). Smoking among these Caucasian light smokers appeared to be less associated with mood states, and does not appear to be just a social activity (Shiffman, 1989; Shiffman and Paty J, 2006). Rather, smoking appears to be often associated with indulgent activities such as relaxation, socialization, eating and drinking alcohol or coffee (Shiffman and Paty J, 2006). It is notable that these studies have only been done in Caucasian light smokers consuming five or fewer CPD and findings may not be extrapolated to light smokers of other racial/ethnic populations. Furthermore, light smokers are a heterogeneous population, and the factors influencing smoking behaviours probably differ between individuals smoking five or fewer CPD compared to those smoking 6 to 10 CPD.

2.4.2 Interventions for smoking cessation

The process of smoking cessation among light smokers is not well understood due to the general misconception that they are able to quit successfully on their own. As such, they have been excluded from nearly all of the clinical trials for smoking cessation published to date. However, it is becoming more evident that these smokers have difficulty achieving and maintaining abstinence. There is currently one published clinical trial that tests the efficacy of 2 mg nicotine gum and counseling specifically in African-Americans smoking 10 or fewer CPD (Ahluwalia, Okuyemi et al., 2006). Nicotine gum did not significantly improve the likelihood of abstinence compared to placebo, although health education counseling resulted in significantly higher quit rates compared to motivational interview counseling (Ahluwalia, Okuyemi et al., 2006). In general, only 15 to 30% of the participants were able to quit
smoking even with formal treatment in this study, which is comparable to the quit rates observed in other clinical trials of Caucasian or African-American moderate to heavy smokers (Ahluwalia et al., 2002; Stead, Perera R et al., 2008).

2.5 African-American light smokers
Tobacco-related health disparities exist between racial/ethnic groups. These include differences in smoking initiation rates, current use, amount consumed, quitting success, and health consequences of smoking. In particular, African-Americans experience disproportionately higher incidences of tobacco-related illnesses such as lung cancer despite their lower self-reported levels of cigarette consumption. Addressing the lack of research and inequalities in health care for this particular group of light smokers is necessary to reduce the disparities that currently exist (Fagan et al., 2007).

2.5.1 Demographics
According to the 2007 United States census, 12.4% of individuals reported themselves as African-American or Black (Grieco, 2009). Eight percent of African-Americans are foreign-born with the majority migrating from the Caribbean or Africa (Grieco, 2009). Current smoking prevalence among African-Americans (21.3%) is similar to that in Caucasians (22.0%) (Figure 1.1) (Centers for Disease Control and Prevention, 2009). However, the proportion of current smokers reporting non-daily use was greater among African-Americans (23.8%) compared to Caucasians (16.6%). The proportion of current smokers who smoke 10 or fewer CPD was also greater among African-Americans (42.7%) compared to Caucasians (23.8%) (Trinidad, Perez-Stable et al., 2009).

2.5.2 Smoking initiation
African-Americans have a delayed onset of smoking initiation, with many experimenting and developing regular smoking patterns after age 18 or during young adulthood, whereas this typically occurs during early- to mid-adolescence for Caucasians (Robinson, Klesges Re et al., 1997; Griesler et al., 1998; Everett et al., 1999; Ellickson et al., 2004; Trinidad et al., 2004; Trinidad et al., 2004; Fagan, Moolchan Et et al., 2007; Centers for Disease Control and Prevention, 2010; Centers for Disease Control and Prevention, 2010). Only 9.5% of African-American high school students reported smoking on at least one day of the past month, compared to 18.0% of Hispanics and 22.5% of Caucasians who reported doing so (Centers for Disease Control and Prevention, 2010). One longitudinal study also found that African-American adolescents had a greater tendency to stop smoking instead of progressing to more regular smoking patterns compared to Caucasians or Hispanics (Ellickson, Orlando et al., 2004).

2.5.3 Cigarette consumption

Among African-Americans adults, more than 40% of males and 60% of females smoke 10 or fewer CPD (Haiman, Stram et al., 2006). However, in spite of the lower cigarette consumption among African-American smokers, they have higher plasma cotinine levels compared to Caucasian smokers (Caraballo et al., 1998; Benowitz, Perez-Stable et al., 1999; Moolchan et al., 2006; Signorello et al., 2009). African-American smokers also have higher plasma cotinine levels compared to Mexican-American smokers who reported similar levels of cigarette consumption (Caraballo, Giovino et al., 1998). One possible explanation is that African-Americans have slower rates of cotinine metabolism, resulting in an accumulation of cotinine per cigarette smoked. There is evidence to suggest that African-Americans have slower rates of cotinine clearance and higher intake of nicotine per cigarette compared to Caucasians (Perez-Stable et al., 1998). African-Americans may also have greater exposure
to tobacco smoke per reported cigarette as a result of deeper inhalation or greater preference for menthol cigarettes that contain higher nicotine and tar content.

2.5.4 Use of menthol cigarettes

Menthol was first used as a cigarette flavoring in the late 1920s, adding a pleasant taste and cooling sensation to cigarette smoke (Garten et al., 2003). Approximately 75% of African-American smokers prefer menthol cigarettes compared to 25 to 30% of Caucasian smokers (Centers for Disease Control and Prevention, 1998; Balbach et al., 2003). This is likely due to advertising campaigns beginning in the 1960s that specifically targets African-American smokers with culturally-sensitive images and messages strongly promoting the use of menthol cigarettes (Balbach, Gasior et al., 2003; Gardiner, 2004). However, this marketing strategy appears to be specific to the United States as only 8% of individuals of Black-African descent in Canada report smoking menthol cigarettes (Mwenifumbo et al., 2008).

Menthol has a short half-life of approximately 1 hour, and it undergoes extensive glucuronidation by UGT1A4 and UGT2B7 (Coffman et al., 1998; Heck, 2010). Menthol does not appear to have any direct toxicological or carcinogenic effects when administered alone (National Cancer Institute, 1979; Murthy et al., 1991; Bhatia et al., 2008), although the cooling and anti-tussive effects of menthol may reduce the irritation by cigarette smoke, allowing for deeper inhalation and larger puff volumes. In addition, it has been proposed that menthol may allow for greater absorption of toxins by increasing the permeability of cell membranes, and that menthol itself may be converted to carcinogenic compounds following pyrolysis (Schmeltz et al., 1968; Kitagawa et al., 1999; Werley et al., 2007). While activation of cold thermoreceptors (e.g. TRPM8) in the respiratory tract by menthol may
result in the perception of increased airflow (Bandell et al., 2006), there does not seem to be any actual changes in nasal patency or resistance to airflow (Garten and Falkner, 2003).

A number of experimental studies have tested the effect of menthol cigarettes on smoking topography (such as puff volume or number of puffs) with inconsistent results. Some studies suggest more intense inhalation (Miller et al., 1994; Ahijevych et al., 1999), others show no change (Ahijevych et al., 1996) and some suggest less intense inhalation among those who smoke menthol versus regular cigarettes (Nil et al., 1989; Jarvik et al., 1994; McCarthy et al., 1995). Menthol cigarette smokers were found to have higher exhaled CO values compared to regular cigarette smokers in some studies (Miller, Jarvik et al., 1994; Clark et al., 1996), although other studies have reported lower exhaled CO levels (Ahijevych, Gillespie et al., 1996), or no differences (Nil and Battig, 1989; Jarvik, Tashkin et al., 1994; McCarthy, Caskey et al., 1995). Similarly, discrepancies in the effect of menthol cigarettes on cotinine levels have been reported. Some studies suggest menthol cigarette smokers have higher cotinine and cotinine per cigarette levels even after adjustment for race (Clark, Gautam et al., 1996; Ahijevych and Parsley, 1999; Wang et al., 2010), whereas other studies did not find such differences (Ahijevych et al., 1994; Ahijevych, Gillespie et al., 1996; Mustonen et al., 2005). It is notable that many of these studies contained small sample sizes and/or also included Caucasian smokers in some cases which may complicate interpretation of the results.

Numerous studies have examined the role of menthol cigarettes on smoking initiation and cessation, although results from these studies have also been inconsistent (Hyland et al., 2002; Muscat et al., 2002; Moolchan, 2004; Okuyemi et al., 2004; Okuyemi et al., 2007; Heck, 2010). The greater use of menthol cigarettes among African-Americans has been
proposed to contribute to their disproportionately higher incidence of smoking-related illnesses. However, a number of case-control studies failed to demonstrate that smoking menthol cigarettes substantially alters the risk of smoking-related illnesses (including cardiovascular and respiratory diseases, lung or esophageal cancers) after adjustment for age, race, gender and amount smoked (Hebert et al., 1989; Kabat et al., 1991; Kabat et al., 1994; Sidney et al., 1995; Carpenter et al., 1999; Stewart Jh 4th, 2001; Brooks et al., 2003; Stellman et al., 2003).

2.5.5 Smoking cessation

More than 70% of African-American adult smokers have indicated that they would like to quit smoking completely (Centers for Disease Control and Prevention, 1998). However, despite their willingness to quit, African-Americans appear to be less successful at quitting smoking compared to Caucasians. Epidemiological studies have found that African-Americans have a lower quit ratio, defined as the proportion of ever smokers who have quit smoking, compared to Caucasians (Novotny et al., 1988; Royce et al., 1993; Centers for Disease Control and Prevention, 1998; King et al., 2004; Haiman, Stram et al., 2006; Fu et al., 2008). This observation that African-Americans were less likely to be former smokers compared to Caucasians remained even after controlling for demographic factors (such as gender, age, marital status, and socioeconomic status) or smoking characteristics (such as amount of cigarettes smoked per day, time to first cigarette in the morning, and age of smoking initiation) (Novotny, Warner et al., 1988; King, Polednak et al., 2004; Fu, Kodl et al., 2008).

2.5.6 Health disparities
The rates of many smoking-related cancers have been declining for all racial/ethnic groups since the early 1990s. However, morbidity and mortality from smoking-related cancers still remain highest among African-Americans despite their lower cigarette consumption and delayed onset of smoking compared to Caucasians. African-Americans have higher incidence and mortality from cancers of the lung, oral cavity, pancreas, esophagus and larynx (Stewart Jh 4th, 2001; Edwards et al., 2005; Haiman, Stram et al., 2006; Fagan, Moolchan Et et al., 2007). In fact, the death rate for all cancers combined is 35% higher in African-American males and 18% higher in African-American females compared to Caucasian males and females, respectively (Ries et al., 2006; American Cancer Society, 2008). The racial/ethnic disparity of lung cancer is particularly evident among African-American males, where incidence and mortality rates were 40 to 60% higher compared to Caucasian males (Stewart Jh 4th, 2001; Haiman, Stram et al., 2006; Ries, Harkins D et al., 2006; Fagan, Moolchan Et et al., 2007; American Cancer Society, 2008).

Racial/ethnic disparities in cancer mortality have been attributed to socioeconomic inequalities that influence cancer prevention, time to detection, and access to quality health care (Centers for Disease Control and Prevention, 1998; Delancey et al., 2008). Differences in environmental correlates of socioeconomic status such as dietary habits, occupational exposure and general health outcomes may, in part, account for the observed disparities (Centers for Disease Control and Prevention, 1998). African-Americans have historically had greater occupational exposure to carcinogens by working in industries such as steel and rubber manufacturing, textiles and shipbuilding (Stewart Jh 4th, 2001). It is also possible that African-Americans have a greater biological sensitivity towards developing tobacco-related cancers. In addition to CYP2A6, other polymorphic xenobiotic metabolizing enzymes such as CYP1A1, CYP2A13, CYP2D6, CYP2E1, UGTs and glutathione S-
transferases may contribute to carcinogenesis by activating procarcinogens or detoxifying carcinogenic and mutagenic agents (El-Zein et al., 1997; Stewart Jh 4th, 2001). Racial/ethnic differences in the activity of these activating or detoxifying enzymes may in part explain the disparities in cancer rates, although studies to support this have not yet been performed.

In addition to cancers, tobacco smoking is the main cause of several non-malignant respiratory illnesses. African-Americans have had lower rates of chronic bronchitis, emphysema and COPD in the past (Centers for Disease Control and Prevention, 1998), although more recent data suggests mortality rates from COPD have been rising faster in African-Americans than in Caucasians (Kirkpatrick et al., 2009). There is evidence that African-Americans with COPD are less likely to utilize medical services and account for lower medical costs compared to Caucasians with COPD, providing a possible explanation for the disparities in mortality rates (Shaya et al., 2009).

SECTION 3: CYP2A6

3.1 CYP2 gene family

The cytochromes P450 (CYP) are a superfamily of heme-containing mono-oxygenases involved in numerous endogenous processes such as the metabolism of fatty acids, cholesterol, steroids and eicosanoids, as well as the detoxification or bioactivation of a wide range of xenobiotics. Members of the same CYP family (e.g. CYP2) share more than 40% amino acid identity while members of the same CYP subfamily (e.g. CYP2A) share more than 55% amino acid identity (Anzenbacher et al., 2001). There are presently 57 active CYP genes identified, with the CYP1, CYP2 and CYP3 families of enzymes having the greatest contribution to xenobiotic metabolism (Nelson et al., 2004; Ingelman-Sundberg, 2005). Of
these, the CYP2 family is the largest and most diverse; the CYP2ABFGST gene cluster is located in an approximately 500 kb region on chromosome 19q13.2 and consists of numerous genes and pseudogenes of high sequence similarity (Figure 3.1).

3.1.1 Evolutionary origins of CYP2ABFGST gene cluster

Various CYP gene subfamilies (e.g. CYP2C, CYP2D, CYP2J, CYP3A, and CYP4F) are scattered across the genome, representing older duplication events that have been separated by chromosomal arrangements over time, while genes within a subfamily tend to be physically clustered as they are derived from a series of more recent tandem duplications (Nelson, Zeldin et al., 2004). However, two CYP gene clusters (CYP2ABFGST and CYP4ABXZ) contain a series of mixed subfamilies that likely arose from more complicated molecular evolutionary events. The human CYP2ABFGST gene cluster is thought to originate from an initial series of tandem duplications followed by inverted duplication, further tandem duplications and insertion of CYP2B6 and CYP2B7P (Hoffman et al., 2001) (Figure 3.1).

Detailed analyses of the CYP2ABFGST cluster in mouse (chromosome 7), rat (chromosome 1), and non-human primates (chromosome 19) have been performed. It is believed that this gene cluster originated in a common ancestor of primates and rodents with a single “founder” locus for each of the six CYP2 subfamilies that duplicated early in mammalian evolution (Hu et al., 2008). Rodents evolutionarily diverged from primates approximately 85 million years ago and accordingly the arrangement of the CYP2ABFGST cluster is more closely related between mice and rats than to humans (Wang et al., 2003; Hu, Wang et al., 2008). Comparison of the gene cluster in humans and chimpanzees, who diverged evolutionarily around 7 million years ago, suggests it has undergone only small-scale chromosomal
rearrangements, while a substantially different arrangement of the locus is found in Rhesus macaques who diverged approximately 25 to 30 million years ago (Hoffman et al., 2007).

Figure 3.1: Human CYP2ABFGST gene cluster on chromosome 19q13.2. Each triangle represents a gene and the orientation of each gene is illustrated with transcription occurring 5’ to 3’ in the direction of each triangle. Shading indicates genes that belong to the same subfamily. A number of inactive pseudogenes are found in this cluster (CYP2T2P, 2F1P, CYP2A7, CYP2G1P, CYP2A18PC/PN, CYP2B7P, CYP2G2P, and CYP2T3P). Insertion of CYP2B6 and CYP2B7P separates the CYP2A18 pseudogene into two segments. Figure is not drawn to scale. Modified from (Hoffman and Nelson Dr, 2001).

3.1.2 CYP2A subfamily

There are three full CYP2A genes found in humans: CYP2A6, CYP2A7, and CYP2A13. The CYP2A6 and CYP2A7 loci arose from tandem duplications; each gene is approximately 6.7 kb and they are physically separated by less than 6 kb of unique sequence (Hoffman and Nelson Dr, 2001). The CYP2A subfamily of genes has an extremely high degree of sequence identity; CYP2A6 has 95% identical gene sequence (including exons and introns) and 94% identical amino acid sequence to CYP2A7 (Hoffman and Nelson Dr, 2001). Similarly, the gene sequence of CYP2A13 is 85% identical to CYP2A6 and 90% identical to CYP2A7, while the amino acid sequence of CYP2A13 is 93% identical to CYP2A6 and 90% identical to CYP2A7 (Hoffman and Nelson Dr, 2001).
3.1.3 CYP2A tissue expression

CYP2A6 is primarily expressed in the liver, representing 1 to 10% of total liver CYP content (Pelkonen, Rautio et al., 2000). Low levels of CYP2A6 mRNA have also been detected in the nasal olfactory mucosa (Su et al., 1996; Koskela et al., 1999), lung, skin, coronary arteries, esophagus, and breast (Hukkanen et al., 2002; Hukkanen, Jacob et al., 2005). Similarly, low levels of CYP2A6 protein have also been detected in the nasal olfactory mucosa (Su, Sheng et al., 1996), lung, larynx, esophagus, and breast (Hukkanen, Pelkonen et al., 2002; Hukkanen, Jacob et al., 2005). Messenger RNA transcripts of CYP2A7 have been found in human livers; however, the protein lacks heme binding ability and is enzymatically inactive (Yamano et al., 1990; Ding et al., 1995). CYP2A13 is primarily expressed in the respiratory tract (nasal epithelium, trachea, and lungs) (Su et al., 2000; Zhu et al., 2006) with very low levels detected in the liver (Koskela, Hakkola et al., 1999). CYP2A13 can also metabolize nicotine and cotinine efficiently in vitro; the Km of CYP2A13 towards nicotine is 20.2 μM compared to 26 μM for CYP2A6, and the Km of CYP2A13 for cotinine is 45.2 μM compared to 265 μM for CYP2A6 (Nakajima, Yamamoto et al., 1996; Bao et al., 2005). However, nicotine undergoes primarily hepatic metabolism and metabolism by CYP2A13 in the lungs is not likely to substantially reduce its systemic levels. This is supported by the observation that individuals completely lacking the CYP2A6 enzyme (e.g. those with CYP2A6*4/*4 genotype) have much higher nicotine plasma levels and AUC values compared to individuals with fully active CYP2A6 (Nakajima, Yamagishi et al., 2000; Xu, Rao et al., 2002; Nakajima and Yokoi T, 2005; Mwenifumbo, Al Koudsi N et al., 2008)

3.1.4 CYP2A6 substrates

In addition to nicotine and cotinine, CYP2A6 contributes to the metabolism of a limited number of compounds including several therapeutic agents and toxins (Table 3.1). CYP2A6
Table 3.1: List of CYP2A6 substrates. Modified from (Raunio et al., 2001).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Source/usage</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>Present in tobacco plants, nicotine replacement therapy</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>Cotinine</td>
<td>Nicotine metabolite</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>Coumarin</td>
<td>Used to treat lymphoedema in some European countries</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>Tegafur</td>
<td>Anti-neoplastic prodrug that is activated to 5-fluorouracil by CYP2A6</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>Letrozole</td>
<td>Anti-neoplastic drug (aromatase inhibitor for breast cancer treatment) that is inactivated by CYP2A6</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>Anesthetic</td>
<td>Dehalogenation</td>
</tr>
<tr>
<td>Halothane</td>
<td>Anesthetic</td>
<td>Reduction</td>
</tr>
<tr>
<td>SM-12502</td>
<td>Novel platelet-activating factor receptor antagonist</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Losigamone</td>
<td>Anti-convulsant</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Anti-convulsant, mood stabilizer</td>
<td>Oxidation</td>
</tr>
<tr>
<td>NNK</td>
<td>Tobacco-specific nitrosamine</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>NNN</td>
<td>Tobacco-specific nitrosamine</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>NDEA</td>
<td>Carcinogen</td>
<td>Mutagenic</td>
</tr>
<tr>
<td>MTBE</td>
<td>Carcinogen</td>
<td>Demethylation</td>
</tr>
<tr>
<td>MOCA</td>
<td>Toxin/suspected carcinogen</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Quinoline</td>
<td>Toxin</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxalen</td>
<td>Selegiline</td>
<td>Tranylcypromine</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Grapefruit juice</td>
<td>Starfruit juice</td>
</tr>
<tr>
<td>Inducers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Oral contraceptives containing estrogen</td>
<td>Broccoli</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Dexamethasone</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: (+)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride (SM-12502); 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and N-nitrosonornicotine (NNN); N- nitrosodiethylamine (NDEA); methyl tert-butyl ether (MTBE); 4,4k-methylenebis(2-chloroaniline) (MOCA). CYP2A6 contributes a large role in the metabolism of nicotine, cotinine, coumarin, tegafur, letrozole and SM-12502. The other compounds listed are likely partially metabolized by CYP2A6 or their metabolic pathways have not been thoroughly studied to date.
has a relatively compact, narrow and hydrophobic active site; as such, the substrates for this enzyme also tend to be small and planar (Yano et al., 2005). There are relatively few clinically used pharmaceutical agents that are metabolized extensively and/or selectively by CYP2A6 in comparison to CYP2D6, CYP3A4 or CYP2C9/18/19, which together are responsible for the metabolism of 80-90% of all clinically used drugs (Wrighton et al., 1996; Anzenbacher and Anzenbacherová, 2001; Zhou et al., 2009). Crystallography and in silico simulations have suggested that CYP2A6 is rather rigid in terms of flexibility and malleability that can limit substrate entry, accommodation into the active site, and product release (Skopalí K et al., 2008). This is in contrast to CYP3A4 which has a greater degree of flexibility and malleability and accordingly, a much broader range of substrates (Skopalí K, Anzenbacher et al., 2008).

3.1.4.1 Tobacco-specific nitrosamines

A number of tobacco-alkaloid derived nitrosamines have been detected in both unburned tobacco and tobacco smoke resulting from combustion. Of these, N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are among the most prevalent and carcinogenic compounds in tobacco smoke (Hecht, 1998). Numerous studies have shown these compounds can induce cancers in a variety of species (Hecht, 1998). In particular, NNK has been shown to be a potent and selective inducer of lung adenocarcinomas and NNN can induce esophageal and nasal tumors in rodents (Hoffmann et al., 1984; Anderson et al., 1989; Padma et al., 1989; Rivenson et al., 1989; Furukawa et al., 1994; Hecht, 1998). These tobacco-specific nitrosamines are procarcinogens and require metabolic activation to form reactive intermediates. NNN can be metabolically activated to reactive intermediates capable of forming DNA adducts via 2’- and 5’-α-hydroxylation (Chen et al., 1978; Hecht et
al., 1986; Hecht, 1998), while NNK and NNAL are metabolized to reactive intermediates via α-hydroxylation (Hecht et al., 1980; Guo et al., 1992; Hecht, 1998). A number of CYP enzymes have been implicated in these reactions; cDNA-expressed CYP2A6 and CYP2A13 catalyzed the 5’-hydroxylation of NNN with similar catalytic efficiency in vitro (Wong et al., 2004). CYP2B6 and CYP2A6 were found to have the highest affinity towards NNK in human liver microsomes (Patten et al., 1996; Dicke et al., 2005). However, the catalytic efficiency of cDNA-expressed CYP2A13 towards NNK is approximately 30 to 50-fold greater than that for CYP2A6 (Su, Bao et al., 2000; He et al., 2004), and the presence of this enzyme in the respiratory system suggests it may contribute significantly to local bioactivation and carcinogenesis. In vitro studies using lung microsomes have shown that CYP2A13 levels but not CYP2A6 were correlated with rates of NNK activation in samples that have high CYP2A13 expression (Zhang et al., 2007). Individuals with higher pulmonary expression of CYP2A13 may be at an increased risk of developing tobacco-related cancers, although it should be noted that CYP2A6 protein was detected in 90% of the samples whereas CYP2A13 was detected in only 12% of the samples, suggesting CYP2A6 may have a greater contribution in most individuals (Zhang, D’agostino et al., 2007).

3.2 Genetic variability in CYP2A6 activity

3.2.1 Inter-individual and inter-ethnic variability

Large inter-individual variability in CYP2A6 activity exists; human liver microsomes have more than 50-fold variation in mRNA, protein expression and activity (Pelkonen, Rautio et al., 2000). Although factors such as gender, and presence of inducers or inhibitors, may contribute to the variation in enzyme activity, the observed variability has mainly been attributed to genetic polymorphisms in CYP2A6. Pharmacokinetic studies in twins have demonstrated a substantial genetic contribution to the variability in the rates of nicotine
clearance, with the heritability of total rates of nicotine clearance and clearance of nicotine via the cotinine pathway estimated at approximately 60% (Swan et al., 2005). Similar studies have shown that approximately 67% of the variability in the plasma ratio of 3HC to cotinine (3HC/COT), a phenotypic indicator of CYP2A6 activity, can be attributed to additive genetic influence (Swan et al., 2009).

CYP2A6 expression and activity vary widely across ethnic groups (Malaiyandi, Sellers et al., 2005; Mwenifumbo and Tyndale, 2007). For example, liver microsomes from Japanese donors have lower levels of CYP2A6 protein and activity compared to Caucasians (Shimada et al., 1996). Furthermore, the rate of nicotine clearance was found to be 18% slower for Asian-Americans (Benowitz et al., 2002) and 13% slower for African-Americans (Benowitz, Perez-Stable et al., 1999) compared with Caucasians. In these studies, the rate of cotinine clearance was also found to be 31% slower for Asian-Americans (Benowitz, Perez-Stable et al., 2002) and 32% slower for African-Americans (Benowitz, Perez-Stable et al., 1999).

3.2.2 Currently identified CYP2A6 variants and their functional consequences

The CYP2A6 gene consists of nine exons and eight introns, encoding 494 amino acids. CYP2A6 is highly polymorphic, and much progress has been made in recent years in the identification of new CYP2A6 genetic variants. There are currently 37 numbered alleles known, compared to the 22 numbered alleles that were recognized back in 2005 (http://www.cypalleles.ki.se/). These genetic variations include single nucleotide polymorphisms (SNPs), nucleotide insertions and deletions, gene conversions, gene deletions and gene duplications. Some alleles encode enzymes that have abolished function (e.g. CYP2A6*2, CYP2A6*4), others reduce activity (e.g. CYP2A6*9, CYP2A6*12) and the gene duplication alleles (CYP2A6*I2A,*I2B) increase activity (Rao et al., 2000; Benowitz et al.,
 Numerous in vitro and in vivo studies have demonstrated that CYP2A6 genetic variants are associated with reduced rates of nicotine clearance (Benowitz, Swan et al., 2006) and lower rates of CYP2A6 activity using phenotypic indicators (Johnstone et al., 2006; Malaiyandi et al., 2006; Malaiyandi et al., 2006; Nakajima, Fukami et al., 2006; Peamkrasatam S et al., 2006; Mwenifumbo Jc et al., 2008). A description of these variants and their effects on enzyme function can be found in Table 3.2, while a summary of their frequencies in various racial/ethnic populations is listed in Table 3.3. The frequency of these alleles varies greatly between racial/ethnic groups, reflecting the large degree of inter-ethnic variability in the rates of nicotine metabolism (Benowitz, Perez-Stable et al., 1999; Benowitz, Perez-Stable et al., 2002; Nakajima, Fukami et al., 2006). The percentage of individuals with slow CYP2A6 metabolism (less than 50% activity), as predicted by frequencies of alleles that encode enzymes with reduced- or loss-of-function, ranges from approximately 10% in Caucasians to approximately 60% in Japanese in accordance with the slower rates of nicotine metabolism observed in Asians compared to Caucasians (Mwenifumbo et al., 2009).

### 3.3 Other influences of CYP2A6 activity

#### 3.3.1 Gender/hormonal effects

In a pharmacokinetic study where participants were given intravenous infusions of isotope-labeled nicotine and cotinine, nicotine clearance rates were 20% higher in females compared to males (Benowitz et al., 2006). This difference was enhanced in females taking estrogen-containing oral contraceptives, who had 44% higher rates of nicotine clearance compared to males, whereas females not taking these drugs had only 13% higher rates of clearance compared to males (Benowitz, Lessov-Schlaggar Cn et al., 2006).
Table 3.2: Description of CYP2A6 alleles and their predicted functional impact

<table>
<thead>
<tr>
<th>Allele</th>
<th>Gene change</th>
<th>Location</th>
<th>Amino acid</th>
<th>Description</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6*1A</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
<td>Normal/wildtype</td>
</tr>
<tr>
<td>CYP2A6*1B</td>
<td>-1013A&gt;G</td>
<td>5'UTR</td>
<td></td>
<td>The -1013A&gt;G SNP is thought to disrupt an enhancer or promoter responsive element in CYP2A6</td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*1C</td>
<td>5717C&gt;T</td>
<td>Exon 8</td>
<td></td>
<td>Synonymous SNP (P408P)</td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*1D</td>
<td>5717C&gt;T,5825A&gt;G</td>
<td>Exon 8, intron 8</td>
<td></td>
<td>SNP located in a putative CYP2A6 promoter region and is thought to disrupt a CCAAT box. In vitro, the transcription of the human CYP2A6 promoter with the -745A&gt;G variant is significantly reduced by 22%</td>
<td>Reduced?</td>
</tr>
<tr>
<td>CYP2A6*1E</td>
<td>-745A&gt;G</td>
<td>5'UTR</td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*1F</td>
<td>-1301A&gt;C, -1289G&gt;A, -1199, -1198ins316bpAlu, -686A&gt;G; 51G; 431C&gt;T; 1620T&gt;C; 1779G&gt;A; 2483G&gt;A; 2994T&gt;C; 3378C&gt;T; 3904G&gt;A; 4074delA; 5668A; 6354T&gt;C; 7160A&gt;G</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1G</td>
<td>Haplotype of various synonymous changes</td>
<td></td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*1H</td>
<td>Gene conversion with CYP2A7 at 7073-7082</td>
<td></td>
<td></td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*1I</td>
<td>Gene duplication</td>
<td></td>
<td></td>
<td></td>
<td>Increased</td>
</tr>
<tr>
<td>CYP2A6*2</td>
<td>1799T&gt;A</td>
<td>Exon 3</td>
<td>L160H</td>
<td>In vitro, the enzyme fails to incorporate heme and is inactive. In vivo, this allele appears inactive.</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*4A-H</td>
<td>Gene deletion</td>
<td></td>
<td></td>
<td>Hybrid allele thought to occur by unequal homologous crossover. A number of crossover regions has been identified within intron 8 to 5.2 – 5.6 kb downstream of the stop codon in the 3’UTR</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*5</td>
<td>6582G&gt;T</td>
<td>Exon 9</td>
<td>G479V</td>
<td>In vitro, expressed enzyme is unstable; only trace amount of enzyme and coumarin 7-hydroxylation activity were detected. In vivo, limited data suggests inactive allele</td>
<td>Inactive</td>
</tr>
<tr>
<td>Allele</td>
<td>Gene change</td>
<td>Location</td>
<td>Amino acid</td>
<td>Description</td>
<td>Activity*</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>----------</td>
<td>------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CYP2A6*6</td>
<td>1703G&gt;A</td>
<td>Exon 3</td>
<td>R128Q</td>
<td>Arg128 is highly conserved among CYP450s and is thought to be important for proper heme binding and holoprotein conformation. <em>In vitro</em>, the expressed enzyme has greatly reduced coumarin 7-hydroxylation activity. <em>In vivo</em>, limited data suggests inactive allele.</td>
<td>Reduced</td>
</tr>
<tr>
<td>CYP2A6*7</td>
<td>6558T&gt;C</td>
<td>Exon 9</td>
<td>I471T</td>
<td>When expressed in <em>E. coli</em> and subjected to heat treatment (37°C), the enzyme encoded by this variant was less stable than its wild type counterpart. Also, the variant lacked nicotine C-oxidase activity while maintaining ~60% of coumarin 7-hydroxylase activity. <em>In vivo</em>, individuals with this allele have decreased nicotine and normal coumarin metabolism.</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*8</td>
<td>6600G&gt;T</td>
<td>Exon 9</td>
<td>R485L</td>
<td><em>In vitro</em>, the expressed enzyme encoded has normal nicotine C-oxidation activity. <em>In vivo</em>, limited data suggests no effect on nicotine metabolism.</td>
<td>Active</td>
</tr>
<tr>
<td>CYP2A6*9A, B</td>
<td>-48T&gt;G</td>
<td>5'TATA box</td>
<td>---</td>
<td><em>In vitro</em>, the SNP resulted in a 50% decrease in transcription activity, and lower mRNA, protein and activity levels were observed in human liver microsomes. <em>In vivo</em>, this allele reduced nicotine metabolism.</td>
<td>Reduced</td>
</tr>
<tr>
<td>CYP2A6*10</td>
<td>6558T&gt;C, 6600G&gt;T</td>
<td>Exon 9</td>
<td>I471T, R485L</td>
<td><em>CYP2A6</em>10 contains variations in amino acid residues lying within or close to substrate recognition site 6 (SRS-6), a highly conserved region. <em>In vitro</em> studies have not been done. <em>In vivo</em>, individuals with this allele have impaired nicotine and coumarin metabolism.</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*11</td>
<td>3391T&gt;C</td>
<td>Exon 5</td>
<td>S224P</td>
<td><em>In vitro</em> the enzyme resulted in ~50% decrease in Vmax when using tegafur as a substrate. <em>In vivo</em>, limited data suggests reduced activity.</td>
<td>Reduced</td>
</tr>
<tr>
<td>CYP2A6*12A-C</td>
<td>Exon 1-2 CYP2A7, Exon 3-9 CYP2A6</td>
<td>---</td>
<td>10 amino acid substitution</td>
<td>This variant result from an unequal crossover event between CYP2A6 and CYP2A7 in intron 2. <em>In vitro</em>, the expressed protein level and coumarin 7-hydroxylation activity was decreased 40-50%. <em>In vivo</em>, this allele appears to reduce function.</td>
<td>Reduced</td>
</tr>
<tr>
<td>CYP2A6*13</td>
<td>13G&gt;A</td>
<td>Exon 1</td>
<td>G5R</td>
<td><em>In vivo</em>, limited data suggests reduced function.</td>
<td>Reduced?</td>
</tr>
<tr>
<td>CYP2A6*14</td>
<td>86G&gt;A</td>
<td>Exon 1</td>
<td>S29N</td>
<td><em>In vivo</em>, limited data suggests no change in function.</td>
<td>Active?</td>
</tr>
<tr>
<td>CYP2A6*15</td>
<td>2134A&gt;G</td>
<td>Exon 4</td>
<td>K194E</td>
<td><em>In vivo</em>, limited data suggests reduced function towards nicotine. <em>In vitro</em>, this allele appears to be fully functional towards coumarin.</td>
<td>Reduced?</td>
</tr>
<tr>
<td>Allele</td>
<td>Gene change</td>
<td>Location</td>
<td>Amino acid</td>
<td>Description</td>
<td>Activity*</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
<td>----------</td>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>CYP2A6*16</td>
<td>2161C&gt;A</td>
<td>Exon 4</td>
<td>R203S</td>
<td>In vitro, expressed enzyme did not alter activity towards nicotine or coumarin. In vivo, limited data suggests no change in function.</td>
<td>Active?</td>
</tr>
<tr>
<td>CYP2A6*17</td>
<td>5065G&gt;A</td>
<td>Exon 7</td>
<td>V365M</td>
<td>In vitro, the expressed enzyme had reduced activity towards nicotine and coumarin. In vivo, this allele appears to be inactive towards nicotine.</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*18A-C</td>
<td>5668A&gt;T</td>
<td>Exon 8</td>
<td>Y392F</td>
<td>In vitro, the expressed enzyme had normal activity towards nicotine but reduced activity towards coumarin. In vivo, limited data suggests normal activity towards nicotine.</td>
<td>Active?</td>
</tr>
<tr>
<td>CYP2A6*19</td>
<td>5668A&gt;T, 6558T&gt;C</td>
<td>Exon 8, 9</td>
<td>Y392F, I471T</td>
<td>In vitro, the expressed enzyme had reduced activity towards nicotine and coumarin. In vivo, limited data suggests this allele have reduced activity towards nicotine.</td>
<td>Reduced?</td>
</tr>
<tr>
<td>CYP2A6*20</td>
<td>2141-2142 AA deletion, premature stop codon</td>
<td>Exon 4</td>
<td>Truncated protein</td>
<td>In vitro, no activity was detected by the expressed enzyme. In vivo, this allele appears to be inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>CYP2A6*21</td>
<td>6573A&gt;G</td>
<td>Exon 9</td>
<td>K476R</td>
<td>In vivo and in vitro, this allele appears to be fully functional</td>
<td>Active</td>
</tr>
<tr>
<td>CYP2A6*22</td>
<td>1794C&gt;G, 1798C&gt;A</td>
<td>Exon 3</td>
<td>D158E, L160I</td>
<td>In vitro, this allele appears to have reduced function towards coumarin.</td>
<td>Reduced?</td>
</tr>
<tr>
<td>CYP2A6*23</td>
<td>2161C&gt;T</td>
<td>Exon 4</td>
<td>R203C</td>
<td>In vitro, this allele appears to have reduced activity towards nicotine and coumarin. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
<tr>
<td>CYP2A6*24</td>
<td>594G&gt;C, 6458A&gt;T</td>
<td>Exon 2, 9</td>
<td>V110L, N438Y</td>
<td>In vitro, the expressed enzyme appears to be unstable. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
<tr>
<td>CYP2A6*25</td>
<td>1672T&gt;C</td>
<td>Exon 3</td>
<td>F118L</td>
<td>In vitro, the expressed enzyme appears to be fully functional. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
<tr>
<td>CYP2A6*26</td>
<td>1672T&gt;C, 1703G&gt;T 1711T&gt;G</td>
<td>Exon 3</td>
<td>F118L, R128L, S131A</td>
<td>In vitro, the expressed enzyme appears to be inactive. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
<tr>
<td>CYP2A6*27</td>
<td>1672T&gt;C 2162 – 2163 GC&gt;A frameshift</td>
<td>Exon 3</td>
<td>F118L, R203 - frameshift</td>
<td>In vitro, the expressed enzyme appears to be inactive. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
<tr>
<td>CYP2A6*28</td>
<td>5745A&gt;G, 5750G&gt;C</td>
<td>Exon 8</td>
<td>N418D, E419D</td>
<td>In vitro, the expressed enzyme appears to be fully functional. In vivo, limited data suggests the allele has reduced activity or is inactive.</td>
<td>Reduced? Inactive?</td>
</tr>
</tbody>
</table>
Table 3.2: Description of CYP2A6 alleles and their predicted functional impact (continued)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Gene change</th>
<th>Location</th>
<th>Amino acid</th>
<th>Description</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6*29</td>
<td>Not released yet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*30</td>
<td>Not released yet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*31</td>
<td>16A&gt;C</td>
<td>Exon 1</td>
<td>M6L</td>
<td>This allele has not been characterized <em>in vitro or in vivo</em></td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*32</td>
<td>Not released yet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*33</td>
<td>Not released yet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*34</td>
<td>Exon 1 – 4 of CYP2A7, Exon 5 – 9 of CYP2A6</td>
<td>---</td>
<td>21 amino acid changes</td>
<td>This allele has not been characterized <em>in vitro or in vivo</em></td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP2A6*35</td>
<td>6458A&gt;T</td>
<td>Exon 9</td>
<td>N438Y</td>
<td><em>In vitro</em>, the expressed enzyme appears to be unstable. <em>In vivo</em>, limited data suggests the allele has reduced activity or is inactive</td>
<td>Reduced? Inactive?</td>
</tr>
</tbody>
</table>

All nucleotide sequence numbering are based on +1 with reference to the ATG start site on the reference genomic sequence NG_000008.7. * Activity towards nicotine C-oxidation. Data modified from (Mwenifumbo et al., 2007; Benowitz et al., 2009; Ho et al., 2009; Tiong Kh et al., 2010).
Table 3.3: *CYP2A6* allele frequencies in various racial/ethnic populations

<table>
<thead>
<tr>
<th><em>CYP2A6</em> allele</th>
<th>non-Hispanic whites</th>
<th>African-Americans</th>
<th>Japanese</th>
<th>Chinese</th>
<th>Koreans</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYP2A6</em>1B1-17</td>
<td>28.8 – 35.0</td>
<td>11.9 – 16.4</td>
<td>48.4 – 54.6</td>
<td>43.2 – 51.3</td>
<td>57.0</td>
</tr>
<tr>
<td><em>CYP2A6</em>2</td>
<td>1.1 – 5.3</td>
<td>0.4 – 0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>4A-H</td>
<td>0 – 4.2</td>
<td>0.9 – 2.7</td>
<td>17.0 – 24.2</td>
<td>4.9 – 15.1</td>
<td>10.8</td>
</tr>
<tr>
<td><em>CYP2A6</em>5</td>
<td>0 – 0.3</td>
<td>0</td>
<td>0</td>
<td>0.5 – 1.2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>CYP2A6</em>6</td>
<td>0</td>
<td>0</td>
<td>0 – 0.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>7</td>
<td>0</td>
<td>0</td>
<td>9.8 – 12.5</td>
<td>5.7 – 9.8</td>
<td>9.4 – 9.8</td>
</tr>
<tr>
<td><em>CYP2A6</em>8</td>
<td>0</td>
<td>0</td>
<td>0 – 1.1</td>
<td>0 – 1.5</td>
<td>0 – 1.2</td>
</tr>
<tr>
<td><em>CYP2A6</em>9A, B</td>
<td>5.2 – 8.0</td>
<td>7.2 – 9.6</td>
<td>19.0 – 20.3</td>
<td>15.6 – 15.7</td>
<td>19.6 – 22.3</td>
</tr>
<tr>
<td><em>CYP2A6</em>10</td>
<td>0</td>
<td>0</td>
<td>2.2 – 3.2</td>
<td>1.7 – 4.3</td>
<td>1.0 – 4.1</td>
</tr>
<tr>
<td><em>CYP2A6</em>11</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td><em>CYP2A6</em>12A-C</td>
<td>0 – 3.0</td>
<td>0 – 0.4</td>
<td>0 – 0.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>13</td>
<td>0</td>
<td>0</td>
<td>1.1 – 1.2</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td><em>CYP2A6</em>14</td>
<td>3.5 – 5.7</td>
<td>1.4 – 2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>15</td>
<td>0</td>
<td>0 – 1.1</td>
<td>1.0 – 2.2</td>
<td>1.4</td>
<td>1.2 – 7.4</td>
</tr>
<tr>
<td><em>CYP2A6</em>16</td>
<td>0.3</td>
<td>1.2 – 1.7</td>
<td>0 – 1.1</td>
<td>1.3</td>
<td>0 – 5.3</td>
</tr>
<tr>
<td><em>CYP2A6</em>17</td>
<td>0</td>
<td>7.1 – 10.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>18A-C</td>
<td>1.1 – 2.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td><em>CYP2A6</em>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>CYP2A6</em>20</td>
<td>0</td>
<td>1.1 – 1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>21</td>
<td>0 – 2.3</td>
<td>0 – 0.6</td>
<td>0</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>22</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>23</td>
<td>0</td>
<td>1.1 – 2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>24</td>
<td>0</td>
<td>0.7 – 2.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>25</td>
<td>0</td>
<td>0.5 – 1.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>26</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>27</td>
<td>0</td>
<td>0.2 – 0.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>28</td>
<td>0</td>
<td>0.9 – 2.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>31</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>CYP2A6</em>34</td>
<td>0</td>
<td>0</td>
<td>Note: Allele was discovered in one Asian individual through sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CYP2A6</em>35</td>
<td>0</td>
<td>2.5 – 2.9</td>
<td>0.8</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

Data modified from (Mwenifumbo and Tyndale, 2007).
Similarly, cotinine clearance rates were 32% higher in all females, 61% higher in females taking estrogen-containing contraceptives and 24% higher in females not taking these drugs compared to males (Benowitz, Lessov-Schlaggar Cn et al., 2006). Nicotine and cotinine half-lives were also shorter in females, with greater effects in those taking estrogen-containing oral contraceptives (Benowitz, Lessov-Schlaggar Cn et al., 2006). A number of other studies have found higher 3HC/COT in females, indicating enhanced CYP2A6 enzyme function (Benowitz, Lessov-Schlaggar Cn et al., 2006; Johnstone, Benowitz et al., 2006; Kandel et al., 2007; Mwenifumbo et al., 2007). A 1.9-fold higher level of CYP2A6 mRNA, 2.0-fold higher level of CYP2A6 protein and 1.5-fold higher rates of nicotine C-oxidation activity were found in human liver microsomes of females compared to males (Al Koudsi et al., 2010).

Neither nicotine nor cotinine pharmacokinetics appear to differ by phases of the menstrual cycle (i.e. follicular vs. luteal) in healthy non-smoking females (Hukkanen et al., 2005). However, pregnancy has a substantial effect on rates of nicotine and cotinine clearance, being increased by 60% and 140%, respectively, compared to post-partum (Dempsey et al., 2002). Together, these results suggest that sex hormones (most likely estrogen) substantially increase CYP2A6 activity and rates of nicotine and cotinine clearance, with fastest activity observed among those taking oral contraceptives or during pregnancy where levels of hormones are highest. Mechanistically, the CYP2A6 promoter region contains a putative estrogen response element at -2.4 kb and it has been demonstrated in vitro that estradiol can induce CYP2A6 in an estrogen receptor-α dependent manner (Higashi et al., 2007).

3.3.2 Age
In general, the capacity for hepatic drug metabolism declines among the elderly, mostly as a result of diminished blood flow and liver volume (Schmucker et al., 1990; Wynne, 2005). This is particularly important for high extraction drugs, such as nicotine, where the liver has great potential capacity to metabolize the compound and clearance rates are dependent on the rate of hepatic blood flow. Indeed, slower rates of nicotine clearance have been found among the elderly (65 to 76 years of age), with total clearance reduced by 23% and renal clearance reduced by 49% compared to young adults (22 to 43 years of age) (Molander et al., 2001). No age-related decreases in CYP2A6 protein or activity levels have been found in human liver microsomes collected from individuals aged 2 to 64 (Parkinson et al., 2004; Al Koudsi, Hoffmann et al.). In vivo, there is also no age-related differences in the 3HC/COT ratio or nicotine pharmacokinetics among individuals of Black-African descent aged 22 to 59 years old (Mwenifumbo, Sellers et al., 2007). However, one study of Caucasians aged 25 to 65 reported the 3HC/COT ratio was significantly associated with age, with the ratio increasing by 2% for every 5-year increase in age (Johnstone, Benowitz et al., 2006).

3.3.3 CYP2A6 inhibitors and inducers

3.3.3.1 Pharmaceutical agents

The prototypical CYP inducers such as rifampicin, dexamethasone, and phenobarbital can induce CYP2A6 in human primary hepatocytes, although there is wide variability in response (Hukkanen, Jacob et al., 2005). Rifampicin is a well-known activator of the nuclear receptors pregnane X receptor (PXR), and phenobarbital is an activator of the constitutive androstane receptor (CAR). These transcription factors recruit other coactivators to enhancer regions of their target genes, mediating gene transcription of numerous xenobiotic-metabolizing enzymes. PXR and CAR can dimerize with retinoid X receptor-α (RXRα) to potentially bind at DR4-like response elements found at -6.6 kb, -5.4 kb and -4.6 kb of
CYP2A6 (Itoh et al., 2006). PXR was shown to interact with peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) at the DR4-like response elements at -5.4 kb and -4.6 kb of CYP2A6 to increase transcription (Itoh, Nakajima et al., 2006). The induction of CYP2A6 by dexamethasone occurs through the glucocorticoid receptor interacting with hepatic nuclear factor 4-α (HNF4-α) at the HNF4-response element located within -95 to +12 kb of the CYP2A6 promoter region (Onica et al., 2008). Another study found response elements for HNF-4α, CCAAT-box/enhancer binding protein α and β (C/EBPα, C/EBPβ), and octamer transcription factor-1 (Oct-1) within -112 to -61 kb of the promoter region that are important in regulating the basal expression of CYP2A6 (Pitarque et al., 2005).

A variety of compounds can inhibit CYP2A6 activity in vitro including methoxsalen (Maenpaa, Sigusch et al., 1993), pilocarpine (Kimonen et al., 1995), clotrimazole (Draper et al., 1997), miconazole (Draper, Madan et al., 1997), tranylcypromine (Zhang et al., 2001) and tryptamine (Zhang, Kilicarslan et al., 2001). Only methoxsalen and tranylcypromine have been shown to inhibit nicotine and coumarin metabolism in vivo (Sellers et al., 2003), although these compounds are not selective and can also inhibit other CYPs (Hukkanen, Jacob et al., 2005).

3.3.3.2 Menthol

Menthol, and several related compounds, can inhibit nicotine C-oxidation and coumarin 7-hydroxylation in vitro using human liver microsomes, albeit modestly (Macdougall et al., 2003). A cross-over study of African-American and Caucasian smokers showed that smoking menthol cigarettes reduces the total and nonrenal nicotine clearance by approximately 10% compared to smoking non-menthol cigarettes (Benowitz et al., 2004). This likely occurred by a reduction in the oxidative metabolism of nicotine via the cotinine
pathway, although nicotine glucuronidation may also be affected (Benowitz, Herrera et al., 2004). Studies in human liver microsomes suggest menthol can inhibit the glucuronidation of NNAL, and menthol cigarette smokers had less NNAL excreted as its glucuronide conjugate via this detoxification pathway (Muscat et al., 2009).

3.3.3.3 Smoking/nicotine

Cigarette smoke increases the metabolism for a number of substrates; it contains polyaromatic hydrocarbons that are known to be strong inducers of CYP1A1 and CYP1A2, with possible effects on CYP2E1 and glucuronidation pathways (Zevin et al., 1999; Kroon, 2007). However, cigarette smokers were found to have slower rates of nicotine and cotinine clearance (Benowitz et al., 1993), suggesting the development of functional tolerance rather than metabolic tolerance contributes to the escalating amount of tobacco consumption that occurs during smoking initiation. Cross-over studies have demonstrated that nicotine clearance was increased by 14% and 36% following four and seven days of smoking abstinence, respectively (Lee et al., 1987; Benowitz et al., 2000). In African-Americans recruited for a nicotine pharmacokinetic study, smokers had slower rates of nicotine metabolism as represented by approximately 25% higher estimated nicotine AUC compared to non-smokers (Mwenifumbo, Sellers et al., 2007). Similarly, smokers have slower rates of coumarin 7-hydroxylation in vivo compared to nonsmokers (Iscan et al., 1994; Poland et al., 2000), suggesting the inhibitory effect of tobacco smoke is specific towards CYP2A6 activity.

Studies have attempted to identify the compound(s) present in tobacco smoke with inhibitory effects on CYP2A6. In non-human primates, nicotine treatment for 21 days resulted in downregulation of CYP2A6 mRNA and protein in the liver, as well as reduced nicotine C-
oxidation activity in liver microsomes (Schoedel et al., 2003). β-Nicotyrine, a minor alkaloid found in tobacco, was also found to be a mechanism-based inhibitor of CYP2A6 \textit{in vitro} (Denton et al., 2004). In contrast, pretreatment with cotinine for four days to achieve levels seen in very heavy smokers (approximately 900 ng/ml) did not alter the rates of nicotine clearance in a crossover study of healthy non-smokers, suggesting cotinine is not inhibiting or decreasing the expression of CYP2A6 (Zevin et al., 1997). Similarly, inhalation of carbon monoxide to achieve carboxyhemoglobin levels similar to those derived from smoking did not alter the pharmacokinetic dispositions of nicotine or cotinine (Benowitz and Jacob, 2000).

3.3.3.4 Dietary

A number of dietary compounds alter CYP activity. Grapefruit juice has a number of clinically relevant drug interactions as it is a strong inhibitor of CYP3A4 and can also inhibit CYP1A1/2 and CYP2C (Nowack, 2008). \textit{In vivo}, grapefruit juice (300 to 1000 ml) inhibited the metabolism of coumarin and delayed the excretion of 7-hydroxycoumarin in the urine (Merkel et al., 1994; Runkel et al., 1997). The exact compound(s) responsible for CYP2A6 inhibition is not yet known, although bergamottin has been shown to be a mechanism-based inhibitor of CYP3A4 (Nowack, 2008).

Other plant products can also alter CYP2A6 activity; consumption of wheatgrass juice by vegans has been associated with reduced coumarin metabolism (Rauma et al., 1996). Isoflavones, which are found mainly in soy products, can inhibit CYP1A1, CYP1B1, CYP2E1 and CYP3A4 (Helsby et al., 1998; Roberts et al., 2004; Nowack, 2008). The isoflavones daidzein, genistein, and glycine inhibited nicotine C-oxidation \textit{in vitro} and soy supplementation was associated with reduced ratio of cotinine to nicotine (COT/NIC), a
phenotype indicator of CYP2A6 activity (Nakajima et al., 2006). Starfruit juice inhibited the activity of a number of CYP enzymes in human liver microsomes with the greatest effect on CYP2A6 (Zhang et al., 2007). CYP2A6 activity was shown to be induced following consumption of a standard diet of broccoli (500 g daily) for six days (Hakooz et al., 2007).

3.4 Other sources of variability in nicotine clearance rates

Hepatic blood flow can be altered by a number of physiological events such as meals, posture, exercise or drugs. As nicotine is a high extraction drug, the rate of nicotine metabolism is also affected with a 17% variation observed over the course of the day (Gries et al., 1996). Hepatic blood flow is increased by 30% and nicotine clearance is increased by 40% following meals, with maximal effects observed at 30 to 60 minutes post-ingestion (Gries, Benowitz et al., 1996). The slowest rates of nicotine clearance are observed during the evening from 6:00 pm to 3:00 am due to reductions in cardiac output and hepatic blood flow from decreased physical activity and the supine position during sleep (Gries, Benowitz et al., 1996).

Polymorphisms in other genes involved in nicotine metabolism (such as UGTs and FMO3) have been identified; however, their contributions to nicotine disposition kinetics have not been investigated to a great extent. Twin studies have also demonstrated significant heritability in the rates of glucuronidation and in the renal clearance of nicotine and cotinine (Benowitz et al., 2008; Lessov-Schlaggar et al., 2009). Pathological conditions such as viral hepatitis and alcoholic liver cirrhosis can impair nicotine and coumarin metabolism (Pasanen et al., 1997; Langmann et al., 2000; Hukkanen, Jacob et al., 2005), while kidney failure decreases both renal and metabolic clearance of nicotine and cotinine by up to 50%
(Molander et al., 2000). This is potentially due to the formation of uremic toxins that may downregulate CYP2A6 expression or act as CYP2A6 inhibitors.

3.5 Phenotypic indicators of CYP2A6 activity

Phenotyping for drug metabolizing enzymes is often used as a non-invasive method to quantitatively assess their activity in vivo. The procedure typically involves administration of a probe drug that is a substrate of the enzyme and detection of the proximal metabolites produced via the pathway of interest at a specific time following drug administration. Several phenotypic indicators have been used to assess CYP2A6 activity.

3.5.1 COT/NIC

The capacity for cotinine formation from nicotine, as represented by the COT/NIC ratio, has been used as a measure of CYP2A6 activity despite a number of limitations. While nicotine and cotinine are found in smokers, the ratio derived from metabolites present during ad libitum smoking is a poor measure of CYP2A6 activity as it is highly dependent on the time of the last cigarette as a result of the long half-life of cotinine (approximately 16 to 19 hours) relative to nicotine (approximately one to two hours) (Benowitz, Jacob P 3rd et al., 1991; Benowitz and Jacob P 3rd, 1993). As such, the levels of nicotine rise and decline rapidly following cigarette smoking whereas the levels of cotinine tend to remain fairly stable (Hukkanen, Jacob et al., 2005). The COT/NIC ratio can be used to assess activity in non-smokers and abstinent smokers by administration of a nicotine dose, typically through chewing of nicotine gum, and detection of metabolites in plasma two hours later (Kwon and Nakajima M, 2001; Nakajima, Kwon et al., 2001). However, nicotine needs to be administered to individuals who have been abstinent from nicotine-containing products (i.e. tobacco, NRTs) for prolonged periods of time (at least 14 days) to allow for the removal of
cotinine derived from these sources, which may be a potential challenge in phenotyping current smokers (Kwon and Nakajima M, 2001; Nakajima, Kwon et al., 2001). In addition, while this ratio measures the metabolites directly related to nicotine metabolism, approximately 10% of cotinine formed from nicotine is mediated by enzymes other than CYP2A6, thus limiting its specificity (Messina, Tyndale et al., 1997; Yamazaki, Inoue et al., 1999).

3.5.2 3HC/COT

The ratio of 3HC to cotinine (3HC/COT) has been used as a proxy measure of CYP2A6 activity as the conversion of cotinine into 3HC is mediated exclusively by CYP2A6 (Nakajima, Yamamoto T et al., 1996; Dempsey et al., 2004; Yamanaka, Nakajima et al., 2004). The half-life of 3HC is approximately 5 hours; however, the half-life of 3HC generated from cotinine is approximately 16 to 19 hours as its elimination rate is formation-dependent and thus matches the longer half-life of cotinine (Benowitz and Peyton J Iii, 2001). As such, the levels of 3HC from cotinine are proportional to its formation rate and independent of its own elimination rate, with the levels of 3HC and cotinine declining in parallel over time. As the formation rate is due to CYP2A6 activity, the ratio is a useful representation of CYP2A6 function. Because of the long half-life of these metabolites, the 3HC/COT ratio can be derived from baseline ad libitum smoking as it is not limited by the time of last cigarette (among regular smokers). Salivary and plasma 3HC/COT derived from either baseline smoking, or administration of isotope-labeled nicotine or cotinine, have been highly correlated with the rates of oral nicotine clearance (r = 0.70 to 0.95) in smokers and non-smokers (Dempsey, Tutka et al., 2004).
The 3HC/COT ratio is generally fairly stable with little intra-individual variation observed due to circadian fluctuations or by sampling times over the course of the day or week, and it is not affected by sample storage at room temperature for up to seven days (Lea et al., 2006). The 3HC/COT ratio is also not greatly altered by variations in smoking patterns or nicotine intake. Using computer modeling and pharmacokinetic parameters derived from experimental studies, the 3HC/COT ratio hypothetically collected from spot saliva samples was predicted to be stable across four different types of daily smoking patterns (Levi et al., 2007). Furthermore, the urinary 3HC/COT ratio from *ad libitum* smoking generally did not change during an eight-week period of smoking reduction (Mooney et al., 2008). The ratio also did not fluctuate substantially when cigarette consumption was gradually reduced by 75% during a NRT-assisted smoking reduction and maintenance phase over a period of 12 weeks (Mooney, Li et al., 2008).

### 3.5.3 Formation of 7-hydroxycoumarin

Coumarin 7-hydroxylation is commonly used *in vitro* and *in vivo* as a probe drug for CYP2A6 activity as this reaction is specifically mediated by this enzyme (Pelkonen, Rautio et al., 2000). Following oral administration, 95 to 98% of coumarin is rapidly excreted as 7-hydroxycoumarin glucuronide with a half-life of approximately one hour (Ritschel et al., 1981). Phenotyping studies of CYP2A6 activity were performed where coumarin was administered either orally or intravenously with detection of total 7-hydroxycoumarin following deconjugation using β-glucuronidase in the urine or plasma over various time points (Rautio et al., 1992; Xu, Rao et al., 2002; Peamkrasatam S, Srivatanakul K et al., 2006). Because of its rapid and extensive metabolism, coumarin is an excellent phenotypic indicator for distinguishing those with enzyme function from those with no activity; however, it is a poor indicator for discriminating among the range of CYP2A6 activity (i.e.
normal vs. intermediate vs. slow) (Xu, Rao et al., 2002; Peamkrasatam S, Sriwatanakul K et al., 2006). Coumarin is used in the treatment of lymphedema although its use has been banned in some countries due to reported cases of rare idiosyncratic hepatotoxicity (Farinola et al., 2005). CYP2A in rats has poor metabolic capacities for coumarin 7-hydroxylolation (Hitoshi et al., 1993; Yamazaki et al., 1994). Rather, coumarin is metabolized via 3,4-epoxidation to form a cytotoxic o-hydroxy-phenylacetylacetalddehyde (o-HPA) metabolite in rats (Lake, 1999; Vassallo et al., 2004). It has been proposed that this alternative routing of coumarin metabolism may be the mechanism of toxicity for coumarin among individuals who lack functioning CYP2A6 (Hadidi et al., 1997).

3.6 Association of CYP2A6 variation with smoking behaviours

Nicotine has an important role in mediating the addictive properties of tobacco smoke; thus, it has been hypothesized that individual differences in the rates of nicotine metabolism will influence tobacco dependence and smoking behaviours. A number of studies have associated genetic variability in CYP2A6 with a range of smoking behaviours.

3.6.1 Smoking initiation

Adult smokers with CYP2A6 genetic variants leading to reduced- or loss-of-function have reported earlier ages of smoking initiation compared to normal metabolizers (13.0 vs.14.3 years of age) (Schoedel et al., 2004). Despite the limitations of retrospective data, only two longitudinal studies examining the effect of CYP2A6 variation on the acquisition of tobacco dependence and smoking behaviours in adolescents have been published to date (O'loughlin et al., 2004; Audrain-McGovern et al., 2007). In a cohort of Caucasian adolescents followed from Grades 7 to 11, CYP2A6 slow metabolizers were found to have 3-fold higher risk of dependence, as measured by International Classification of Diseases – 10 (O'loughlin,
Paradis et al., 2004; Karp I, 2006). However, a second prospective study of Caucasian adolescents followed from Grades 9 to 12 found CYP2A6 slow metabolizers progressed in levels of dependence, as measured by the modified Fagerström Tolerance Questionnaire, at slower rates with the increase in dependence levels reaching a plateau faster compared to normal metabolizers (Audrain-McGovern, Koudsi et al., 2007). This may be due to different measures of nicotine dependence, methods of analyses, or baseline characteristics between the two samples. Interestingly, both of these studies report that once dependent, adolescents with slow CYP2A6 activity were associated with lower levels of cigarette consumption, resembling the observation in adults (see section 3.6.2) (O’Loughlin, Paradis et al., 2004).

3.6.2 Cigarette consumption

It has been hypothesized that individuals with slow CYP2A6 activity will need to smoke less often in order to maintain nicotine levels in the body so as to avoid withdrawal symptoms. Indeed, a number of studies have found an association between reduced CYP2A6 activity and lower cigarette consumption. This finding has been reported primarily in moderate to heavy smokers of Caucasian (Pianezza et al., 1998; Rao, Hoffmann et al., 2000; Benowitz et al., 2003; Schoedel, Hoffmann Eb et al., 2004; Johnstone, Benowitz et al., 2006; Malaiyandi, Lerman et al., 2006; Derby et al., 2008) and Japanese ancestry (Minematsu et al., 2003; Fujieda et al., 2004; Kubota et al., 2006; Minematsu et al., 2006; Derby, Cuthrell et al., 2008). A meta-analysis found a significant association of CYP2A6 slow metabolism group with reduced cigarette consumption (Munafo et al., 2004) even though not all studies have replicated these findings (Zhang et al., 2001; Ando et al., 2003). In addition, moderate to heavy Caucasian smokers who were CYP2A6 slow metabolizers had lower levels of exhaled CO and cotinine (Rao, Hoffmann et al., 2000), and significantly smaller puff volumes (Strasser et al., 2007) compared to normal metabolizers.
3.6.3 Smoking cessation

3.6.3.1 Case-control gene association studies

Several case-control studies have reported a significantly higher prevalence of *CYP2A6* slow metabolizers among non-smokers, typically defined as having ever tried smoking but never becoming dependent, compared to smokers (Pianezza, Sellers et al., 1998; Iwahashi et al., 2004; Schoedel, Hoffmann Eb et al., 2004). These results from cross-sectional studies can be interpreted in several ways. *CYP2A6* slow metabolizers may be less likely to become dependent regular smokers, although more research is needed to address this possibility. It may also be that *CYP2A6* slow metabolizers are more likely to quit, and indeed several case-control studies have found a higher prevalence of *CYP2A6* slow metabolizers among former smokers (Gu et al., 2000; Tang et al., 2009). While it is not yet clear how CYP2A6 activity influences smoking acquisition or cessation processes, there is evidence to suggest *CYP2A6* slow metabolizers may be less dependent. Among adult smokers, *CYP2A6* slow metabolizers have lower FTND scores (Kubota, Nakajima-Taniguchi et al., 2006) and were less likely to smoke the first cigarette of the day within five minutes of waking (Kubota, Nakajima-Taniguchi et al., 2006; Malaiyandi, Lerman et al., 2006).

3.6.3.2 Clinical trials

There are three smoking cessation clinical trials published to date where CYP2A6 activity was associated with quitting success. Lerman et al. reported that among individuals assigned nicotine transdermal patch treatment, those with 3HC/COT ratios in the slowest quartile (i.e. the lowest 25th percentile, indicative of slowest CYP2A6 activity) were significantly more likely to quit compared to those with 3HC/COT ratios in the fastest quartile at both week 8 end-of-treatment (46% vs. 28%) and 6-months follow-up (30% vs. 11%) (Figure 3.3A) (Lerman et al., 2006). This finding was further replicated in an independent sample
comparing standard (8 weeks) versus extended (24 weeks) nicotine transdermal patch treatment (Lerman et al., 2010). Following 8 weeks of standard treatment, individuals with 3HC/COT ratios in the slowest quartile had higher quit rates (42%) than those with faster CYP2A6 activity (27 to 30%) (Schnoll et al., 2009).

The greater smoking cessation success among CYP2A6 slow metabolizers may be due in part to their higher plasma nicotine levels obtained from the nicotine patch in comparison to normal metabolizers (Malaiyandi, Lerman et al., 2006). Interestingly, CYP2A6 slow metabolizers achieved similar quit rates as normal metabolizers when assigned to nicotine nasal spray treatment, a form of NRT where dosages can be titrated according to needs (Figure 3.3B) (Lerman, Tyndale et al., 2006). CYP2A6 slow metabolizers reported using fewer doses of the nasal spray per day and attained similar nicotine plasma levels as normal metabolizers (Malaiyandi, Lerman et al., 2006).

In the placebo treatment arm of a clinical trial testing the efficacy of bupropion, individuals with 3HC/COT ratios in the slowest quartile had the highest quit rates (32%) compared to those with 3HC/COT ratios in the fastest quartile (10%) at end-of-treatment (Figure 3.3C) (Patterson et al., 2008). In contrast, the quit rates did not differ by CYP2A6 activity for individuals taking bupropion (Figure 3.3D) (Patterson, Schnoll et al., 2008). At 6-months follow-up, similar trends were observed where individuals with 3HC/COT ratios in the slowest quartile had higher quit rates compared to those with 3HC/COT in the fastest quartile for placebo treatment, although this no longer remained significant (Patterson, Schnoll et al., 2008).

It is notable that these three clinical trials included primarily Caucasian moderate to heavy
Fig 3.2: Quit rates by 3HC/COT quartiles in clinical trials for smoking cessation. A) CYP2A6 slow metabolizers (3HC/COT in the 1st quartile) had significantly higher quit rates at both end-of-treatment and at 6 months follow-up (p = 0.005) (Lerman, Tyndale et al., 2006). B) The 3HC/COT ratio was not significantly associated with smoking cessation at either end-of-treatment or 6 months follow-up for individuals treated with nicotine spray (p = 0.68) (Lerman, Tyndale et al., 2006). C, D) CYP2A6 slow metabolizers (3HC/COT in the 1st quartile) had significantly higher quit rates (p = 0.02), and there was a significant interaction between treatment arm and the 3HC/COT ratio at end-of-treatment (p = 0.04). Bupropion significantly improved quit rates compared to placebo for individuals with fastest rates of CYP2A6 activity (3HC/COT in the 4th quartile) at both end-of-treatment (p = 0.005) and 6 months follow-up (p = 0.02) (Patterson, Schnoll et al., 2008).
smokers. Total withdrawal scores and dependence scores at baseline did not significantly differ by CYP2A6 activity in these studies. However, among those who were abstinent from smoking following one week of nicotine transdermal patch treatment, individuals with higher 3HC/COT ratios reported having more intense cravings for cigarettes (Lerman, Tyndale et al., 2006).

3.6.3.3 CYP2A6 inhibition to aid smoking cessation

CYP2A6 inhibitors have clinical utility as they may be used to phenocopy the effects of CYP2A6 genetic slow metabolizers and increase the likelihood of smoking cessation. The limited efficacy of currently available NRT formulations are likely due in part to the low-level of nicotine replacement with large variability observed in plasma drug levels (Benowitz, Jacob P 3rd et al., 1987; Benowitz, Jacob P 3rd et al., 1991; Compton, Sandborn Wj et al., 1997; Hukkanen, Jacob et al., 2005), as well as the poor adherence rates reported (Shiffman et al., 2002; World Health Organization, 2003; Schneider et al., 2004; Lam et al., 2005; Schneider et al., 2005; Okuyemi et al.). The use of orally ingested nicotine as a form of pharmacotherapy, which may enhance adherence rates, is possible with the concurrent administration of a CYP2A6 inhibitor in order to reduce the substantial first-pass effect of nicotine (Sellers et al., 2003).

CYP2A6 inhibition has the additional benefit of reducing exposure to toxins in tobacco smoke among current smokers by decreasing the amount of cigarettes consumed and decreasing bioactivation of tobacco-specific nitrosamines procarcinogens (Sellers, Tyndale et al., 2003). Administration of the CYP2A6 inhibitor methoxalen concurrently with 4 mg of oral nicotine resulted in plasma nicotine levels that were 70 to 80% higher those when nicotine was administered with placebo (Sellers, Kaplan et al., 2000). In addition,
methoxalen co-administered with a 4 mg oral nicotine dose decreased the desire to smoke, decreased the amount smoked (as indicated by 24% reduction in number of cigarettes smoked, 24% reduction in grams of tobacco burned and 50% reduction in exhaled CO), and decreased the total number of puffs taken by 25% compared to the oral nicotine given alone (Sellers, Kaplan et al., 2000; Sellers et al., 2000). Furthermore, smokers receiving methoxalen while maintaining their regular smoking patterns had significantly more NNK metabolized to the NNAL-glucuronide metabolite compared to those receiving placebo, suggesting a rerouting of CYP2A6-mediated NNK bioactivation through this detoxification pathway (Sellers, Ramamoorthy et al., 2003).

3.6.4 Lung Cancer

CYP2A6 slow metabolizers may have reduced bioactivation of tobacco-specific procarcinogens, and they may also have lower exposure to harmful substances in tobacco smoke due to reduced cigarette consumption and shorter lifetime smoking duration (Schoedel, Hoffmann Eb et al., 2004). To date, 13 studies have examined whether CYP2A6 genetic polymorphisms were associated with altered lung cancer risk. In general, CYP2A6 alleles that reduce or abolish enzyme activity appear to have a protective effect against lung cancer (Rossini et al., 2008; Rodriguez-Antona et al., 2010). A meta-analysis found the CYP2A6*4 deletion allele was protective against tobacco-related cancers compared to wildtype CYP2A6*1 individuals among Asian populations (OR = 0.25, 95% CI = 0.16–0.39) (Rodriguez-Antona, Gomez et al., 2010). A few studies have also examined the association of CYP2A6 slow metabolism towards esophageal squamous cell carcinoma, and oral, stomach, liver, colorectal, nasopharyngeal and neuroblastoma cancers (Rossini, De Almeida Simao et al., 2008). While these studies have not consistently reported a protective effect, this may be attributed to a lack of sufficient statistical power in some cases.
STATEMENT OF RESEARCH HYPOTHESES

The reduced rates of nicotine metabolism in populations of Black-African descent cannot be explained by currently known $CYP2A6$ genetic variants. In Chapter 1, we hypothesize there are unidentified and uncharacterized SNPs with important functional consequences still present among populations of Black-African descent. Specifically, we hypothesize the novel $CYP2A6^{*23}$ allele, a nonsynonymous SNP (2161C>T) encoding an amino acid change of Arg203Cys, will reduce enzyme function.

The biological factors associated with light smoking behaviours are not well known. Chapter 2 examines the association of $CYP2A6$ genetic variants with smoking behaviours in a clinical trial of African-American light smokers. We hypothesize that recently identified and established $CYP2A6$ variants categorized into predicted $CYP2A6$ genotype groups (normal, intermediate or slow metabolizers) will be significantly associated with the 3HC/COT ratio. We also hypothesize that females will have higher 3HC/COT, indicative of faster CYP2A6 activity, smokers of menthol cigarettes will have lower 3HC/COT, and body mass index (BMI) and age will not alter CYP2A6 activity. Finally, we hypothesize that CYP2A6 slow metabolizers will smoke fewer cigarettes per day and be more successful at smoking cessation.

The light and sporadic smoking patterns, and slower rates of cotinine metabolism in this population, will likely have important implications on the utility of biomarkers of smoking exposure. In Chapter 3, we hypothesize that exhaled carbon monoxide will be a poor biomarker for distinguishing between smokers and non-smokers, that exhaled carbon monoxide and plasma cotinine will be weakly correlated with self-reports of cigarette consumption, and that CYP2A6 activity, gender, smoking menthol cigarettes, BMI, and age will alter levels of self-reported cigarette consumption, exhaled CO or plasma cotinine.
CHAPTER 1: A NOVEL CYP2A6 ALLELE, CYP2A6*23, IMPAIRS ENZYME FUNCTION IN
VITRO AND IN VIVO AND DECREASES SMOKING IN A POPULATION OF BLACK-
AFRICAN DESCENT

Man Ki Ho, Jill C. Mwenifumbo, Bin Zhao, Elizabeth M.J. Gillam, Rachel F. Tyndale

Reprinted with permission from Wolters Kluwer Health: Pharmacogenetics and Genomics,
18(1):67-76, 2008. © 2010 All rights reserved.

Dr. Rachel F. Tyndale designed the human experimental study with the original goal of studying
the effect of genetic polymorphisms in CYP2A6 on nicotine pharmacokinetics. Dr. Elizabeth
M.J. Gillam kindly provided the bicistronic expression construct containing the full-length
cDNA of CYP2A6 and human NADPH-CYP reductase. Bin Zhao performed the HPLC
analyses to detect product formation from nicotine C-oxidation and coumarin 7-hydroxylation
assays. Dr. Jill C. Mwenifumbo provided guidance for database management of participants in
the human experimental study and extracted the DNA from blood samples with the aid of Ewa
B. Hoffmann and Qian Zhou. All other experimental work including development of genotyping
assays and screening of DNA samples, DNA sequencing, site-directed mutagenesis, expression
of CYP2A6 construct, and in vitro enzyme activity assays was performed by MKH. Data
analysis and manuscript writing were performed by MKH.
Abstract

Objectives: CYP2A6 is the main enzyme involved in nicotine metabolism in humans. We have identified a novel allele, CYP2A6*23 (2161C>T, R203C), in individuals of Black-African descent and investigated its impact on enzyme activity and association with smoking status.

Methods: Wildtype and variant enzymes containing amino acid changes R203C (CYP2A6*23), R203S (CYP2A6*16) and V365M (CYP2A6*17) were expressed in Escherichia coli. The effect of CYP2A6*23 in vivo was examined in individuals of Black-African descent given 4 mg oral nicotine.

Results: CYP2A6*23 occurred at an allele frequency of 2.0% in individuals of Black-African descent (N = 560 alleles, 95% confidence interval 0.8% - 3.1%) and was not detected in Caucasians (N = 334 alleles), Chinese (N = 288 alleles) or Japanese (N = 104 alleles). In vitro, CYP2A6.23 had greatly reduced activity towards nicotine C-oxidation similar to CYP2A6.17, as well as reduced coumarin 7-hydroxylation. Conversely, CYP2A6.16 did not differ in activity compared to the wildtype enzyme. The trans-3’-hydroxycotinine to cotinine ratio, a phenotypic measure of CYP2A6 activity in vivo, was lower in CYP2A6*1/*23 and CYP2A6*23/*23 individuals (mean adjusted ratio of 0.60, n = 5) compared to CYP2A6*1/*1 individuals (mean adjusted ratio of 1.21, n = 150) (p < 0.04). CYP2A6*23 trended towards a higher allele frequency in nonsmokers (3.1%, N = 9/286 alleles) compared to smokers (0.7%, N = 2/274 alleles) (p = 0.06).

Conclusions: These results suggest the novel CYP2A6*23 allele impairs enzyme function in vitro and in vivo and trends toward an association with lower risk of smoking.
**Introduction**

Nicotine is responsible for the majority of the psychoactive and highly addictive properties of cigarettes (2000). In humans, ~80% of absorbed nicotine is converted into cotinine and the majority of this reaction (~90%) is mediated by the hepatic enzyme cytochrome P450 2A6 (CYP2A6) (Benowitz and Jacob P 3rd, 1994; Messina, Tyndale et al., 1997). Cotinine is further metabolized to trans-3’-hydroxycotinine and this reaction is entirely mediated by CYP2A6 (Nakajima, Yamamoto T et al., 1996). CYP2A6 is also the main enzyme metabolizing therapeutic compounds such as coumarin (Pelkonen, Rautio et al., 2000), tegafur (Ikeda et al., 2000), and SM-12502 (Nunoya et al., 1996), and it can bioactivate tobacco smoke procarcinogens such as N’-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Yamazaki et al., 1992).

Large interindividual and interethnic differences in nicotine metabolism have been reported *in vitro* and *in vivo* (reviewed in (Malaiyandi, Sellers et al., 2005)). CYP2A6 is highly polymorphic, with 22 numbered alleles and numerous single nucleotide polymorphisms (SNPs) identified so far (http://www.cypalleles.ki.se/cyp2a6.htm). Characterization of CYP2A6 variants is needed because CYP2A6 polymorphisms altering nicotine metabolism may be important determinants of smoking behaviours. Several, but not all, studies have found that individuals with CYP2A6 alleles impairing enzyme activity are less likely to be current smokers (Schoedel, Hoffmann Eb et al., 2004; Malaiyandi, Sellers et al., 2005).

Differences in nicotine metabolism, smoking behaviours and incidence of tobacco-related illnesses have been observed between ethnic groups. Compared to Caucasians, individuals of Black-African descent have a significantly reduced fractional conversion of nicotine to cotinine, reduced metabolic clearance of nicotine to cotinine and higher plasma levels of cotinine for
similar cigarette consumption (Perez-Stable, Herrera et al., 1998; Benowitz, Perez-Stable et al., 1999). Together, this suggests reduced nicotine and cotinine metabolism, likely as a result of genetic variations in \textit{CYP2A6}. Interestingly, populations of Black-African descent are also characterized by unique smoking patterns. Despite having a similar prevalence of current smoking as Caucasians (Centers for Disease Control and Prevention, 2005), populations of Black-African descent report later ages of smoking initiation (Trinidad, Gilpin et al., 2004; Trinidad, Gilpin et al., 2004) and lower cigarette consumption (Centers for Disease Control and Prevention, 1998; National Survey on Drug Use and Health, 2006). Furthermore, populations of Black-African descent suffer from disproportionately higher rates of most tobacco-related illnesses (Centers for Disease Control and Prevention, 1998), such as lung cancer (National Center for Chronic Disease Prevention and Health Promotion, 1998; Haiman, Stram et al., 2006), despite their lower levels of smoking.

In this study, we identified a novel \textit{CYP2A6} allele (\textit{CYP2A6*23}) with a SNP at 2161C>T (GenBank accession number NG_000008.7) in exon 4 corresponding to an amino acid change R203C in a population of Black-African descent. We discovered this variant while designing a genotyping assay for the previously identified \textit{CYP2A6*16} allele (Kiyotani et al., 2002), which occurs at the same locus (2161C>A, R203S). Primers were designed for both the C>A and C>T nucleotide change as there had been initial confusion to the specific change found in \textit{CYP2A6*16}. The effect of \textit{CYP2A6*23} on enzyme function was determined \textit{in vitro} using an \textit{Escherichia coli} (\textit{E.coli}) heterologous expression system. We compared it to \textit{CYP2A6*16}, which encodes a different amino acid change at the same position, and to \textit{CYP2A6*17}, a previously characterized reduced-activity allele (Fukami et al., 2004). Furthermore, we examined the effect of \textit{CYP2A6*23} on nicotine metabolism \textit{in vivo} and its association with smoking status.
Materials and Methods

Participants

Adults of Black-African descent were recruited from Toronto, Ontario; detailed descriptions of the population and experimental protocol can be found elsewhere (Mwenifumbo, Sellers et al., 2007). Briefly, male and female current smokers and tried non-smokers were recruited. Smokers were defined as smoking $\geq 100$ lifetime cigarettes and currently smoking at least 5 days of the week while tried non-smokers had smoked 1 – 99 lifetime cigarettes but were never regular smokers. Plasma nicotine, cotinine (COT) and $\text{trans}$-3’-hydroxycotinine (3HC) levels were measured following administration of an oral nicotine dose (4 mg capsule). The 3HC/COT ratio, calculated from plasma levels collected at 270 minutes after dosing, has been previously verified as an indicator of CYP2A6 activity (Dempsey, Tutka et al., 2004). Caucasian (n = 167), Chinese (n = 144) and Japanese (n = 52) individuals recruited for a separate study on CYP2A6 genetic variation and smoking behaviours (Schoedel, Hoffmann Eb et al., 2004) were also genotyped for CYP2A6*23. The research protocol was approved and monitored by the University of Toronto Ethics Review Office and the Institutional Review Board Services (Aurora, ON).

Genotyping assay

Blood samples were stored at -20°C, and genomic DNA was extracted and stored at -20°C until use (GenElute Mammalian Genomic DNA Kit, Sigma-Aldrich Co., Mississauga, ON). A novel two-step allele specific polymerase chain reaction (PCR) assay was developed for CYP2A6*23. A fragment of CYP2A6 between intron 3 to intron 5 was amplified using the primers: forward (2A6exin3F) 5’ – GGC ACT GGC GGT GAG CAG – 3’ and reverse (2A6in5R) 5’ – GGC CTG TGT CAT CTG CCT – 3’. The reaction mixture contained: 50 ng of genomic DNA, 1X Taq buffer with KCl, 200 $\mu$M deoxyribonucleoside triphosphates (dNTPs), 1.5 mM MgCl2, 125 nM
of each primer, 1.25 U of *Taq* Polymerase (MBI Fermentas, Burlington, ON) and H₂O for a total volume of 25 μl. The reaction conditions were as follows: initial denaturation at 95°C for 1 min., denaturation at 95°C for 15 sec., annealing at 58°C for 30 sec., and extension at 72°C for 2 min. for 30 cycles, followed by final extension step at 72°C for 7 min. (MJ Research PCR Cycler PTC 200; Waltham, MA). The product from this first amplification served as the template for a second allele-specific reaction using the primers: forward (2A6in3F) 5’ – CTG CCT CCT GGA ATT CTG AC – 3’ and a reverse primer specific to 2161C (2A6ex42161AWR, 5’ – GGA AGA TTC CTA GCA TCA TGC G – 3’) or to 2161T (2A6ex42161AVR, 5’ – GGA AGA TTC CTA GCA TCA TGC A – 3’). The reaction mixture contained: 0.8 μl of template, 1X *Taq* buffer with (NH₄)₂SO₄, 1.0 mM of MgCl₂, 75 nM of each primer, 1.25 U of *Taq* Polymerase and H₂O for a total volume of 25 μl. The reaction conditions were: initial denaturation at 95°C for 1 min., denaturation at 95°C for 15 sec., annealing at 57°C for 10 sec., and extension at 72°C for 30 sec. for 20 cycles. The PCR products were separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Subjects had been genotyped for known *CYP2A6* variants (*CYP2A6*1B,*2,*4A & D, *9,*12,*14,*15,*17,*20,*21,*24,*25,*26,*27,*28,*29).

**DNA sequence analyses**

We confirmed the detection of *CYP2A6*23 by our genotyping assay, and determined its haplotype by sequencing all *CYP2A6* exons and exon-intron borders in two heterozygous individuals (*CYP2A6*1/*23) and a homozygous individual (*CYP2A6*23/*23). A 9.2 kb fragment spanning -1.4 kb upstream and 7.8 kb downstream of the +1 ATG start site of *CYP2A6* was amplified from genomic DNA using long-PCR. The primers for long-PCR were: forward
(2A65Pr1F) 5' – ACC TAG ACT TAA TCT TCC CGT ATA C – 3' and reverse (2A6R0) 5' –
AGG TCA TCT AGA TTT TCT CCT ACA – 3’. The product was subcloned into pCR®-XL-
TOPO plasmid (TOPO® XL PCR Cloning Kit, Invitrogen Canada Inc., Burlington, ON). DNA
sequencing was performed with an ABI 3730XL DNA analyzer at the Centre for Applied
Genomics (Toronto, ON).

**Construction of CYP2A6 expression plasmids**

The bicistronic construct with the full length cDNA of CYP2A6 and human NADPH-CYP
reductase (hNPR) inserted into the pCW expression vector (8537 bp) (Gillam et al., 1999) was
derived from the cognate monocistronic construct prepared by Soucek *et al.* (Soucek, 1999), in
which the native CYP2A6 sequence was changed to encode Ala at the second position with only
silent nucleotide changes elsewhere in the N-terminus. The SNPs 2161C>T (*CYP2A6*23),
2161C>A (*CYP2A6*16) and 5065G>A (*CYP2A6*17) were introduced into the expression
constructs using the QuikChange® II XL Site-Directed Mutagenesis Kits (Stratagene, La Jolla,
CA). Primers used in the mutagenesis reactions were: 2161C>T (5' – AGT TCC TGT CAC TGT
TG T GCA TGA TGC TAG GAA TC – 3', 5' – GAT TCC TAG CAT CAT GC TAA CAG
TGA CAG GAA CT – 3'), 2161C>A (5' – AGT TCC TGT CAC TGT TG A GCA TGA TGC
TAG GAA TC – 3', 5' – GAT TCC TAG CAT CAT GA TAA CAG TGA CAG GAA CT – 3'),
5065G>A (5' – ATC CAA AGA TTT GGA GAC ATG ATC CCC ATG AGT TTG G – 3', 5' –
CCA AAC TCA TGG GGA TCA TG T CTC CAA ATC TTT GGA T – 3'). A negative control
was created using *BamHI* to remove 802 bp from the 5’ end of the *CYP2A6* cDNA (total length
of 1485 bp). The products were separated by gel electrophoresis and the remaining plasmid
(7735 bp) was extracted and re-ligated. The variant constructs were confirmed by sequencing
using an ABI 3730XL DNA analyzer at the Centre for Applied Genomics (Toronto, ON).
Expression of CYP2A6 constructs in *E.coli*

The constructs encoding CYP2A6 and hNPR were expressed in *E.coli* as described previously (Pritchard Mp et al., 2006). DH5α cells (Invitrogen, Burlington, ON) were transformed with wildtype and variant constructs and a starter culture was grown in Luria-Bertani medium containing ampicillin (100 μg/ml) overnight at 37°C with shaking at 200 rpm. The starter culture was diluted (1:100) in 100 ml of terrific broth containing ampicillin (100 μg/ml), 1.0 mM thiamine, and 0.5 mM δ-aminolevulinic acid. The cultures were incubated at 30°C with shaking at 120 rpm for 4 – 6 hours, with induction initiated by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside. The cultures were incubated for a further 19 – 22 hours at 30°C and shaking at 120 rpm. Membrane fractions were prepared as described in (Pritchard Mp, Mclaughlin L et al., 2006) with minor modifications. Briefly, the pelleted bacteria were resuspended in buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4) and digested with lysozyme (0.25 mg/ml) for 60 min. The spheroplast was pelleted, sonicated, and centrifuged at 12,000 x g for 12 min. The supernatant was centrifuged for 110,000 x g for 90 min, and the pellet containing the membrane fraction was resuspended in 1.15% KCl and stored at -80°C until use. All constructs were initiated for expression concurrently and processed for immunoblotting and activity at the same time. Batch processing was performed to reduce construct to construct differences in degradation.

Immunoblotting

Total amount of membrane protein was measured by the Bradford protein assay (Bio-Rad Labs, Mississauga, ON) and the amount of CYP2A6 protein in the membrane preparations was determined by immunoblotting as previously described (Schoedel, Sellers et al., 2003). A
standard curve was constructed using CYP2A6 expressed by a baculovirus-infected insect cell system (BD Gentest, San Jose, CA). The bacteria membrane preparations and baculovirus-expressed CYP2A6 were serially diluted to establish linear range of detection.

**Enzyme assays for nicotine and coumarin**

Nicotine C-oxidation and coumarin 7-hydroxylation were determined as previously described (Nakajima, Yamamoto et al., 1996; Siu et al., 2006). Briefly, the reaction mixture contained 20 nM of CYP2A6, 20 nM of expressed cytochrome b5 (Invitrogen, Burlington, ON), 50 mM Tris-HCl buffer (pH 7.4) and substrate. Two substrate concentrations of nicotine (30 and 300 μM) or coumarin (5 and 50 μM) were initially used to screen for catalytic activity. Subsequently, full kinetic analyses were performed with nicotine, our main substrate of interest, ranging from 1 – 500 μM. Mouse liver cytosol (1.2 mg protein/ml) was added to the nicotine C-oxidation reaction as a source of aldehyde oxidase; this was added in excess so that CYP-mediated oxidation would be rate-limiting (Cashman et al., 1992; Siu, Wildenauer Db et al., 2006). The mixture was pre-warmed for 2 min. and the reaction was initiated by the addition of 1 mM NADPH. The mixture was incubated at 37°C for 45 min. for nicotine C-oxidation and 30 min. for coumarin 7-hydroxylation, and the reaction was stopped with 4% Na2CO3. The amount of cotinine and trans-3’-hydroxycotinine formed were detected by high-pressure liquid chromatography (HPLC) as previously described (Siu, Wildenauer Db et al., 2006).

To detect 7-hydroxycoumarin formation, 5 μl of 20% (w/v) of trichloroacetic acid was added to the samples. 10 μl of 4-hydroxycoumarin (1 mg/ml) was also added as an internal standard. The samples were centrifuged at 13,000 rpm for 10 min. and 100 μl of the supernatant was analyzed by HPLC. Coumarin and its metabolite 7-hydroxycoumarin were separated on the ZORBAX SB
C18 Column (250 × 4.6 mm I.D.; particle size, 5 μm) from Agilent Technologies Inc. (Mississauga, ON) at a flow-rate of 1 ml/min., with a mobile phase of acetonitrile, water and acetic acid (25 : 75 : 0.1, v/v) and detected at 315 nm. The concentrations of coumarin and 7-hydroxycoumarin were determined from standard curves created by spiking drug-free bacterial membrane preparations with known amounts of coumarin (0.2 – 10 μg/ml) and 7-hydroxycoumarin (50 – 1000 ng/ml). The within-day precisions were 2.4 – 12.8% for coumarin and 2.6 – 8.3% for 7-hydroxycoumarin, and the between-day precisions were 4.2 – 7.7% for coumarin and 5.9 – 13.7% for 7-hydroxycoumarin.

**Bioinformatics**

Linkage disequilibrium of CYP2A6*23 to other genotyped CYP2A6 variants was determined using Haploview (version 3.32) (Barrett et al., 2005). We used SIFT and PMUT, bioinformatics programs that predict whether nonsynonymous SNPs will affect enzyme function or structure based on physicochemical properties and evolutionary conservation of the amino acid changes, to examine how CYP2A6*23 may be affecting enzyme function (Ng et al., 2001; Ferrer-Costa et al., 2005).

**Statistics**

The catalytic activities of the in vitro expressed wildtype and variant enzymes were compared using one-way ANOVA with the Bonferroni correction for post-hoc analyses. In ethnic groups where the allele frequency of CYP2A6*23 was zero, the score method was used to calculate confidence intervals (Wilson, 1927). A comparison of the CYP2A6*23 allele frequencies between ethnic groups, differences in the distribution of the CYP2A6*23 allele in nonsmokers versus smokers, and the Hardy-Weinberg equilibrium were calculated using Fisher’s Exact Test. Consistent with previous studies, gender (Benowitz, Lessov-Schlaggar Cn et al., 2006) and
smoking status (Benowitz and Jacob P 3rd, 1993; Benowitz and Jacob, 2000) were found to affect the rate of nicotine metabolism in our population of Black-African descent, as indicated by the 3HC/COT ratio (Mwenifumbo, Sellers et al., 2007). Thus, the metabolic ratio was adjusted by dividing the value from each individual with the overall mean from their respective group (e.g. female nonsmokers, male nonsmokers, female smokers, male smokers). The effect of genotype on the adjusted 3HC/COT ratio was examined by a two-tailed independent t-test. A multiple linear regression model was also used to examine the impact of \textit{CYP2A6*23} genotype on the unadjusted 3HC/COT ratio while controlling for the effect of smoking status and gender. The unadjusted 3HC/COT ratio was not normally distributed according to the Kolmogorov-Smirnov test and was log-transformed for the regression model. All statistical analyses were performed using SPSS (Windows version 14.0) and GraphPad Prism (Windows version 2.0).

\textbf{Results}

\textit{CYP2A6*23 was found in populations of Black-African descent}

Among the individuals of Black-African descent genotyped for \textit{CYP2A6*23} (n = 280), nine heterozygous and one homozygous individuals were found. Thus, \textit{CYP2A6*23} occurred at an allele frequency of 2.0\% (95\% confidence interval 0.8 – 3.1\%, Table 1); genotype frequencies did not deviate from Hardy-Weinberg equilibrium ($p = 0.821$). Five of the ten individuals with \textit{CYP2A6*23} had other \textit{CYP2A6} variants (Table 2); \textit{CYP2A6*23} was not found in linkage disequilibrium with any of the other \textit{CYP2A6} variants genotyped in this population when analyzed with Haploview (version 3.32) (Barrett, Fry B et al., 2005), and no other nonsynonymous SNPs were found in linkage with \textit{CYP2A6*23} in the samples sequenced. \textit{CYP2A6*23} was not detected in Caucasian (N = 334 alleles), Chinese (N = 288 alleles) or Japanese individuals (N = 104 alleles) (Table 1). The allele frequency of \textit{CYP2A6*23} in
individuals of Black-African descent significantly differed from Caucasians and Chinese \((p < 0.05, \text{Table 1})\).

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Allele frequency (%)</th>
<th>Total # of alleles</th>
<th>95% Confidence Intervals</th>
<th>p-values (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black-African descent</td>
<td>2.0</td>
<td>560</td>
<td>0.8 – 3.1%</td>
<td>---</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0</td>
<td>334</td>
<td>0 – 1.1%</td>
<td>0.01</td>
</tr>
<tr>
<td>Chinese</td>
<td>0</td>
<td>288</td>
<td>0 – 1.3%</td>
<td>0.02</td>
</tr>
<tr>
<td>Japanese</td>
<td>0</td>
<td>104</td>
<td>0 – 3.6%</td>
<td>0.23</td>
</tr>
</tbody>
</table>

\(^a\) The allele frequency of \(CYP2A6*23\) in each ethnic group was compared against the value found in individuals of Black-African descent.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Genotype</th>
<th>n</th>
<th>Mean adjusted 3HC/COT</th>
<th>SD</th>
<th>% of wildtype</th>
<th>p-values (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*23</td>
<td>*1/*1</td>
<td>150</td>
<td>1.210</td>
<td>0.634</td>
<td>100</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>*1/*23</td>
<td>4</td>
<td>0.756</td>
<td>0.540</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*23/*23</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>*17</td>
<td>*1/*1</td>
<td>150</td>
<td>1.210</td>
<td>0.634</td>
<td>100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>*1/*17</td>
<td>19</td>
<td>0.672</td>
<td>0.468</td>
<td>55.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*17/*17</td>
<td>3</td>
<td>0.109</td>
<td>0.095</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>&gt;1 variant</td>
<td>*1/*1</td>
<td>150</td>
<td>1.210</td>
<td>0.634</td>
<td>100</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>*17/*23</td>
<td>2</td>
<td>0.259</td>
<td>0.366</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*20/*23</td>
<td>2</td>
<td>0.555</td>
<td>0.512</td>
<td>45.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*9/*23</td>
<td>1</td>
<td>1.032</td>
<td>0</td>
<td>85.2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The adjusted 3HC/COT ratio was compared between wildtype individuals \((CYP2A6*1/*1)\) to other genotype groups combined using a two-tailed independent t-test.
CYP2A6*23 reduced enzyme activity towards nicotine and coumarin in vitro

The specific content of CYP2A6 was similar between CYP2A6.1 (0.11 pmol CYP2A6/µg membrane protein), CYP2A6.16 (0.12 pmol CYP2A6/µg of membrane protein) and CYP2A6.17 (0.12 pmol CYP2A6/µg membrane protein), although CYP2A6.23 was expressed at lower levels (0.07 pmol CYP2A6/µg membrane protein) (Fig. 1A). The negative construct did not produce any detectable CYP2A6 protein. Subsequent enzyme assays with the wildtype and variant constructs were performed using equivalent amounts of CYP2A6 (20 nM).

We initially screened the activities of the wildtype and variant constructs towards nicotine and coumarin at two concentrations (Fig. 1B, C). There was a significant difference in activities of the constructs at 30 µM of nicotine ($F = 29.0, p < 0.001$), 300 µM of nicotine ($F = 35.9, p < 0.001$), and 50 µM of coumarin ($F = 28.3, p < 0.01$). Both CYP2A6.23 and CYP2A6.17 had significantly lower activity towards nicotine compared to CYP2A6.1 at 30 µM ($p < 0.01$) and 300 µM ($p < 0.001$) of substrate. CYP2A6.23 also had significantly reduced activity towards 50 µM of coumarin ($p < 0.01$) while CYP2A6.17 retained similar activity as the wildtype enzyme. CYP2A6.16 had similar activities as CYP2A6.1 towards nicotine and coumarin at both concentrations tested (Fig. 1B, C).

We then performed full kinetic analyses on nicotine, our main substrate of interest. CYP2A6.23 and CYP2A6.17 had reduced activity towards nicotine in vitro while CYP2A6.16 had similar activity as CYP2A6.1 (Fig. 2). The apparent $K_m$ values did not significantly differ between the wildtype and variant constructs ($F = 3.0, p = 0.083$), though it trended towards higher values for CYP2A6.17 ($p = 0.09$, Table 3). However, there was a significant difference in $V_{max}$ ($F = 35.2, p < 0.001$) and $V_{max}/K_m$ ($F = 19.0, p < 0.001$) between the constructs. $V_{max}$ was significantly
lower for CYP2A6.23 ($p < 0.001$) and CYP2A6.17 ($p < 0.01$) compared to CYP2A6.1. Likewise, $V_{\text{max}}/K_{\text{m}}$ was significantly lower for CYP2A6.23 ($p < 0.001$) and CYP2A6.17 ($p < 0.01$) compared to CYP2A6.1. There was no significant difference in $V_{\text{max}}$ and $V_{\text{max}}/K_{\text{m}}$ between CYP2A6.23 and CYP2A6.17, or between CYP2A6.16 and CYP2A6.1.
Figure 1: CYP2A6.23 had substantially reduced catalytic activity towards nicotine and coumarin in vitro. A) Immunoblot shows the expression of CYP2A6 protein from the wildtype and variant constructs. The amount of total membrane protein loaded is as labeled and ranged from 0.5 – 2.0 μg for CYP2A6.1, CYP2A6.16 and CYP2A6.17, while CYP2A6.23 was loaded at 0.75 – 4.0 μg. Detection of CYP2A6 constructs was linear by immunoblotting at lower amounts loaded, and was used to calculate the amount of CYP2A6 detected by comparison to the standard (not shown). B) CYP2A6.17 and CYP2A6.23 have reduced cotinine formation from nicotine (30 and 300 μM) while CYP2A6.16 had similar activity as CYP2A6.1. C) CYP2A6.23 had reduced 7-hydroxycoumarin formation from coumarin (5 and 50 μM) while CYP2A6.16 and CYP2A6.17 had similar activity as CYP2A6.1. No product formation was found using the negative construct. The data are presented as mean ± SEM for nicotine C-oxidation (n = 3) and coumarin 7-hydroxylation (n = 2). *p < 0.01, **p < 0.001 when compared to CYP2A6.1.
Figure 2: CYP2A6.17 and CYP2A6.23, but not CYP2A6.16, have reduced \textit{in vitro} nicotine C-oxidation. A representative plot is shown, and the curve was fitted to the Michaelis-Menten equation using non-linear regression in GraphPad Prism (version 2.0).

Table 3: Kinetic parameters of CYP2A6 wildtype and variant constructs for nicotine

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol•min$^{-1}$•pmol CYP2A6$^{-1}$)</th>
<th>$V_{max}/K_m$ (nL•min$^{-1}$•pmol CYP2A6$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6.1</td>
<td>58.3 ± 6.5</td>
<td>5.5 ± 0.5</td>
<td>97.3 ± 11.6</td>
</tr>
<tr>
<td>CYP2A6.16</td>
<td>73.6 ± 6.1</td>
<td>5.1 ± 0.2</td>
<td>71.4 ± 7.7</td>
</tr>
<tr>
<td>CYP2A6.17</td>
<td>88.9 ± 6.8</td>
<td>2.6 ± 0.2 *</td>
<td>29.6 ± 4.2 *</td>
</tr>
<tr>
<td>CYP2A6.23</td>
<td>77.7 ± 10.4</td>
<td>1.4 ± 0.2 **</td>
<td>18.4 ± 2.3 **</td>
</tr>
</tbody>
</table>

$^a$Kinetic parameters were calculated using non-linear regression in GraphPad Prism (version 2.0). Data are presented as mean ± SEM of parameters calculated from three or four independent experiments. * $p < 0.01$, ** $p < 0.001$ when compared to CYP2A6.1.
**CYP2A6*23 decreased the rate of nicotine metabolism in vivo in a population of Black-African descent**

The 3HC/COT is a validated phenotypic measure of CYP2A6 activity and rates of nicotine metabolism (Dempsey, Tutka et al., 2004), with previous studies finding a significant association between the ratio and CYP2A6 genotype (Benowitz, Swan et al., 2006; Malaiyandi, Goodz et al., 2006; Malaiyandi, Lerman et al., 2006). Because the 3HC/COT ratio did not differ significantly from CYP2A6*1/*1 individuals in this population of Black-African descent, the wildtype group (*1/*1) included individuals with the CYP2A6*1B allele. The adjusted 3HC/COT ratio was significantly lower in individuals with at least one CYP2A6*23 allele and no other variants (n = 5) in comparison to CYP2A6*1/*1 individuals (n = 150) (p < 0.04, Fig. 3). A gene-dose effect was observed such that CYP2A6*1/*23 heterozygous individuals had ~40% loss in enzyme activity compared to CYP2A6*1/*1 individuals, while one CYP2A6*23/*23 homozygous individual did not produce any 3HC (Fig. 3). A similar impact of CYP2A6*23 was observed in a multivariate linear regression model with the log(3HC/COT) as the dependent variable and including smoking status and gender as predicting variables (R² = 0.355, p < 0.04). It is notable that the five individuals with CYP2A6*23 in addition to other genetic variants also had significantly lower 3HC/COT ratios compared to the wildtype group (p < 0.02, Table 2).

**CYP2A6*23 was associated with a lower likelihood of being a current adult smoker**

CYP2A6*23 trended towards a higher frequency in nonsmokers (3.1%, N = 9/286 alleles) compared to smokers (0.7%, N = 2/274 alleles) (p = 0.06, Fig. 4). Individuals with CYP2A6*23 were approximately 4 – 5 times less likely to be smokers when all individuals with CYP2A6*23 were taken into account (odds ratio = 0.23). The magnitude of the effect was the same when analyses were restricted to individuals with only CYP2A6*1/*1, CYP2A6*1/*23, and CYP2A6*23/*23 (odds ratio = 0.18, p = 0.11).
Figure 3: *CYP2A6*23 decreased the rates of nicotine metabolism *in vivo*, as measured by the 3HC/COT ratio. The number of individuals in each genotype group is shown in parentheses on the x-axis. The data are presented as mean ± SD. Individuals with other *CYP2A6* genetic variants (*CYP2A6*2, *4A & D, *9, *12, *14, *15, *17, *20, *21, *24, *25, *26, *27, *28, *29) were excluded from the wildtype and *CYP2A6*23 genotype groups.

Figure 4: Individuals with the *CYP2A6*23 allele trended to having a lower likelihood of being current smokers. The allele frequency of *CYP2A6*23 was calculated from the 280 genotyped individuals in the study, including 143 nonsmokers and 137 smokers.
Discussion

We have identified a novel CYP2A6 allele, CYP2A6*23, which is found in populations of Black-African descent but not in Caucasians, Chinese and Japanese. Two recently described alleles, CYP2A6*17 and CYP2A6*20, have also been identified exclusively in this population (Fukami, Nakajima et al., 2004; Fukami et al., 2005). These genetic variants may help explain the reduced rates of nicotine metabolism in individuals of Black-African descent compared to Caucasians (Perez-Stable, Herrera et al., 1998; Benowitz, Perez-Stable et al., 1999).

We have demonstrated that CYP2A6*23 (2161C>T, R203C) impairs both nicotine C-oxidation and coumarin 7-hydroxylation in vitro. CYP2A6.23 had a significantly reduced V_max towards nicotine, and the intrinsic clearance (V_max/K_m) was reduced to 19% of the wildtype enzyme. This is in agreement with the observation that in vivo, the 3HC/COT ratio is reduced in individuals with the CYP2A6*23 allele. CYP2A6*23 may be affecting enzyme function through alteration in substrate binding. Molecular modeling indicates Arg203 may be important in the orientation of Phe209, a residue critical for coumarin binding and possibly involved in nicotine binding (Lewis et al., 1999; Kiyotani et al., Oct 23-27, 2005). In addition, CYP2A6.23 had a lower level of expression in vitro compared to the other constructs used in this study, which may also contribute to the lower activity observed in vivo.

In contrast to CYP2A6*23 (2161C>T, R203C), CYP2A6*16 (2161C>A, R203S) did not appear to affect enzyme function. Our in vitro data suggest CYP2A6.16 has similar rates of nicotine and coumarin metabolism as the wildtype enzyme. Furthermore, Nakajima et al. recently reported that CYP2A6*16 did not affect rates of nicotine metabolism in vivo (Nakajima, Fukami et al., 2006). These data suggest the functional impact of CYP2A6*23 (Cys203) differs from that of CYP2A6*16 (Ser203). The wildtype residue (Arg203) is positively charged and hydrophilic.
while Cys203 is neutral and hydrophobic and Ser203 is neutral and hydrophilic. Furthermore, the bioinformatics program SIFT and PMUT (Ng and Henikoff, 2001; Ferrer-Costa, Gelpi et al., 2005) predicted the amino acid change in CYP2A6*23 as not tolerated while CYP2A6*16 was predicted to be benign.

Interestingly, CYP2A6*23 impaired nicotine C-oxidation to at least the same extent as CYP2A6*17 both in vitro and in vivo. Similar to expressed CYP2A6.23, CYP2A6.17 had a significantly reduced $V_{\text{max}}$ towards nicotine, with $V_{\text{max}}/K_{m}$ reduced to 30% of the wildtype enzyme. This is in agreement with a previous study where CYP2A6.17 expressed in E.coli did not alter $K_{m}$ but reduced $V_{\text{max}}$ towards nicotine, with $V_{\text{max}}/K_{m}$ reduced to 40% of the wildtype construct (Fukami, Nakajima et al., 2004). In vivo, the 3HC/COT ratio in heterozygous individuals for CYP2A6*17 is reduced to approximately 55% of wildtype activity, while homozygous individuals of CYP2A6*17 had approximately 9% of wildtype activity. This is consistent with previous studies using the COT/NIC ratio as a biomarker (Nakajima, Fukami et al., 2006), and together these data strongly suggest the CYP2A6*17 allele results in reduced nicotine metabolism. We also observed that the impact of CYP2A6.17 is substrate-dependent such that it did not differ in coumarin 7-hydroxylation compared to the wildtype enzyme. Accordingly, a previous study found CYP2A6.17 expressed in E.coli had an increased $K_{m}$ but no change in $V_{\text{max}}$ towards coumarin (Fukami, Nakajima et al., 2004).

A trend was observed where individuals with CYP2A6*23 were less likely to be current adult smokers. Several case-control studies have associated CYP2A6 genetic variations leading to impaired activity with a lower likelihood of smoking (Pianezza, Sellers et al., 1998; Tyndale et al., 2001; Schoedel Ka et al., 2004), though negative findings have also been reported (Tan et al., 2001; Zhang, Amemo K et al., 2001; Ando, Hamajima et al., 2003). A greater understanding of
CYP2A6 genetic variation through identification and characterization of novel variants, particularly among different ethnic groups, will allow for better replication of genetic case-control association studies.

In summary, we have discovered a novel CYP2A6 allele, occurring predominantly in a population of Black-African descent, which impairs enzyme activity in vitro and in vivo and may be associated with a lower risk of smoking. An understanding of the genetic factors that contribute to nicotine pharmacokinetics in populations of Black-African descent is important given their unique smoking patterns and higher incidence of tobacco-related illnesses.

**Significance to thesis**

In this chapter, a new CYP2A6 genetic variant was identified among individuals of Black-African descent that reduces enzyme activity in vitro and in vivo. Much progress has been made in recent years towards our characterization of CYP2A6 genetic variants, particularly among populations of Black-African descent. In addition to CYP2A6*23, our group identified and determined the functional impact of six new CYP2A6 alleles consisting of nonsynonymous SNPs that were found predominantly in populations of Black-African descent. Many of these alleles reduced or completely abolished enzyme function (Appendix C) (Mwenifumbo et al., 2008; Ali Koudsi et al., 2009; Mwenifumbo et al., 2010).

This chapter adds to our current understanding of the genetic factors that contribute to the observed variability in rates of nicotine metabolism among populations of Black-African descent. Identification of CYP2A6 genetic variants that are detrimental to enzyme function will help explain the slower rates of nicotine and cotinine clearance observed in this population (Benowitz, Perez-Stable et al., 1999). In addition, determining the functional impact of CYP2A6
genetic variants will help reduce the heterogeneity of assigned \textit{CYP2A6} genotype groups for genetic association studies of smoking behaviours performed in populations of Black-African descent. This will improve our ability to replicate and interpret the results from these studies, allowing us to gain a better understanding of how genetic variability in \textit{CYP2A6} influences smoking behaviours in this group.
CHAPTER 2: ASSOCIATION OF NICOTINE METABOLITE RATIO AND CYP2A6 GENOTYPE WITH SMOKING CESSATION TREATMENT IN AFRICAN-AMERICAN LIGHT SMOKERS

Man Ki Ho, Jill C. Mwenifumbo, Nael Al Koudsi, Kolawole S. Okuyemi, Jasjit S. Ahluwalia, Neal L. Benowitz, Rachel F. Tyndale


Drs. Kolawole Okuyemi, Jasjit Ahluwalia, Neal Benowitz and Rachel Tyndale designed and recruited the participants in this clinical trial with the primary aim of testing the efficacy of 2 mg nicotine gum and two different counseling methods for smoking cessation in a population of African-American light smokers. The levels of nicotine, cotinine and trans-3-hydroxycotinine in plasma samples were determined in the laboratory of Dr. Neal Benowitz. MKH performed a substantial portion of the DNA extraction and CYP2A6 genotyping, with the assistance of Dr. Jill Mwenifumbo, Dr. Nael Al Koudsi, Ewa Hoffmann, and Qian Zhou. MKH also identified the key research questions to be addressed, performed all of the statistical analyses and wrote the manuscript.
Abstract
Cytochrome P450 2A6 (CYP2A6) is the main nicotine metabolizing enzyme in humans. We investigated the relationships between CYP2A6 genotype, baseline plasma 3HC/COT (a phenotypic marker of CYP2A6 activity), and smoking behaviours in African-American light smokers. Cigarette consumption, age of initiation, and dependence scores did not differ between 3HC/COT quartiles or CYP2A6 genotype groups. Slow metabolizers (both genetic and phenotypic) had significantly higher plasma nicotine levels suggesting cigarette consumption was not reduced to adjust for slower rates of nicotine metabolism. Individuals in the slowest 3HC/COT quartile had higher quit rates with both placebo and nicotine gum treatments (OR 1.85, 95% CI 1.08-3.16, p = 0.03). Similarly, the slowest CYP2A6 genotype group had higher quit rates, although this did not reach significance (OR 1.61, 95% CI 0.95-2.72, p = 0.08). 3HC/COT ratio, and possibly CYP2A6 genotype, may be useful in the future for personalizing the choice of smoking cessation treatment for African-American light smokers.

Introduction
While overall smoking rates in North America have declined considerably, there are a growing number of smokers who maintain low levels of cigarette consumption. Light smoking is more prevalent particularly among adolescents, females and some racial/ethnic minority groups. For example, the majority of African-Americans consume ≤10 cigarettes per day (CPD) (Kandel et al., 2000), yet they report high levels of dependence and have difficulty quitting (Centers for Disease Control and Prevention, 1993; Royce, Hymowitz et al., 1993). Further, African-Americans have disproportionately higher incidences of tobacco-related illnesses such as lung cancer (Centers for Disease Control and Prevention, 1998; Haiman, Stram et al., 2006), which may be partly explained by their greater use of mentholated cigarettes with higher nicotine and tar yields (Us Department of Health and Human Services, 1998), deeper inhalation patterns
(Clark, Gautam et al., 1996), and altered rates of nicotine and nitrosamine metabolism (Benowitz, Perez-Stable et al., 1999; Muscat et al., 2005).

Nicotine is primarily responsible for the highly addictive properties of tobacco smoke (Benowitz, 1999). The majority (~80%) of nicotine (NIC) is inactivated to cotinine (COT) (Benowitz and Jacob P 3rd, 1994), and ~90% of this reaction is mediated by the hepatic enzyme cytochrome P450 2A6 (CYP2A6) (Messina, Tyndale et al., 1997). Cotinine is further metabolized to trans-3'-hydroxycotinine (3HC) primarily by CYP2A6 (Nakajima, Yamamoto T et al., 1996; Dempsey, Tutka et al., 2004). In addition, CYP2A6 can bioactivate tobacco-specific precarcinogens including (methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) (Yamazaki, Inui et al., 1992).

The gene encoding CYP2A6 is highly polymorphic, with 36 numbered alleles and numerous single nucleotide polymorphisms (SNPs) identified so far (http://www.cypalleles.ki.se/cyp2a6.htm). Large inter-ethnic and inter-individual variations in CYP2A6 activity have been reported (Mwenifumbo and Tyndale, 2007). This has been mainly attributed to genetic variations in CYP2A6, although environmental influences (e.g. diet) may also contribute through enzyme induction or inhibition. For example, estrogen has been found to induce CYP2A6 in vitro (Higashi, Fukami et al., 2007), and females have faster rates of in vivo nicotine metabolism compared to males (Benowitz, Lessov-Schlaggar Cn et al., 2006).

Since dependent smokers regulate the amount smoked to maintain plasma and brain nicotine levels (Mcmorrow and Foxx Rm, 1983), variations in CYP2A6 activity is predicted to alter smoking behaviors. Individuals with CYP2A6 alleles encoding enzymes with decreased- or loss-of-function metabolize nicotine at a slower rate (Rao, Hoffmann et al., 2000; Benowitz, Swan et
al., 2006; Mwenifumbo, Al Koudsi et al., 2008), and have been associated with lower risk of being a smoker in adults (Schoedel, Hoffmann Eb et al., 2004), lower cigarette consumption (Rao, Hoffmann et al., 2000; Schoedel, Hoffmann Eb et al., 2004), reduced smoking intensity (Rao, Hoffmann et al., 2000; Strasser, Malaiyandi et al., 2007), and reduced withdrawal symptoms during abstinence (Kubota, Nakajima-Taniguchi et al., 2006). In addition, CYP2A6 slow metabolizers achieved higher quit rates in clinical trials (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Schnoll et al., 2009), were more likely to be former smokers in a case-control study (Gu, Hinks et al., 2000), and had shorter durations of smoking suggestive of greater quit success (Schoedel, Hoffmann Eb et al., 2004). However, these studies have been mainly in heavy smoking individuals of European- or Asian-ancestry. There is a paucity of research on the role of CYP2A6 variation and smoking in light smoking populations such as African-Americans. Interestingly, African-Americans have slower rates of nicotine and cotinine metabolism compared to European-Americans (Benowitz, Perez-Stable et al., 1999), and our lab recently identified novel CYP2A6 variants in this population that are associated with lower enzyme activity (Mwenifumbo, Al Koudsi et al., 2008). It follows that variation in CYP2A6 activity may also contribute to the unique smoking patterns observed in this group.

The large variation in CYP2A6 activity and numerous CYP2A6 alleles indicates the need for a phenotypic measure for population studies. The 3HC/COT ratio has been validated as a phenotypic marker of CYP2A6 activity as the conversion of COT to 3HC is specific to CYP2A6 (Nakajima, Yamamoto T et al., 1996; Dempsey, Tutka et al., 2004). The ratio of these nicotine metabolites is highly correlated with rates of nicotine clearance (r = 0.70 - 0.95) (Dempsey, Tutka et al., 2004), and has been associated with CYP2A6 genotype in several studies (Johnstone, Benowitz et al., 2006; Malaiyandi, Goodz et al., 2006; Mwenifumbo, Al Koudsi et al., 2008). However, these studies have been primarily in heavy smoking individuals of European-ancestry,
and it is unknown whether the 3HC/COT ratio derived from baseline ad libitum smoking will also be a good phenotypic measure of CYP2A6 activity in African-American light smokers where smoking can be both low and irregular in frequency.

Few smoking cessation clinical trials have focused on African-Americans as treatment studies typically exclude light smokers. A recent study, entitled Kick it at Swope-II (KIS-II), evaluated the efficacy of nicotine replacement therapy (2 mg nicotine gum vs. placebo) and counseling sessions (health education (HE) vs. motivational interviewing (MI)) in 755 African-American light smokers (Ahluwalia, Okuyemi et al., 2006). Nicotine gum did not increase quit rates compared to placebo at week 26 follow-up, although those receiving HE were significantly more likely to quit compared to those receiving MI. Thus, further investigations of the biological factors underlying smoking behaviors in light smoking populations are warranted.

Here, we first investigated whether CYP2A6 genotype is related to the 3HC/COT ratio derived from baseline smoking in treatment seeking African-American light smokers enrolled in KIS-II. We then determined whether CYP2A6 variation, as indicated by genotype and the 3HC/COT ratio, is associated with baseline smoking behaviors and treatment outcomes. This is the largest study to date examining the role of CYP2A6 variation in smoking behaviors among African-American light smokers, with the additional benefit of having genetic and phenotypic measures available.

**Materials and Methods**

**Study design**

A detailed description of the study design and participant recruitment has been described elsewhere (Ahluwalia, Okuyemi et al., 2006). Briefly, individuals (n = 755) self-identified as
“African-American” or “Black” were randomized into a double-blind, placebo-controlled study at a community health center in Kansas City, Missouri. This study consisted of four treatment arms (n ~189 each) of either placebo or nicotine gum (2 mg), along with either HE or MI counseling. Inclusion criteria included: ≥18 years of age, smoked ≤10 CPD for at least 6 months prior to enrolment, smoked at least 25 of the last 30 days, and interested in quitting smoking in the next 2 weeks.

Drug treatment continued for 8 weeks and the dosing regimen was based on the participant’s baseline cigarette consumption as described previously (Ahluwalia, Okuyemi et al., 2006). Six counseling sessions were provided, and all participants were followed for a total of 26 weeks. The research protocol was approved and monitored by the University of Toronto Ethics Review Office and the University of Kansas Human Subjects Committee.

Measurements

Demographic information, smoking history, and severity of nicotine dependence were collected at randomization. Plasma sample was also taken at randomization and the levels of NIC, COT and 3HC from baseline smoking were determined by methods described previously (Dempsey, Tutka et al., 2004).

CYP2A6 genotyping assays

Blood samples for genetic analyses were available from 618 of the 755 participants who consented to genetic testing. Established CYP2A6 alleles (CYP2A6*1B, *2, *4, *9, *12, *17, *20) and novel alleles that were recently identified in a population of Black-African descent (CYP2A6*23, *24, *25, *26, *27, *28, *35) were genotyped using a two-step allele-specific polymerase chain reaction assay as previously described (Goodz et al., 2002; Ho et al., 2008;...
Mwenifumbo Jc, Al Koudsi N et al., 2008; Al Koudsi, Ahluwalia Js et al., 2009). The genotyping assay for *CYP2A6*<sup>1B</sup> was revised because SNPs occurring at a high frequency in this population were found at the location of the primers used in the previous assay (Mwenifumbo et al., 2010). An assay that detects *CYP2A6*<sup>4A</sup> and *4D*, as well as the recently identified gene deletions *CYP2A6*<sup>4E – H</sup>, will be reported elsewhere (Mwenifumbo, Zhou et al., 2010).

**Statistical analyses**

The 3HC/COT ratio was not normally distributed and was log-transformed for all statistical analyses. Chi-squared test was used to test for Hardy-Weinberg equilibrium, to examine differences in allele frequencies in this sample compared to previously reported values in a population of Black-African descent, and to test for differences in quit rates by CYP2A6 activity groups. Univariate ANOVA was used to assess differences in 3HC/COT between *CYP2A6* genotype groups, and to test the association of CYP2A6 activity with CPD, CO levels, nicotine plasma levels, age of regular smoking, and dependence scores. Univariate ANOVA was also used to examine if differences in baseline variables exists between the total participants and those with 3HC/COT data, genotype data, and both 3HC/COT and genotypes. Bonferroni correction was used for post-hoc analyses. Multiple logistic regression models were used to assess the association of *CYP2A6* genotype and 3HC/COT quartiles on quit rates at EOT and follow-up. All statistical analyses of treatment effects were performed on an intent-to-treat basis and those lost during follow-up were counted as smokers.
Results

Participant characteristics

There was no significant difference in participant characteristics (gender, age, body mass index (BMI), smoking mentholated cigarettes, CPD, exhaled carbon monoxide (CO), baseline plasma NIC and COT, age of regular smoking, or dependence severity) between the total population (n = 755), the subset of participants with 3HC/COT data (n = 646), those with genotype data (n = 588), and those with both genotype and 3HC/COT data (n = 495) (Supplementary Table 1, pg. 126).

Variables that influence the 3HC/COT ratio

To examine other factors that may influence the 3HC/COT ratio independent of CYP2A6 genetic variation, an analysis was performed including only CYP2A6*1/*1 individuals (n = 246). The 3HC/COT ratio was significantly higher in females, increased with age, and was higher in those with lower BMI (Table 1). Individuals who smoked mentholated cigarettes also had significantly lower 3HC/COT ratios (p < 0.05). In a multivariate regression model, gender, age and BMI remained independently associated with the 3HC/COT ratio (p < 0.05, Table 1).

CYP2A6 genotypes and associations with 3HC/COT

CYP2A6*1B has been associated with faster enzyme activity in European-Americans (Mwenifumbo et al., 2007), although no difference was observed previously in individuals of Black-African descent (Mwenifumbo Jc, Al Koudsi N et al., 2008). We used a newly revised CYP2A6*1B genotyping assay to account for SNPs occurring at a high frequency in this population that confounded our previous assay (Mwenifumbo, Zhou et al., 2010), and found individuals with CYP2A6*1B had significantly higher 3HC/COT ratios even after controlling for gender, age and BMI as covariates (F(2, 238) = 5.1, p < 0.01, Table 2).
Table 1: Factors that influence the 3HC/COT in CYP2A6*1/*1 individuals (n = 246)

<table>
<thead>
<tr>
<th></th>
<th>Mean 3HC/COT</th>
<th>SD</th>
<th>N</th>
<th>p – value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analyses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.37</td>
<td>0.19</td>
<td>79</td>
<td>0.02</td>
</tr>
<tr>
<td>Females</td>
<td>0.46</td>
<td>0.32</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 – 29</td>
<td>0.35*</td>
<td>0.20</td>
<td>22</td>
<td>0.002</td>
</tr>
<tr>
<td>30 – 39</td>
<td>0.37*</td>
<td>0.20</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>40 – 49</td>
<td>0.43</td>
<td>0.24</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>50 – 59</td>
<td>0.48</td>
<td>0.35</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>60 – 77</td>
<td>0.63</td>
<td>0.43</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.22</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 29.7)</td>
<td>0.46</td>
<td>0.3</td>
<td>122</td>
<td>0.05</td>
</tr>
<tr>
<td>High (≥ 29.7)</td>
<td>0.40</td>
<td>0.3</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>-0.16</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Smoke mentholated cigarettes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.41</td>
<td>0.27</td>
<td>190</td>
<td>0.02</td>
</tr>
<tr>
<td>No</td>
<td>0.50</td>
<td>0.31</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td><strong>Multivariate regression analyses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.15</td>
<td>0.005 – 0.044</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.24</td>
<td>0.001 – 0.002</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>-0.16</td>
<td>-0.003 – 0.000</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Mentholated cigarettes</td>
<td>-0.08</td>
<td>-0.036 – 0.008</td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>

1 BMI was categorized by the median value of 29.7. r = Spearman’s correlation coefficient between BMI and 3HC/COT. BMI data were not available for two participants.

2 R² = 0.12. Age and BMI were included as continuous variables.

* denotes significant difference (p < 0.05) from the oldest age group (60 – 77). r = Spearman’s correlation coefficient between age and 3HC/COT.
To examine the functional impact of other CYP2A6 variants, individuals with CYP2A6*1B were included in the wildtype reference group, as this allows us to compare our results with those reported previously (Mwenifumbo Jc, Al Koudsi N et al., 2008), and this genotype was not associated here with altered baseline smoking variables or treatment outcomes.

Consistent with earlier studies (Benowitz, Swan et al., 2006; Mwenifumbo Jc, Al Koudsi N et al., 2008), heterozygous individuals with established alleles, CYP2A6*4, *17 and *20, had ~40 – 60% activity remaining, while heterozygous individuals with CYP2A6*9 and *12 had ~50 – 75% activity remaining (Fig. 1A, Table 2). Heterozygous individuals with alleles recently identified in individuals of Black-African descent (CYP2A6*25, *26, *27 and *35) (Mwenifumbo Jc, Al Koudsi N et al., 2008) also had ~40 – 60% activity remaining (Fig. 1A, Table 2). Given the low prevalence of these novel variants, the larger size of this current study provides further evidence of their in vivo functional impact. CYP2A6*2, *23, *24 and *28 were associated with 3HC/COT ratios higher than expected (Fig. 1A, Table 2). Thus, in agreement with previous studies in other racial/ethnic groups (Johnstone, Benowitz et al., 2006; Malaiyandi, Goodz et al., 2006), there is generally a good concordance between CYP2A6 genotype and 3HC/COT as a phenotypic measure. The frequencies of the genotypes did not deviate significantly from Hardy-Weinberg equilibrium (p > 0.10). CYP2A6 allele frequencies in this sample of African-Americans did not significantly differ from those reported in Canadian individuals of Black-African descent, with the exception of CYP2A6*28 (Table 3).
Table 2: *CYP2A6* genotypes and associated 3HC/COT ratios

<table>
<thead>
<tr>
<th>Allele</th>
<th>Genotype</th>
<th>N</th>
<th>Mean 3HC/COT</th>
<th>SD</th>
<th>%</th>
<th>p – value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYP2A6</em>1B</td>
<td>*<em>1/<em>1</em></em></td>
<td>169</td>
<td>0.40</td>
<td>0.29</td>
<td>100</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>*<em>1/<em>1B</em></em></td>
<td>62</td>
<td>0.49</td>
<td>0.26</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*<em>1B/<em>1B</em></em></td>
<td>15</td>
<td>0.50</td>
<td>0.26</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>*<em>1/<em>1</em></em></td>
<td>246</td>
<td>0.43</td>
<td>0.28</td>
<td>100</td>
<td>---</td>
</tr>
<tr>
<td><em>CYP2A6</em>2</td>
<td>*<em>1/<em>2</em></em></td>
<td>5</td>
<td>0.31</td>
<td>0.16</td>
<td>72</td>
<td>0.29</td>
</tr>
<tr>
<td><em>CYP2A6</em>4</td>
<td>*<em>1/<em>4</em></em></td>
<td>14</td>
<td>0.21</td>
<td>0.09</td>
<td>49</td>
<td>0.001</td>
</tr>
<tr>
<td><em>CYP2A6</em>9</td>
<td>*<em>1/<em>9</em></em></td>
<td>70</td>
<td>0.32</td>
<td>0.29</td>
<td>74</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*<em>9/<em>9</em></em></td>
<td>10</td>
<td>0.15</td>
<td>0.10</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td><em>CYP2A6</em>12</td>
<td>*<em>1/<em>12</em></em></td>
<td>4</td>
<td>0.23</td>
<td>0.11</td>
<td>53</td>
<td>0.06</td>
</tr>
<tr>
<td><em>CYP2A6</em>17</td>
<td>*<em>1/<em>17</em></em></td>
<td>49</td>
<td>0.26</td>
<td>0.15</td>
<td>61</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*<em>17/<em>17</em></em></td>
<td>5</td>
<td>0.06</td>
<td>0.03</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><em>CYP2A6</em>20</td>
<td>*<em>1/<em>20</em></em></td>
<td>13</td>
<td>0.17</td>
<td>0.11</td>
<td>40</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*<em>20/<em>20</em></em></td>
<td>1</td>
<td>0.18</td>
<td>---</td>
<td>---</td>
<td>42</td>
</tr>
<tr>
<td><em>CYP2A6</em>23</td>
<td>*<em>1/<em>23</em></em></td>
<td>7</td>
<td>0.38</td>
<td>0.12</td>
<td>88</td>
<td>0.56</td>
</tr>
<tr>
<td><em>CYP2A6</em>24</td>
<td>*<em>1/<em>24</em></em></td>
<td>3</td>
<td>0.37</td>
<td>0.16</td>
<td>86</td>
<td>0.50</td>
</tr>
<tr>
<td><em>CYP2A6</em>25</td>
<td>*<em>1/<em>25</em></em></td>
<td>7</td>
<td>0.22</td>
<td>0.06</td>
<td>51</td>
<td>0.10</td>
</tr>
<tr>
<td><em>CYP2A6</em>26</td>
<td>*<em>1/<em>26</em></em></td>
<td>4</td>
<td>0.23</td>
<td>0.11</td>
<td>54</td>
<td>0.09</td>
</tr>
<tr>
<td><em>CYP2A6</em>27</td>
<td>*<em>1/<em>27</em></em></td>
<td>8</td>
<td>0.18</td>
<td>0.07</td>
<td>42</td>
<td>0.001</td>
</tr>
<tr>
<td><em>CYP2A6</em>28</td>
<td>*<em>1/<em>28</em></em></td>
<td>16</td>
<td>0.73</td>
<td>0.71</td>
<td>170</td>
<td>0.007</td>
</tr>
<tr>
<td><em>CYP2A6</em>35</td>
<td>*<em>1/<em>35</em></em></td>
<td>20</td>
<td>0.26</td>
<td>0.09</td>
<td>61</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*<em>35/<em>35</em></em></td>
<td>1</td>
<td>0.18</td>
<td>---</td>
<td>---</td>
<td>42</td>
</tr>
<tr>
<td>&gt; 1 variant</td>
<td>---</td>
<td>39</td>
<td>0.15</td>
<td>0.08</td>
<td>35</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

1 Univariate analyses including gender, age, and BMI as covariates. Comparisons were made with the *CYP2A6*1/*1 individuals as the reference group as was done previously (Mwenifumbo Jc, Al Koudsi N et al., 2008). In cases where there is only one individual who is homozygous for the variant, they are combined with the heterozygous variant group for analyses.

2 Individuals with *CYP2A6*1B were included in the reference group.

3 Compound heterozygotes, such as individuals with *CYP2A6*4/*17 genotypes, were grouped as having > 1 variant.
Table 3: *CYP2A6* allele frequencies in African-Americans in this population compared to our previous study in individuals of Black-African descent

<table>
<thead>
<tr>
<th><em>CYP2A6</em> allele</th>
<th>African-Americans (n = 1236 alleles)</th>
<th>Black-African descent&lt;sup&gt;1&lt;/sup&gt; (n = 560 alleles)</th>
<th>p – value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td></td>
</tr>
<tr>
<td><em>CYP2A6</em>&lt;sup&gt;1B&lt;/sup&gt;</td>
<td>18.2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>18.3</td>
<td>0.95</td>
</tr>
<tr>
<td><em>CYP2A6</em>2</td>
<td>0.9</td>
<td>0.4</td>
<td>0.22</td>
</tr>
<tr>
<td><em>CYP2A6</em>4</td>
<td>1.9</td>
<td>2.7</td>
<td>0.33</td>
</tr>
<tr>
<td><em>CYP2A6</em>9</td>
<td>9.6</td>
<td>7.2</td>
<td>0.09</td>
</tr>
<tr>
<td><em>CYP2A6</em>12</td>
<td>0.4</td>
<td>0.0</td>
<td>0.33</td>
</tr>
<tr>
<td><em>CYP2A6</em>17</td>
<td>8.0</td>
<td>7.3</td>
<td>0.61</td>
</tr>
<tr>
<td><em>CYP2A6</em>20</td>
<td>1.5</td>
<td>1.1</td>
<td>0.51</td>
</tr>
<tr>
<td><em>CYP2A6</em>23</td>
<td>1.1</td>
<td>2.0</td>
<td>0.16</td>
</tr>
<tr>
<td><em>CYP2A6</em>24</td>
<td>0.7</td>
<td>1.3</td>
<td>0.28</td>
</tr>
<tr>
<td><em>CYP2A6</em>25</td>
<td>0.9</td>
<td>0.5</td>
<td>0.43</td>
</tr>
<tr>
<td><em>CYP2A6</em>26</td>
<td>0.7</td>
<td>0.7</td>
<td>0.97</td>
</tr>
<tr>
<td><em>CYP2A6</em>27</td>
<td>0.7</td>
<td>0.2</td>
<td>0.15</td>
</tr>
<tr>
<td><em>CYP2A6</em>28</td>
<td>2.4</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td><em>CYP2A6</em>35</td>
<td>2.9</td>
<td>2.5</td>
<td>0.62</td>
</tr>
</tbody>
</table>

<sup>1</sup>Data previously published in (Mwenifumbo Jc, Al Koudsi N et al., 2008) and (Al Koudsi, Ahluwalia Js et al., 2009)

<sup>2</sup>*CYP2A6*<sup>1B</sup> was genotyped only in individuals without other *CYP2A6* variants examined in this study (n = 297).
**Fig. 1A:** CYP2A6 genotypes and their associated unadjusted 3HC/COT ratios. Each dot represents an individual and the line represents the mean 3HC/COT ratio in each genotype group. The CYP2A6*1/*1 group (n = 246) includes individuals with the CYP2A6*1B allele. The >1 variant group represents compound heterozygote individuals (e.g. CYP2A6*4/*17).
**CYP2A6 genotype grouping strategy**

Due to the large number of low prevalence *CYP2A6* alleles, participants were categorized by the predicted rate of CYP2A6 activity based on their genotype. This was done according to the grouping strategy used in previous studies (Benowitz, Swan et al., 2006; Mwenifumbo Jc, Al Koudsi N et al., 2008). Individuals with *CYP2A6*\(^*28\) were excluded from this analysis due to extreme range in 3HC/COT values (Fig. 1A), suggesting some may also have gain-of-function copy number variants which is under current investigation. *CYP2A6* gene duplications (*CYP2A6*\(^*1x2A, *1x2B\)) leading to increased enzyme function have been described (Rao, Hoffmann et al., 2000; Fukami et al., 2007). However, these duplications are rare in African-Americans, occurring at < 2% allele frequencies. We genotyped 343 samples for *CYP2A6*\(^*1x2A\), and 28 samples with high 3HC/COT ratios for *CYP2A6*\(^*1x2B\) but did not find any individuals with these gene duplication alleles.

Individuals with one copy of the decrease-of-function alleles (*CYP2A6*\(^*9\) and \(^*12\)) were grouped as IMs (n = 78). Individuals with two copies of the decrease-of-function alleles, one or two copies of loss-of-function alleles (*CYP2A6*\(^*2, *4, *17, *20, *23, *24, *25, *26, *27\) and \(^*35\)), or one decrease-of-function allele with one loss-of-function allele were grouped as SMs (n = 213). Although the genotype for *CYP2A6*\(^*2, *23\) and \(^*24\) were not in agreement with the phenotype measure (3HC/COT), these individuals were categorized as SMs based on previous studies demonstrating their functional impact (Benowitz, Swan et al., 2006; Ho, Mwenifumbo Jc et al., 2008; Mwenifumbo Jc, Al Koudsi N et al., 2008).

**3HC/COT was associated with CYP2A6 genotype**

The 3HC/COT ratio was significantly associated with the *CYP2A6* genotype groupings (F(2, 492) = 52.6, \(p < 0.001\)), and remained significant after controlling for gender, age and BMI as
covariates ($F(2, 485) = 58.7$, $p < 0.001$). The mean 3HC/COT ratio (± 95% CI) in the $CYP2A6$ genotype groups were: NM = 0.43 ± 0.04, IM = 0.32 ± 0.07, SM = 0.22 ± 0.02. The 3HC/COT ratio was significantly lower in intermediate and slow metabolizers (IMs and SMs, respectively) compared to the normal metabolizers (NMs) ($p < 0.001$, Fig. 1B). When we adjusted the ratio for gender (mean ± 95% CI: NM = 1.27 ± 0.10, IM = 0.95 ± 0.20, SM = 0.64 ± 0.06, Fig 1C), we observed similar adjusted values as were reported previously in our population of Black-African descent (mean ± 95% CI: NM = 1.18 ± 0.10, IM = 0.77 ± 0.15, SM = 0.51 ± 0.10) (Mwenifumbo Jc, Al Koudsi N et al., 2008).

**Fig 1B** The unadjusted 3HC/COT ratio was significantly associated with $CYP2A6$ genotype groupings. Statistical analyses were performed on the log-transformed ratio with gender, age and BMI as covariates. It should be noted that since only 18 individuals were predicted to be poor metabolizers (completely lacking $CYP2A6$ function due to having two copies of loss-of-function alleles), they were combined with those predicted to have 10-50% activity to form the SM group. **Fig 1C** The 3HC/COT ratio was also adjusted by gender as illustrated in our previous papers (Ho, Mwenifumbo Jc et al., 2008; Mwenifumbo Jc, Al Koudsi N et al., 2008). Adjustments were made by dividing each ratio by the mean value of their respective gender. For example, a male individual with an adjusted ratio greater than one indicates values that are higher than the mean ratio of all males. NM = normal metabolizers, IM = intermediate metabolizers, SM = slow metabolizers. The 3HC/COT when adjusted for the covariates (gender, BMI, and age) found to be significant in this population, using regression analyses, is as follows (mean ± 95%CI): NM = 0.44 ± 0.03, IM = 0.31 ± 0.05, SM = 0.22 ± 0.03). *** $p < 0.001$ when compared to the NMs. ## $p < 0.01$ when compared to IMs. The number of individuals are listed on the x – axis.
CYP2A6 activity and baseline smoking behaviours

The self-reported cigarettes per day (CPD) was not associated with CYP2A6 genotype groups (F(2,585) = 0.26, p = 0.77) or 3HC/COT quartiles (F(3, 642) = 0.25, p = 0.86) (Fig. 2A, B). Exhaled CO levels, a biochemical measure of cigarette smoke exposure, was also not associated with CYP2A6 genotype groups (F(2, 584) = 3.0, p = 0.05) or 3HC/COT quartiles (F(3, 641) = 1.6, p = 0.18) (Fig. 2C, D).

In contrast to observations in heavy smokers (Schoedel, Hoffmann Eb et al., 2004), slow CYP2A6 activity was associated with higher plasma NIC levels suggesting individuals were not altering their cigarette intake to compensate for different rates of NIC metabolism. CYP2A6 SMs had significantly higher baseline plasma nicotine levels compared to NMs (F (2, 585) = 6.0, p = 0.003) (Fig. 2E). Similarly, plasma NIC levels progressively increased corresponding to 3HC/COT quartiles (that is, with decreasing CYP2A6 activity), such that levels are lowest in the first quartile (fastest CYP2A6 activity) and highest in the fourth quartile (slowest CYP2A6 activity) (F(3, 642) = 9.8, p < 0.001) (Fig. 2F). Plasma nicotine levels were significantly higher among slow metabolizers in both males and females.

Neither CYP2A6 genotype (F(2, 584) = 0.08, p = 0.92) nor the 3HC/COT quartiles (F(3, 640) = 0.43, p = 0.73) were associated with age of onset of regular smoking. Tobacco dependence was assessed by the Cigarette Dependence Scale (CDS) (Etter et al., 2003) and the Fagerström Test for Nicotine Dependence (FTND) (Heatherton, Kozlowski Lt et al., 1991). CYP2A6 genotype was not associated with scores from the CDS (F(2, 585) = 0.33, p = 0.72) or FTND (F(2, 585) = 0.37, p = 0.69). The 3HC/COT quartiles were also not associated with scores from the CDS (F(3, 642) = 0.39, p =0.76) or FTND (F(3, 642) = 0.24, p = 0.87). Gender, age and BMI did not alter any of the relationships listed above when included separately in the analysis as covariates.
Fig. 2: Association of CYP2A6 activity with smoking indices. **A, B** CYP2A6 genotype and 3HC/COT were not associated with self-reported CPD. **C, D** CYP2A6 genotype and 3HC/COT were not associated with expired CO. **E** CYP2A6 genotype was associated with nicotine plasma levels. **p < 0.01 when compared to NMs. F** 3HC/COT was associated with nicotine plasma levels. *p < 0.05, **p < 0.01 and *** p < 0.001 when compared to the 1st quartile. Individuals with 3HC/COT values in the 1st quartile have the fastest CYP2A6 activity, while those in the 4th quartile have the slowest CYP2A6 activity. Data are presented as mean ± 95% confidence interval. The number of individuals are listed on the x-axis.
CYP2A6 activity and smoking abstinence

Seven-day point prevalence abstinence – defined as having smoked no cigarettes, not even a puff – for the previous 7 days, was assessed at week 8 (end-of-treatment, EOT) and week 26 (follow-up), and verified by exhaled CO levels ($\leq$ 10 ppm). Variables previously reported to be predictors of abstinence in this population (counseling, gender, income, age, and BMI) (Nollen et al., 2006), as well as drug treatment, were included in a multiple logistic regression model. Consistent with previous analyses in this population (Nollen, Mayo Ms et al., 2006), MI counseling and lower income ($\leq$ $1,800) were associated with lower odds of quitting while male gender, older age and higher BMI were associated with greater odds of quitting at both EOT and follow-up (Table 4). CYP2A6 SMs trended towards being significantly more likely to quit smoking at both EOT (p = 0.10) and follow-up (p = 0.08) compared to IMs and NMs combined (Table 4, Fig. 3A). Individuals with the slowest 3HC/COT quartile trended towards having greater odds of quitting at EOT (p = 0.08) and were significantly more likely to quit at follow-up (p = 0.03) (Table 4, Fig. 3B). The effect of CYP2A6 activity on quit success appeared to be more pronounced in females (Fig. 3C, D), and was observed in both the placebo and nicotine gum arms among females (Fig. 3 E, F), but the gender interaction was not significant.
Fig. 3: Association of CYP2A6 activity and smoking abstinence. A, B) Association of CYP2A6 genotype and 3HC/COT quartiles with CO-verified quit rates at EOT and follow-up. The NMs and IMs were pooled for analyses and compared to SMs. Individuals with highest 3HC/COT ratios in quartiles 1st to 3rd were pooled for analyses, and compared to individuals with 3HC/COT ratios in the lowest 25th percentile (4th quartile, slowest activity). C, D) Association of CYP2A6 genotype and 3HC/COT quartiles with CO-verified quit rates at EOT and follow-up by gender. E, F) Association of CYP2A6 genotype and 3HC/COT quartiles with CO-verified quit rates at EOT and follow-up by treatment arm. Only data in females is shown for Fig. 3E and F. The p-values listed were derived from univariate analyses of quit rates by categories of CYP2A6 activities; the results from the multivariate analysis is presented in Table 4.
Table 4: Logistic regression analyses of predictors of CO-verified quit rates at EOT (week 8) and follow-up (week 26)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>EOT</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>A) Genotype, n = 588</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype (SM)</td>
<td>1.54</td>
<td>0.92 – 2.57</td>
</tr>
<tr>
<td>Drug (placebo)</td>
<td>0.62</td>
<td>0.41 – 0.93</td>
</tr>
<tr>
<td>Counseling (MI)</td>
<td>0.61</td>
<td>0.41 – 0.92</td>
</tr>
<tr>
<td>Gender (males)</td>
<td>1.68</td>
<td>0.99 – 2.85</td>
</tr>
<tr>
<td>Age</td>
<td>1.04</td>
<td>1.02 – 1.06</td>
</tr>
<tr>
<td>BMI</td>
<td>1.03</td>
<td>1.00 – 1.05</td>
</tr>
<tr>
<td>Income (≤ $1800)</td>
<td>0.59</td>
<td>0.39 – 0.88</td>
</tr>
<tr>
<td>B) 3HC/COT, n = 646</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quartile (slowest)</td>
<td>1.61</td>
<td>0.94 – 2.76</td>
</tr>
<tr>
<td>Drug (placebo)</td>
<td>0.76</td>
<td>0.51 – 1.11</td>
</tr>
<tr>
<td>Counseling (MI)</td>
<td>0.72</td>
<td>0.49 – 1.06</td>
</tr>
<tr>
<td>Gender (males)</td>
<td>1.59</td>
<td>0.98 – 2.58</td>
</tr>
<tr>
<td>Age</td>
<td>1.04</td>
<td>1.02 – 1.06</td>
</tr>
<tr>
<td>BMI</td>
<td>1.01</td>
<td>0.99 – 1.04</td>
</tr>
<tr>
<td>Income (≤ $1800)</td>
<td>0.63</td>
<td>0.43 – 0.93</td>
</tr>
</tbody>
</table>

1 The odds ratio provided refers to the variable listed in brackets beside each categorical predictor.
2 The NM and IM group were combined for analyses and compared against the SM group.
3 Individuals in quartiles 1 to 3 (highest 3HC/COT ratios) were combined for analyses and compared against individuals in the 4th quartile (lowest 3HC/COT ratios).
Discussion

This is the first study examining the relationship between CYP2A6 activity and smoking behaviours in African-American light smokers. We provided further evidence that the 3HC/COT ratio derived from *ab libitum* smoking is a good phenotypic measure of CYP2A6 activity in this population as there was a good concordance with *CYP2A6* genotypes. Our results also suggest that in this sample of light smokers, cigarette consumption was not lowered to fully compensate for slower rates of nicotine metabolism, and rather, higher plasma nicotine levels were observed in slow metabolizers. The group with the slowest CYP2A6 activity trended towards increased cessation success at EOT which reached significance at follow-up. This suggests that slow CYP2A6 activity, and the resulting higher plasma nicotine levels, may influence some aspect of the addiction process leading to greater cessation.

The 3HC/COT ratio is particularly useful as a phenotypic measure of CYP2A6 activity because COT has a relatively long elimination half-life (~13 – 19 hours) and the level of 3HC is formation-dependent (Benowitz and Jacob P 3rd, 1994). The 3HC/COT ratio, where metabolites were derived from *ab libitum* smoking, is independent of the time of sampling (Lea, Dickson S et al., 2006), does not vary widely within individuals over time (Lea, Dickson S et al., 2006; Mooney, Li et al., 2008), and has been associated with *CYP2A6* genotypes in heavy smoking individuals of European-ancestry (Malaiyandi, Goodz et al., 2006). Our results and those in our previous study (Mwenifumbo, Sellers et al., 2007; Mwenifumbo Jc, Al Koudsi N et al., 2008) also indicate the ratio has good concordance with *CYP2A6* genotype and rates of nicotine metabolism in light smoking populations of Black-African descent. Our observation that the *CYP2A6* *2, *23, *24 and *28 alleles were associated with 3HC/COT ratios higher than predicted is likely due to other sources of variability in genotype and phenotype, combined with the small number of individuals with these alleles. We estimated that to detect a 50% reduction in
3HC/COT from the wildtype reference group (mean = 0.43) with a power of 0.80, assuming a standard deviation of 0.25, we would need 11 individuals who have the variant of interest. There may also be other unidentified genetic variation, such as gene duplications, and/or individuals may have been exposed to CYP2A6 inducers. Information regarding the use of oral contraceptives, a known CYP2A6 inducer (Benowitz, Lessov-Schlaggar Cn et al., 2006), was not collected in this study.

The finding that the 3HC/COT ratio is higher in females is in agreement with previous data (Benowitz, Lessov-Schlaggar Cn et al., 2006; Mwenifumbo, Sellers et al., 2007). Our observation that the 3HC/COT ratio increases with age suggests CYP2A6 activity may be increased among the elderly (Johnstone, Benowitz et al., 2006). BMI has also been negatively correlated with the 3HC/COT ratio previously (Mooney, Li et al., 2008; Swan Ge et al., 2008), though the relationship between obesity and CYP2A6 activity or nicotine pharmacokinetics have not been well examined. Mentholated cigarettes have been shown to reduce nicotine metabolism in smokers (Benowitz, Herrera et al., 2004). It is notable that mentholated cigarette smokers were younger than non-mentholated cigarette smokers (42.5 vs. 50.0, p < 0.001). Thus, the modest reduction in 3HC/COT among mentholated cigarette smokers may be attributed to an age effect on CYP2A6 activity. Smoking menthol cigarettes did not significantly affect the ratio after controlling for age, gender and BMI. In summary, in addition to CYP2A6 genetic variation, male gender, younger age and higher BMI were also associated with slower CYP2A6 activity.

Because of the short half-life of nicotine, heavy smokers smoke regularly over the course of the day to maintain sufficient levels in the body to avoid withdrawal symptoms. Accordingly, smokers change their smoking behaviour when nicotine yield of cigarettes or rates of nicotine elimination are altered experimentally (Scherer, 1999). However, light smokers, including our
study population, are not smoking at regular intervals over the course of the day, and thus, their plasma nicotine levels likely fluctuate widely. Accordingly, there was no relationship between cigarette consumption and CYP2A6 activity, and SMs had higher plasma nicotine levels than NMs, indicating a lack of full compensation for altered rates of nicotine metabolism. There is evidence that smoking in chippers (≤ 5 CPD) is under greater stimulus control, i.e. smoking is highly influenced by social and sensory motives and tends to be associated with specific activities, such as after meals or drinking alcohol (Shiffman and Paty J, 2006). However, these studies of chippers represent a distinct subset of smokers of European-ancestry, and further investigation into the factors driving light smoking in African-Americans is necessary.

We did not observe a relationship between CYP2A6 activity and age of onset of regular smoking. In any case, this is a relatively weak measure because it is generally limited by recall bias. Similarly, while we did not see a relationship between CYP2A6 activity and CDS or FTND scores, tobacco dependence is a complex, multi-dimensional construct and there are likely aspects of dependence that were not captured by these scales, such as cravings to smoke. It was recently reported that those with slow CYP2A6 activity may smoke less in response to cravings and were less influenced by smoking-related cues (Piper, Mccarthy et al., 2008). Given the large sample size of our study, it is unlikely that our negative findings result from a lack of statistical power since associations have been reported in smaller studies of heavy smokers of European-descent (Schoedel, Hoffmann Eb et al., 2004; Kubota, Nakajima-Taniguchi et al., 2006).

The finding that individuals with slow CYP2A6 activity had greater success at quitting (significant at follow-up) is in agreement with recent findings in clinical trials of heavy smoking individuals of European-ancestry. Individuals with 3HC/COT ratios in the slowest quartile had significantly greater odds of quitting in the placebo arm of one study (Patterson, Schnoll et al.,
2008), which was further augmented by treatment with transdermal nicotine patch in another study (Lerman, Tyndale et al., 2006) that was recently replicated (Schnoll, Patterson et al., 2009). The observation of slow CYP2A6 activity and higher quit rates in the placebo arm of this and the previous study suggests greater likelihood of cessation even in the absence of nicotine. This is consistent with the observation that CYP2A6 slow metabolizers were more likely to be former smokers (Gu, Hinks et al., 2000) and had shorter smoking durations (Schoedel, Hoffmann Eb et al., 2004). The higher quit rates among CYP2A6 slow metabolizers were observed primarily in females in this study. Because our sample had disproportionately more females (67%), further replication of this effect in a sample with a larger number of males is necessary.

It is notable that significant associations between CYP2A6 activity and quit rates were observed when individuals were identified by the 3HC/COT quartiles ($p = 0.03$ at follow-up, Table 4), and only trended towards significance when categorized by CYP2A6 genotypes ($p = 0.10$ at EOT and $p = 0.08$ at follow-up, Table 4). Given the large number of participants and the substantial portion of African-Americans defined as genetically slow metabolizers (~36%), our study should have been sufficiently powered. The difference between these two measures may be that the ratio takes into account other sources of variation and/or because of additional unidentified alleles. Large variation in the phenotype is observed among those defined as CYP2A6*1/*1 (i.e. those without identified variants, Fig. 1A), suggesting additional variants may still be present. Thus, CYP2A6 genotypes may have similar utility as 3HC/COT quartiles in predicting smoking cessation in African-American light smokers in the future, particularly as we gain a better understanding of the genetic variations in CYP2A6 in this population.

The results from the current and other recent retrospective studies (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Schnoll, Patterson et al., 2009) suggest that CYP2A6 activity may
have important clinical utility in determining the type of smoking cessation treatment prescribed. For example, individuals with faster CYP2A6 activity might be encouraged to use pharmacotherapy to aid their quit attempts, given their lower quit rates on placebo compared to slower metabolizers. In contrast, slow metabolizers may do well with behavioural therapy alone and/or with nicotine patch treatment but may not benefit greatly from bupropion (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Schnoll, Patterson et al., 2009). Prospective clinical trials have not yet been performed to directly assess whether the rate of CYP2A6 activity will have utility in the personalization of smoking cessation therapy.

One of the strengths of this study is that we were able to associate CYP2A6 activity with smoking behaviours and treatment outcomes using both genotype and phenotype measures in a large population. A limitation of this study is that it was secondary analyses of a clinical trial designed to test the efficacy of nicotine gum and counseling, and there was an overrepresentation of females. In addition, this treatment-seeking sample of African-American light smokers may not be representative of the general population as they are likely smokers who had difficulty quitting in the past. Further studies are also necessary to determine whether the results are applicable to other racial/ethnic populations, such as Hispanics, where light smoking is also prevalent (Office of Applied Studies and Substance Abuse and Mental Health Services Administration, 2006).

In conclusion, this study provides further evidence that the 3HC/COT ratio can serve as a phenotypic marker of CYP2A6 activity, and gives new insights into the role of CYP2A6 in light smoking in African-Americans. Confirmation of the validity of the 3HC/COT ratio derived from baseline smoking as a phenotypic marker in light smoking populations will expand its utility for future studies. Secondly, the ability of some individuals to maintain low levels of cigarette
consumption contradicts classical theories of tobacco addiction as driven by physical
dependence, and further research is needed to examine the biological and psychosocial context
underlying light smoking behaviours. There is no safe level of smoking, and a better
understanding of the phenomenon will lead to more effective intervention methods for this
unique group of smokers.

**Significance to thesis**

In our large sample of African-American light smokers, we confirmed the association between
*CYP2A6* genotype groups and the 3HC/COT ratio derived from *ad libitum* smoking. This
provides further support that 3HC/COT is a good indicator of CYP2A6 activity in this
population. Furthermore, females, individuals with lower BMI, and those in the oldest age group
(aged 60 to 77) had significantly higher 3HC/COT, while this ratio did not differ between those
who smoke menthol or regular cigarettes. Identifying sources of variability in rates of CYP2A6
activity is important in helping explain the slower rates of nicotine and cotinine metabolism in
this population.

This study is the first to examine the role of *CYP2A6* on smoking behaviours in African-
American light smokers. The ability of these individuals to maintain low levels of consumption
cannot be explained by prevailing theories of tobacco dependence, and very little is known about
the biological factors associated with smoking behaviours in this population. In contrast to
moderate to heavy smokers of Caucasian ancestry, CYP2A6 activity was not a predictor of the
amount of cigarettes smoked. However, similar to previous findings in moderate to heavy
smokers of Caucasian ancestry, slow CYP2A6 activity was associated with increased likelihood
of smoking cessation among this sample of African-American light smokers. Thus, these
smokers do not appear to be altering cigarette consumption to maintain nicotine levels, yet
CYP2A6 activity remained a predictor of smoking cessation. This suggests the processes underlying tobacco dependence differ between CYP2A6 slow and normal metabolizers, presumably as a result of varying nicotine levels, although the precise mechanism(s) by which this is occurring is not yet clear.

An additional benefit of this study is that both CYP2A6 genotype and phenotype measures were available as indicators of CYP2A6 activity. Similar results were observed using either measures of CYP2A6 activity, although the effect of CYP2A6 genotype on smoking abstinence only trended towards significance. Determining whether genotype or phenotype measures are better predictors of smoking cessation is important as the rate of CYP2A6 activity may potentially be used to optimize treatment paradigms for African-American light smokers in the future. This treatment-seeking population of African-American light smokers achieved poor quit rates in spite of their high motivation to quit, providing further support that these smokers have difficulty quitting despite their lower levels of cigarette consumption. This highlights the complexity of tobacco dependence and the need to develop successful smoking cessation intervention programs for this understudied group of smokers.
**Supplementary information**

Supplementary Table 1: Participant demographics. Data are presented as mean (SD).

<table>
<thead>
<tr>
<th></th>
<th>Total n = 755</th>
<th>3HC/COT n = 646</th>
<th>Genotype n = 588</th>
<th>Genotype and 3HC/COT n = 495</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, % females</td>
<td>66.9%</td>
<td>68.0%</td>
<td>67.5%</td>
<td>69.7%</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>45.1 (10.7)</td>
<td>45.1 (10.9)</td>
<td>44.9 (10.9)</td>
<td>45.0 (11.2)</td>
</tr>
<tr>
<td>Range 19 – 81</td>
<td>19 – 81</td>
<td>19 – 81</td>
<td>19 – 81</td>
<td>19 – 81</td>
</tr>
<tr>
<td>BMI</td>
<td>30.6 (8.1)</td>
<td>30.5 (8.0)</td>
<td>30.6 (8.0)</td>
<td>30.5 (8.0)</td>
</tr>
<tr>
<td>Range 14 – 74</td>
<td>14 – 74</td>
<td>14 – 74</td>
<td>14 – 74</td>
<td>14 – 74</td>
</tr>
<tr>
<td>Menthol cigarettes, % yes</td>
<td>81.7%</td>
<td>80.3%</td>
<td>82.8%</td>
<td>81.0%</td>
</tr>
<tr>
<td>CPD</td>
<td>7.6 (3.2)</td>
<td>7.6 (3.1)</td>
<td>7.5 (3.4)</td>
<td>7.6 (3.3)</td>
</tr>
<tr>
<td>Range 0 – 30</td>
<td>0 – 30</td>
<td>0 – 30</td>
<td>0 – 30</td>
<td>0 – 30</td>
</tr>
<tr>
<td>Baseline CO, ppm</td>
<td>13.9 (8.9)</td>
<td>13.8 (8.8)</td>
<td>14.0 (9.2)</td>
<td>13.9 (9.3)</td>
</tr>
<tr>
<td>Range 0 – 69</td>
<td>0 – 69</td>
<td>0 – 69</td>
<td>0 – 69</td>
<td>0 – 69</td>
</tr>
<tr>
<td>Baseline NIC, ng/ml</td>
<td>12.1 (8.9)</td>
<td>12.3 (8.9)</td>
<td>12.2 (8.9)</td>
<td>12.4 (9.0)</td>
</tr>
<tr>
<td>Range 0.5 – 46</td>
<td>0.5 – 46</td>
<td>0.5 – 46</td>
<td>0.5 – 46</td>
<td>0.5 – 46</td>
</tr>
<tr>
<td>Baseline COT, ng/ml</td>
<td>244.2 (154.4)</td>
<td>249.8 (153.6)</td>
<td>245.5 (157.5)</td>
<td>252.9 (158.4)</td>
</tr>
<tr>
<td>Range 5 – 938</td>
<td>5 – 938</td>
<td>5 – 938</td>
<td>5 – 938</td>
<td>5 – 938</td>
</tr>
<tr>
<td>Age of regular smoking</td>
<td>21.1 (6.9)</td>
<td>21.1 (6.8)</td>
<td>21.2 (7.1)</td>
<td>21.2 (6.9)</td>
</tr>
<tr>
<td>CDS</td>
<td>15.0 (3.7)</td>
<td>15.0 (3.7)</td>
<td>15.0 (3.7)</td>
<td>15.0 (3.7)</td>
</tr>
<tr>
<td>FTND</td>
<td>2.9 (1.8)</td>
<td>2.9 (1.8)</td>
<td>2.9 (1.8)</td>
<td>2.9 (1.8)</td>
</tr>
<tr>
<td>Range 0 – 9</td>
<td>0 – 9</td>
<td>0 – 9</td>
<td>0 – 9</td>
<td>0 – 9</td>
</tr>
</tbody>
</table>
CHAPTER 3: UTILITY AND RELATIONSHIPS OF BIOMARKERS OF SMOKING IN AFRICAN-AMERICAN LIGHT SMOKERS

Man Ki Ho, Babalola Faseru, Won S. Choi, Nicole L. Nollen, Matthew S. Mayo, Janet L. Thomas, Kolawole S. Okuyemi, Jasjit S. Ahluwalia, Neal L. Benowitz, Rachel F. Tyndale

Reprinted with permission from American Association for Cancer Research: Cancer Epidemiology Biomarkers and Prevention, 18(12): 3426-3434, 2009. © 2010 All rights reserved.

Drs. Kolawole Okuyemi, Jasjit Ahluwalia, Neal Benowitz and Rachel Tyndale designed and recruited the participants in this clinical trial with the primary aim of testing the efficacy of 2 mg nicotine gum and two different counseling methods for smoking cessation in a population of African-American light smokers. The levels of nicotine, cotinine and trans-3-hydroxycotinine in plasma samples were determined in the laboratory of Dr. Neal Benowitz. Drs. Babalola Faseru, Won S. Choi, Nicole L. Nollen, Matthew S. Mayo, Janet L. Thomas were responsible for storing and maintaining the database of information collected from participants, and provided helpful feedback for the preparation of the manuscript. MKH performed a substantial portion of the DNA extraction and CYP2A6 genotyping, was responsible for determining the research questions of interest, performed all statistical analyses and wrote the manuscript.
Abstract
While expired carbon monoxide (CO) and plasma cotinine (COT) have been validated as biomarkers of self-reported cigarettes per day (CPD) in heavy smoking Caucasians, their utility in light smokers is unknown. Further, variability in CYP2A6, the enzyme that mediates formation of COT from nicotine (NIC) and its metabolism to trans-3’-hydroxycotinine (3HC), may limit the usefulness of COT. We assessed whether CO and COT are correlated with CPD in African-American light smokers (≤10CPD, n=700), a population with known reduced CYP2A6 activity and slow COT metabolism. We also examined whether gender, age, BMI, smoking mentholated cigarettes or rate of CYP2A6 activity, by genotype and phenotype measures (3HC/COT), influence these relationships. At baseline, many participants (42%) exhaled CO ≤10ppm, the traditional cutoff for smoking, while few (3.1%) had COT below the cutoff of ≤14ng/ml; thus COT appears to be a better biomarker of smoking status in this population. CPD was weakly correlated with CO and COT (r = 0.32–0.39, p<0.001), and those reporting fewer CPD had higher CO/cigarette and COT/cigarette, although the correlation coefficients between these variables were also weak (r = -0.33 and -0.08, p < 0.05). The correlation between CPD and CO was not greatly increased when analyzed by CYP2A6 activity, smoking mentholated cigarettes or age, although it appeared stronger in females (r = 0.38 vs.0.21, p<0.05) and obese individuals (r = 0.38 vs.0.24, p<0.05). Together, these results suggest that CO and COT are weakly associated with self-reported cigarette consumption in African-American light smokers, and that these relationships are not substantially improved when variables previously reported to influence these biomarkers are considered.

Introduction
Biomarkers of cigarette smoke exposure have been utilized to confirm self-reported smoking measurements. Two commonly used measures are cotinine (COT) and exhaled carbon
Carbon monoxide (CO). COT, a metabolite of nicotine (NIC), is detected in a number of biological fluids (i.e. plasma, saliva and urine) and is highly specific to NIC exposure (Benowitz, Peyton J Iii et al., 2002). However, COT is not specific to cigarette smoke as individuals exposed to alternative sources of tobacco or nicotine replacement therapy will also have detectable COT (Benowitz, Peyton J Iii et al., 2002). The traditional cut-off value for differentiating smokers from non-smokers is plasma or salivary COT levels of $\leq 14 \text{ ng/ml}$ (Jarvis et al., 1987; Benowitz, Peyton J Iii et al., 2002). COT has a relatively long half-life (13 – 19 hrs) and reflects exposure to tobacco within the past 3 – 4 days (Benowitz, Peyton J Iii et al., 2002; Hukkanen, Jacob et al., 2005). CO is a byproduct of the combustion of tobacco, and a cutoff value for CO of $\leq 10 \text{ ppm}$ is traditionally used to distinguish smokers from non-smokers (Benowitz, Peyton J Iii et al., 2002; Scherer, 2006). However, CO is not specific to tobacco smoke exposure and contributions from environmental sources (such as vehicle exhaust) and endogenous formation of CO from heme catabolism can limit its use (Scherer, 2006). CO has a short half-life of approximately 1 – 4 hours (Scherer, 2006) and reflects more recent exposure to smoking; it is highly dependent on the time of the last cigarette.

Several prior studies have confirmed the utility of both COT and CO levels to verify self-reported cigarette consumption, with correlation coefficients ranging from 0.3 – 0.8 (Perez-Stable et al., 1995; Domino et al., 2002; Mustonen, Spencer Sm et al., 2005; Scherer, 2006). However, these studies have been performed primarily among Caucasian moderate to heavy smokers. It is unknown whether these biomarkers are representative of cigarette consumption among light smokers where smoking levels are lower and occur at irregular intervals. In particular, the short half-life of CO, and its lack of specificity, may make it difficult to differentiate light smoking from non-smoking. While the longer half-life of COT may make it a suitable marker among light smokers, it may be influenced by variable rates of its metabolism.
COT is the main proximate metabolite of nicotine (NIC) (Benowitz and Jacob P 3rd, 1994), and COT itself is further metabolized to trans-3’-hydroxycotinine (3HC) (Nakajima, Yamamoto T et al., 1996; Dempsey, Tutka et al., 2004). The conversion of NIC to COT, and COT to 3HC, are primarily mediated by the enzyme cytochrome P450 2A6 (CYP2A6) (Messina, Tyndale et al., 1997). Large interindividual variability in CYP2A6 activity has been reported (Mwenifumbo and Tyndale, 2007). The gene encoding CYP2A6 is highly polymorphic, with 38 numbered alleles identified so far (http://www.cypalleles.ki.se/cyp2a6.htm). To aid in population studies, the 3HC/COT ratio (which can be measured in plasma, saliva or urine), has been validated as a phenotypic indicator of CYP2A6 activity (Dempsey, Tutka et al., 2004). COT levels are potentially influenced by a number of factors that alter CYP2A6 activity, including genetic variation, the presence of enzyme inducers or inhibitors, body mass index (BMI), age, and gender. For instance, African-Americans have higher COT plasma levels than Caucasian smokers even after controlling for number of cigarettes smoked (Caraballo, Giovino et al., 1998). This can be partly attributed to their slower rates of COT metabolism (Benowitz, Perez-Stable et al., 1999); approximately 50% of African-Americans have decrease- or loss-of-function CYP2A6 genetic variants compared to approximately 20% of Caucasians (Mwenifumbo and Tyndale, 2007; Ho, Mwenifumbo et al., 2009). Furthermore, a large proportion of African-Americans smoke mentholated cigarettes, and it has been suggested that the cooling sensation may result in deeper, longer inhalation and larger puff volumes (Werley, Coggins et al., 2007). Some studies, but not all, have found higher CO and COT levels among mentholated cigarette users (Werley, Coggins et al., 2007).

There is also evidence menthol can inhibit CYP2A6 in vitro and mentholated cigarette smokers have slower rates of NIC metabolism (Macdougall, Fandrick et al., 2003; Benowitz, Herrera et
Individuals with higher BMI have been found to have lower COT levels (Perez-Stable, Benowitz Nl et al., 1995; Ahijevych et al., 2002), and older age has been associated with higher levels of COT (Swan et al., 1993; Patterson et al., 2003). Females are known to have faster rates of NIC and COT metabolism (Benowitz, Lessov-Schlaggar Cn et al., 2006), with estrogen being an inducer of CYP2A6 (Higashi, Fukami et al., 2007).

In this study, we examined whether biomarkers derived from ad libitum smoking are associated with self-reported cigarette consumption in a population of African-American light smokers, a population with unique smoking characteristics. The need for validated biomarkers for this specific population is warranted as the prevalence of light smoking is particularly common among African-Americans, with up to 50% reporting ≤ 10 CPD compared to 18-20% of the general smoking population (Okuyemi Ks, Harris Kj et al., 2002). We also examined whether variables previously known to affect biomarker levels, such as rate of CYP2A6 activity (by genotype and 3HC/COT phenotype measure), gender, smoking mentholated cigarettes, BMI or age influenced these relationships. While CYP2A6 genotype was not found to substantially influence the relationships between biomarkers and cigarette consumption in a previous study of Caucasian heavy smokers (Malaiyandi, Goodz et al., 2006), the proportion of individuals with CYP2A6 genetic variants was low. It is possible that the high prevalence of CYP2A6 decrease- or loss-of function genetic variants and slower rates of COT metabolism among African-Americans may play a greater role in this population.

**Materials and Methods**

**Study design**
A detailed description of the study design and participant recruitment can be found elsewhere (Ahluwalia, Okuyemi et al., 2006; Ho, Mwenifumbo et al., 2009). Briefly, participants (n = 755) were randomized into a double-blind, placebo-controlled smoking cessation study at a community health centre in Kansas City, Missouri. Participants (≥ 18 years of age) self-identified as “African-American” or “Black”, smoked ≤10 CPD for at least 6 months prior to enrollment, and smoked at least 25 of the past 30 days were recruited. The research protocol was approved by the University of Toronto Ethics Review Office and the University of Kansas Human Subjects Committee.

**Baseline assessment**

Information regarding the participant’s demographic, smoking and psychosocial characteristics have been described in detail elsewhere (Nollen, Mayo Ms et al., 2006). Age, gender, and BMI were collected at randomization. Participants were asked about their smoking patterns, including the number of CPD, mentholated vs. non-mentholated cigarette use, depth of inhalation, and number of years smoked. Participants were asked to estimate their cigarette consumption by the question: “During the past 7 days, on those days that you smoked, what was the average number of cigarettes smoked per day?” Although all of the participants recruited into the clinical trial reported smoking ≤ 10 CPD on the eligibility questionnaire, a small subset of participants reported consuming zero or > 10 CPD during the past week during the randomization assessment (n = 55) and thus were excluded from analyses in the current study.

**Biochemical measures**

A blood sample was collected at randomization to determine the levels of NIC and its metabolites, and for genotyping purposes. Plasma levels of NIC, COT and 3HC were determined using the methods described elsewhere (Dempsey, Tutka et al., 2004), although 3HC
levels were only available for a subset of the participants (n = 602 out of 700). Expired CO levels were measured by a handheld portable CO monitor (Bedfont Micro Smokerlyzer, Kent, England).

**CYP2A6 genotyping**

*CYP2A6* genotyping for this population was performed using two-step allele-specific polymerase chain reaction assays as described previously (Ho, Mwenifumbo et al., 2009). A subset of the total participants consented to have their blood sampled for genetic analyses, and genotyping data was available for 570 of the 700 participants. Participants were genotyped for *CYP2A6*1B, *2, *4, *9, *12, *17, *20, *23, *24, *25, *26, *27, *28, and *35 (Ho, Mwenifumbo et al., 2009). Individuals were categorized into groups based on their predicted effects of their *CYP2A6* genotypes on rates of activity as previously described (Ho, Mwenifumbo et al., 2009). Those with one copy of the decrease-of-function alleles (*CYP2A6*9 and *12) were grouped as intermediate metabolizers (n = 70). Individuals with two copies of the decrease-of-function alleles, one or two copies of loss-of-function alleles (*CYP2A6*2, *4, *17, *20, *23, *24, *25, *26, *27 and *35), or one decrease-of-function allele with one loss-of-function allele were grouped as slow metabolizers (n = 197). Normal metabolizers (n = 275) were individuals without these genetic variants. Those with *CYP2A6*1B (n = 89) were also included in the normal metabolizer group as previously described (Ho, Mwenifumbo et al., 2009). Individuals with *CYP2A6*28 (n = 28) were excluded from analyses due to extreme range in 3HC/COT values, suggesting some may also have gain-of-function copy number variants which is currently under investigation.

**Statistical analyses**
Statistical analyses were performed using SPSS statistical software, version 16.0. The data (CPD, CO, NIC, COT, 3HC, 3HC/COT, BMI) were not normally distributed according to the Kolmogorov-Smirnov test and were log-transformed when appropriate. Pearson’s correlation coefficient was used to examine the relationships between log-transformed CPD, CO, and COT with CO/cigarette, COT/cigarette. Differences in log-transformed CPD, CO or COT between gender, use of mentholated cigarettes, and BMI as categorized by those considered obese (BMI ≥ 30) versus non-obese (BMI < 30), were tested using the t-test for independent samples. Differences in CPD, CO or COT between CYP2A6 genotype groups, 3HC/COT quartiles, and age categories were examined using univariate analysis of variance with Bonferroni correction for post hoc analyses. Pearson’s correlations between log-transformed CPD, CO or COT with age and log-transformed 3HC/COT and BMI as continuous variables were also examined. Differences between Pearson’s correlation coefficients were tested using the Fisher r-to-z transformation. A multiple linear regression model was used to examine the predictors of baseline CPD, CO and COT. Variables included in the model were significant in univariate analyses (p < 0.10), and variables that were not normally distributed (CPD, CO, COT, BMI, 3HC/COT) were log-transformed in the analyses.

**Results**

**Participant demographics**

A summary of the participant demographics, smoking history and biochemical measures can be found in Table 1. The study sample was overrepresented by females (66.7%), and the majority smoked menthol cigarettes (81.3%). A histogram of the CPD, expired CO and plasma COT from baseline smoking is found in Fig. 1 A-C. Many participants (42%, n = 294) had expired CO values of ≤ 10 ppm, the traditional cutoff for differentiating between smokers from non-smokers. In contrast, few individuals had plasma COT levels below the widely used cutoff value
of 14 ng/ml (3.1%, n = 22) (Benowitz, Peyton J Iii et al., 2002; Hukkanen, Jacob et al., 2005), or below the cutoff of 20 ng/ml used to verify smoking abstinence in this clinical trial (3.9%, n = 27) (Ahluwalia, Okuyemi et al., 2006).

Table 1: Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>N§</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD</td>
<td>700</td>
<td>7.2</td>
<td>2.4</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Expired CO (ppm)</td>
<td>699</td>
<td>13.7</td>
<td>8.7</td>
<td>0.0</td>
<td>69.0</td>
</tr>
<tr>
<td>Plasma NIC (ng/ml)</td>
<td>699</td>
<td>12.1</td>
<td>8.8</td>
<td>0.5</td>
<td>46.4</td>
</tr>
<tr>
<td>Plasma COT (ng/ml)</td>
<td>699</td>
<td>243.7</td>
<td>152.8</td>
<td>5.0</td>
<td>937.8</td>
</tr>
<tr>
<td>Plasma 3HC (ng/ml)</td>
<td>602</td>
<td>74.5</td>
<td>63.7</td>
<td>1.2</td>
<td>720.0</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>698</td>
<td>45.0</td>
<td>10.7</td>
<td>19.1</td>
<td>81.3</td>
</tr>
<tr>
<td>BMI</td>
<td>697</td>
<td>30.5</td>
<td>8.0</td>
<td>14.0</td>
<td>73.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N§</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>233</td>
<td>33.3</td>
</tr>
<tr>
<td>Females</td>
<td>467</td>
<td>66.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mentholated cigs</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>569</td>
<td>81.3</td>
</tr>
<tr>
<td>No</td>
<td>131</td>
<td>18.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Depth of inhalation</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep into chest</td>
<td>163</td>
<td>23.4</td>
</tr>
<tr>
<td>Partly into chest</td>
<td>220</td>
<td>31.5</td>
</tr>
<tr>
<td>To back of throat</td>
<td>143</td>
<td>20.5</td>
</tr>
<tr>
<td>To back of mouth</td>
<td>129</td>
<td>18.5</td>
</tr>
<tr>
<td>Puff only</td>
<td>43</td>
<td>6.2</td>
</tr>
</tbody>
</table>

§ Number of participants with data available for each variable. SD = standard deviations

**Relationship between expired CO, plasma COT and CPD**

CPD was significantly, albeit weakly, correlated with expired CO (Pearson’s r = 0.32, p < 0.001) and with plasma COT (Pearson’s r = 0.39, p < 0.001) (Fig 1D, E). Expired CO and plasma COT were also significantly correlated with each other (Pearson’s r = 0.60, p < 0.001) (fig 1F). We examined the ratio of expired CO or plasma COT to self-reported values of cigarettes smoked as indicators of the extent of inhalation. CPD was poorly correlated with CO/cigarette (Pearson’s r = -0.33, p < 0.001, fig 1G) and COT/cigarette (Pearson’s r = -0.08, p < 0.05, fig 1H).
Variables that influence CPD, expired CO and plasma COT

CYP2A6 slow metabolizers, as defined by genotype, had significantly higher plasma COT levels compared to normal metabolizers (p < 0.01), although CYP2A6 genotype was not associated with CPD or expired CO levels (Table 2, Fig. 2 A-C). Similarly, individuals with 3HC/COT ratios in quartile 1, representing those with slowest rate of CYP2A6 activity, also had significantly higher plasma COT levels compared to those in quartiles 2 – 4 (p < 0.001), although the 3HC/COT ratio was not significantly associated with either CPD or expired CO (Table 2, Fig. 2 D-F). CYP2A6 slow metabolizers by genotype were also found to have significantly higher plasma NIC (p < 0.001) and 3HC levels (p < 0.001), and those in the slowest 3HC/COT quartile had significantly higher plasma NIC and 3HC levels compared to those with the fastest metabolic activity (p < 0.001).

No gender difference was found for CPD or expired CO, though females trended towards higher plasma COT levels (p = 0.09, Table 2). Those who smoked mentholated cigarettes trended towards reporting fewer CPD compared to those who did not (p = 0.05), although no difference was found for expired CO or plasma COT levels between mentholated and non-mentholated cigarette smokers. Obese individuals (BMI ≥ 30) smoked fewer cigarettes (p < 0.05) and had
Fig 1 (A – C): Histogram of self-reported CPD and the biomarkers expired CO and plasma COT for study participants ($n = 700$). Cutoff values of expired CO at $\leq 10$ ppm and plasma COT levels of $\leq 14$ ng/ml have been traditionally used to differentiate smokers from non-smokers. Correlations are weak but significant between expired CO with CPD (D), plasma COT with CPD (E), and plasma COT with CO (F). Correlations are also significant between CO/cigarette with CPD (G) and COT/cigarette with CPD (H). Each point represents an individual. $r =$ Pearson’s correlation coefficient. Analyses were performed on log-transformed variables (CPD, expired CO, plasma COT) although raw data is plotted.
Table 2: Variables that influence CPD, expired CO or plasma COT levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>CPD Mean</th>
<th>CPD SD</th>
<th>CPD N</th>
<th>p-value</th>
<th>Expired CO (ppm) Mean</th>
<th>Expired CO (ppm) SD</th>
<th>Expired CO (ppm) N</th>
<th>p-value</th>
<th>Plasma COT (ng/ml) Mean</th>
<th>Plasma COT (ng/ml) SD</th>
<th>Plasma COT (ng/ml) N</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A6 genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>7.04</td>
<td>2.51</td>
<td>275</td>
<td>0.85</td>
<td>14.33</td>
<td>9.24</td>
<td>274</td>
<td>0.13</td>
<td>221.74</td>
<td>146.31</td>
<td>275</td>
<td>0.002</td>
</tr>
<tr>
<td>IM</td>
<td>6.97</td>
<td>2.56</td>
<td>70</td>
<td></td>
<td>11.94</td>
<td>6.90</td>
<td>70</td>
<td></td>
<td>249.17</td>
<td>149.06</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>7.14</td>
<td>2.44</td>
<td>197</td>
<td></td>
<td>13.53</td>
<td>9.22</td>
<td>197</td>
<td></td>
<td>272.63*</td>
<td>165.17</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>3HC/COT ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlations</td>
<td>0.001</td>
<td>0.98</td>
<td></td>
<td>-0.06</td>
<td>0.12</td>
<td>-0.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Categorical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fastest - Quartile 4</td>
<td>7.22</td>
<td>2.50</td>
<td>151</td>
<td>0.98</td>
<td>12.95</td>
<td>9.35</td>
<td>151</td>
<td>0.28</td>
<td>189.25</td>
<td>127.20</td>
<td>151</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>7.28</td>
<td>2.48</td>
<td>150</td>
<td></td>
<td>14.45</td>
<td>9.72</td>
<td>150</td>
<td></td>
<td>255.45</td>
<td>147.85</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>7.07</td>
<td>2.35</td>
<td>151</td>
<td></td>
<td>14.10</td>
<td>8.52</td>
<td>151</td>
<td></td>
<td>265.94</td>
<td>146.61</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Slowest - Quartile 1</td>
<td>7.22</td>
<td>2.44</td>
<td>150</td>
<td></td>
<td>12.89</td>
<td>6.88</td>
<td>150</td>
<td></td>
<td>278.62†</td>
<td>170.57</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>7.04</td>
<td>2.55</td>
<td>233</td>
<td>0.27</td>
<td>13.42</td>
<td>8.19</td>
<td>233</td>
<td>0.63</td>
<td>231.79</td>
<td>150.28</td>
<td>233</td>
<td>0.09</td>
</tr>
<tr>
<td>Females</td>
<td>7.20</td>
<td>2.38</td>
<td>467</td>
<td></td>
<td>13.88</td>
<td>8.98</td>
<td>466</td>
<td></td>
<td>249.60</td>
<td>153.84</td>
<td>466</td>
<td></td>
</tr>
<tr>
<td>Menthol cigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7.07</td>
<td>2.46</td>
<td>569</td>
<td>0.05</td>
<td>13.49</td>
<td>8.28</td>
<td>569</td>
<td>0.51</td>
<td>242.93</td>
<td>150.55</td>
<td>568</td>
<td>0.55</td>
</tr>
<tr>
<td>No</td>
<td>7.53</td>
<td>2.32</td>
<td>131</td>
<td></td>
<td>14.74</td>
<td>10.42</td>
<td>131</td>
<td></td>
<td>246.84</td>
<td>162.71</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlations</td>
<td>-0.03</td>
<td>0.39</td>
<td></td>
<td>-0.11</td>
<td>0.004</td>
<td><strong>0.003</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Categorical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 30.0)</td>
<td>7.35</td>
<td>2.41</td>
<td>381</td>
<td><strong>0.02</strong></td>
<td>14.35</td>
<td>8.74</td>
<td>380</td>
<td><strong>0.01</strong></td>
<td>268.19</td>
<td>156.37</td>
<td>380</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High (≥ 30.0)</td>
<td>6.90</td>
<td>2.46</td>
<td>316</td>
<td></td>
<td>12.97</td>
<td>8.70</td>
<td>316</td>
<td></td>
<td>213.57</td>
<td>142.65</td>
<td>316</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Variable</th>
<th>CPD</th>
<th>Expired CO (ppm)</th>
<th>Plasma COT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson’s r</td>
<td>p-value</td>
<td>Pearson’s r</td>
</tr>
<tr>
<td>Correlations</td>
<td>0.05</td>
<td>0.21</td>
<td>-0.001</td>
</tr>
<tr>
<td>Categorical</td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>19 – 29</td>
<td>6.32</td>
<td>2.27</td>
<td>60</td>
</tr>
<tr>
<td>40 – 49</td>
<td>7.16</td>
<td>2.48</td>
<td>280</td>
</tr>
<tr>
<td>50 – 59</td>
<td>7.41</td>
<td>2.45</td>
<td>158</td>
</tr>
<tr>
<td>60 – 77</td>
<td>7.04</td>
<td>2.51</td>
<td>52</td>
</tr>
<tr>
<td>Depth of Inhalation</td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Into chest</td>
<td>7.31</td>
<td>2.45</td>
<td>383</td>
</tr>
<tr>
<td>Puff/throat only</td>
<td>6.96</td>
<td>2.43</td>
<td>315</td>
</tr>
<tr>
<td>Other smokers at home</td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Yes</td>
<td>7.00</td>
<td>2.37</td>
<td>426</td>
</tr>
<tr>
<td>No</td>
<td>7.24</td>
<td>2.48</td>
<td>274</td>
</tr>
</tbody>
</table>

* denotes statistical significance in plasma COT levels in NM vs. SM; † denotes statistical significance in plasma COT levels in Quartile 4 (fastest activity) vs. Quartile 1 (slowest activity). No significant differences in plasma COT levels were found between IM vs. NM or SMs, or between the other quartiles. Raw data are listed although statistical analyses were performed on log-transformed data for CPD, expired CO and plasma COT. NM = normal metabolizers, IM = intermediate metabolizers, SM = slow metabolizers, SD = standard deviation.
Fig 2: Relationship between CYP2A6 activity, CPD, expired CO, and plasma COT. Self-reported CPD and expired CO did not differ by CYP2A6 genotype (A, B) or 3HC/COT quartiles (D, E), while CYP2A6 slow metabolizers had significantly higher plasma COT levels compared to normal metabolizers (* p < 0.01, C) and those in the slowest 3HC/COT quartile (1st quartile) had significantly higher plasma COT levels compared to those in the fastest quartile (4th quartile) (#, p < 0.001, F). NM = normal metabolizers, IM = intermediate metabolizers, SM = slow metabolizers. Analyses were performed on log-transformed variables (CPD, expired CO, plasma COT) although raw data are plotted.
lower levels of expired CO (p < 0.05) and COT (p < 0.001). BMI was not significantly correlated with CPD, but a negative correlation was found between BMI and expired CO (p < 0.01), and between BMI and plasma COT (p < 0.001). CPD, expired CO and plasma COT were not significantly associated with age, either by correlation analyses or when examined categorically. We did not find significant differences in CPD, expired CO or plasma COT by self-reported inhalation patterns or the presence of other smokers in the home (Table 2).

**Variables that influence relationships between CPD and expired CO with NIC and its metabolites**

The correlation coefficients between CPD and expired CO with plasma NIC and its metabolites were not greatly altered by *CYP2A6* genotype or 3HC/COT quartiles (Table 3). Similarly, these relationships were generally not altered when analyzed separately by gender, mentholated cigarettes, BMI or age. However, it is notable that the correlation between CPD and expired CO was stronger in females compared to males (Pearson’s r = 0.38 vs. 0.21, respectively, p < 0.05) and in obese (BMI ≥ 30) compared to non-obese individuals (Pearson’s r = 0.38 vs. 0.24, respectively, p < 0.05). No difference was observed for the correlations between CPD and plasma COT by gender or BMI.

In a multiple regression model including the predictors of CPD that were significant in univariate analyses (p < 0.10), plasma COT (β = 0.31) and expired CO (β = 0.13) remained significant (p < 0.01) while age and BMI were no longer significant, and the trend of mentholated cigarette use associating with fewer CPD remained (Table 4). Together, these predictors accounted for 17% of the variance in CPD.
Table 3: Correlations (r) between biomarkers and cigarette consumption by variables

<table>
<thead>
<tr>
<th></th>
<th>Total population (n = 700)</th>
<th>CYP2A6*1/*1 only (n = 275)</th>
<th>CYP2A6 variants only (n = 267)</th>
<th>3HC/COT§ Quartiles 2 – 4 (n = 451)</th>
<th>Slowest quartile§ (n = 151)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expired CO</td>
<td>0.32</td>
<td>0.30</td>
<td>0.33</td>
<td>0.28</td>
<td>0.36</td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.31</td>
<td>0.33</td>
<td>0.33</td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.39</td>
<td>0.40</td>
<td>0.44</td>
<td>0.39</td>
<td>0.33</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.31</td>
<td>0.36</td>
<td>0.31</td>
<td>0.39</td>
<td>0.27</td>
</tr>
<tr>
<td>Expired CO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.63</td>
<td>0.65</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.60</td>
<td>0.62</td>
<td>0.59</td>
<td>0.59</td>
<td>0.50</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.45</td>
<td>0.52</td>
<td>0.39</td>
<td>0.54</td>
<td>0.44</td>
</tr>
<tr>
<td>CPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expired CO</td>
<td>0.32</td>
<td>0.21</td>
<td>0.38</td>
<td>0.38</td>
<td>0.30</td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
<td>0.32</td>
<td>0.31</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.39</td>
<td>0.36</td>
<td>0.41</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.31</td>
<td>0.21#</td>
<td>0.36</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>Expired CO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.65</td>
<td>0.62</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.60</td>
<td>0.63</td>
<td>0.59</td>
<td>0.59</td>
<td>0.61</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.45</td>
<td>0.33</td>
<td>0.50</td>
<td>0.46</td>
<td>0.45</td>
</tr>
<tr>
<td>CPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expired CO</td>
<td>0.32</td>
<td>0.24</td>
<td>0.38</td>
<td>0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.31</td>
<td>0.27</td>
<td>0.34</td>
<td>0.28</td>
<td>0.34</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.39</td>
<td>0.34</td>
<td>0.43</td>
<td>0.36</td>
<td>0.42</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.31</td>
<td>0.29</td>
<td>0.33</td>
<td>0.31</td>
<td>0.32</td>
</tr>
<tr>
<td>Expired CO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NIC</td>
<td>0.63</td>
<td>0.61</td>
<td>0.64</td>
<td>0.66</td>
<td>0.59</td>
</tr>
<tr>
<td>Plasma COT</td>
<td>0.60</td>
<td>0.54</td>
<td>0.65</td>
<td>0.61</td>
<td>0.59</td>
</tr>
<tr>
<td>Plasma 3HC§</td>
<td>0.45</td>
<td>0.40</td>
<td>0.51</td>
<td>0.54</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Values listed are Pearson’s correlation coefficients calculated on log-transformed variables (CPD, expired CO, plasma NIC, COT, 3HC); all were significant at p < 0.001 with the exception of the value marked as #, which was significant at p < 0.01. §3HC data were available for a subset of the participants only.
Table 4: Multiple linear regression models of the predictors of CPD, expired CO and plasma COT

<table>
<thead>
<tr>
<th>Dependent variable: CPD (n = 700), R² = 0.17</th>
<th>Predictor</th>
<th>B</th>
<th>95% CI</th>
<th>Standardized β</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma COT</td>
<td>0.15</td>
<td>0.11 – 0.19</td>
<td>0.32</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Expired CO</td>
<td>0.09</td>
<td>0.03 – 0.15</td>
<td>0.12</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Mentholated cigs</td>
<td>-0.03</td>
<td>-0.06 – 0.00</td>
<td>-0.07</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.08</td>
<td>-0.04 – 0.20</td>
<td>0.05</td>
<td>0.21</td>
<td></td>
</tr>
</tbody>
</table>

Non-mentholated cigarette users were coded as 0. Variables that were not normally distributed (CPD, expired CO, plasma COT, BMI) were log-transformed in the analyses.

Discussion

In this population of African-American light smokers, where approximately one-third consume ≤ 5 CPD, two commonly used biomarkers of cigarette smoke exposure, expired CO and plasma COT, were significantly correlated with self-reported CPD. However, the strength of the correlations were relatively weak (r ~ 0.31 – 0.37), and in a multiple regression model, only ~17% of the variance in CPD was explained by plasma COT and expired CO. This is in contrast to heavy smoking Caucasian populations, where correlation coefficients from 0.3 – 0.8 have been reported (Perez-Stable, Benowitz Ni et al., 1995; Domino and Ni L, 2002; Mustonen, Spencer Sm et al., 2005; Scherer, 2006). Self-reported number of cigarettes smoked per day is a limited indicator of exposure as there is a nonlinear relationship between biomarkers and CPD, with a plateau observed at higher levels of consumption (>20 – 25 CPD) (Joseph et al., 2005). Heavy smokers reporting consumption at these levels appear to smoke each cigarette with less intensity (Joseph, Hecht et al., 2005; Malaiyandi, Goodz et al., 2006). Thus, self-reported measures of CPD may not be representative of exposure particularly at extremely high or low levels of smoking.

It would have been ideal to compare our findings with a matched group of Caucasian light smokers from a clinical trial (e.g. treatment seekers) to determine whether the weaker
correlations between the biomarkers and self-reported cigarette consumption in this study were reflective of variables that were specific to African-Americans, or resulted from the narrow range in cigarettes consumed. However, established light smoking patterns among adults are less common among Caucasians, and the clinical trial from which participants in the current study was drawn is the only published one to date to have recruited specifically light smokers (≤ 10 CPD) (Stead, Perera R et al., 2008). To partially address this issue, we analyzed a subset of Caucasian smokers that reported ≤ 10 CPD in our previously published biomarkers paper (Malaiyandi, Goodz et al., 2006). Despite the considerably smaller numbers in this subset analyses (N = 40 vs. 152) the correlation coefficients appeared higher in the Caucasian light smokers. Specifically, the Pearson’s correlation coefficient between CO with CPD (r = 0.37, p = 0.02) was similar, while the correlations between COT with CPD (r = 0.51, p < 0.001), and CO with COT (r = 0.77, p < 0.001) were stronger than in African American light smokers (r = 0.32, 0.39 and 0.60 respectively, Fig 1). The correlations between CO and CPD were improved when the total sample of Caucasians in that study (n = 152, mean CPD = 19.4) (Malaiyandi, Goodz et al., 2006) was examined (r = 0.60, p < 0.001), although the relationships between COT and CPD (r = 0.53, p < 0.001) and CO with COT (r = 0.74, p < 0.001) remained similar. Thus, it appears that COT may be a poorer biomarker of cigarette consumption in African-American light smokers compared to Caucasians, and as expected CO appears poorly correlated with CPD in all light smokers. Furthermore, in a subset of heavy-smoking, treatment-seeking African-Americans, in which these variables were available, recruited for a clinical trial testing the efficacy of bupropion (n = 93) (Ahluwalia, Harris et al., 2002), both CO and COT were poorly correlated with self-reported cigarette consumption (r = 0.20, p = 0.05 for CO with CPD, and r = 0.05, p = 0.62 for COT with CPD). Together this suggests that CO is a poor correlate of cigarette consumption in light smokers in general, while COT is a poor correlate of cigarette consumption among African-American smokers.
Traditional cutoff levels of expired CO and plasma COT for differentiating between smokers and nonsmokers have previously been determined primarily in heavy smoking Caucasian populations (Jarvis, Tunstall-Pedoe H et al., 1987; Waage et al., 1992). Our results suggest that using expired CO $\leq$ 10 ppm to verify smoking status in light smokers may result in misclassification of smokers as nonsmokers, as ~40% of our treatment-seeking sample of smokers had expired CO levels below this limit. In contrast, very few individuals (3.1%) had plasma COT levels below the traditional cutoff of 14 ng/ml. This cutoff value was determined more than 20 years ago when there were high levels of secondhand smoke (Jarvis, Tunstall-Pedoe H et al., 1987).

Recently, it was suggested that the plasma COT cutoff should be further reduced to 3 ng/ml, with optimal cutoff revised to 6 ng/ml for African-Americans (Benowitz et al., 2009). This revised cutoff of 6 ng/ml would misclassify only 2.5% of smokers as nonsmokers in this sample. While further studies will be needed to precisely determine the optimal cutoff points for expired CO and plasma COT among African-American light smokers, our study suggests plasma COT may be a better indicator of smoking status than expired CO.

The second objective of this study was to determine whether other variables (i.e. CYP2A6 activity, gender, age, BMI, smoking mentholated cigarettes) influence biomarker levels (expired CO, plasma COT) or their relationships to self-reported CPD. Individuals with slow CYP2A6 activity, as indicated by genotype and 3HC/COT, had significantly higher plasma COT levels despite similar intake as represented by self-reported CPD and expired CO values. COT clearance rates were previously found to be reduced by ~35% in individuals with CYP2A6 genetic variants (Benowitz, Swan et al., 2006), and in this study, COT levels are ~20 – 30% higher in individuals with slow CYP2A6 activity. Despite its substantial effect on COT levels however, CYP2A6 activity did not greatly alter the correlations between NIC or its metabolites
with self-reported CPD or expired CO levels, similar to what was observed in our previous study of Caucasian heavy smokers (Malaiyandi, Goodz et al., 2006).

Individuals with higher BMI were found to have significantly lower plasma COT levels (r = -0.19), as well as lower NIC (r = -0.16) and 3HC (r = -0.26) in this study. A negative correlation between BMI and COT levels has been previously reported (r = -0.16 to -0.36) (Perez-Stable, Benowitz Nl et al., 1995; Ahijevych, Tyndale Rf et al., 2002). It is possible that differences in BMI may result in altered volumes of distribution for NIC and its metabolites, thus resulting in altered plasma levels. The volume of distribution for NIC and COT have been correlated with total body weight and lean body mass (r = 0.23 – 0.67), although no significant correlation was found between the volume of distribution with adipose mass (Benowitz, Perez-Stable et al., 1999). BMI has also been negatively associated with the 3HC/COT ratio (Mooney, Li et al., 2008; Swan Ge, Lessov-Schlaggar Cn et al., 2008; Ho, Mwenifumbo et al., 2009), suggesting obesity and rate of CYP2A6 activity may be related, although this has not been examined explicitly. While the lower plasma COT levels in individuals with higher BMI may also be interpreted as lower exposure to cigarette smoke, this is unlikely as BMI was not significantly associated with expired CO or CPD in multiple regression analyses. As such, it is yet unclear whether the relationship between BMI and COT represents altered rates of COT metabolism (altered CYP2A6 activity), or volume of distribution, or a combination of both. Interestingly, expired CO appeared to be a better measure of exposure in obese individuals (BMI ≥ 30.0) as the correlation to CPD appeared stronger in these individuals.

It has been proposed that the cooling sensation associated with smoking mentholated cigarettes allows for increased smoke intake. Thus, this may contribute to the higher COT levels and disproportionately higher incidences of tobacco-related illnesses among African-Americans, who
because of the influence of marketing campaigns in the 1960s, predominantly smoke mentholated cigarettes (Balbach, Gasior et al., 2003). However, while some cross-sectional and experimental studies have found higher CO and COT levels among mentholated cigarette smokers, this has not been consistently replicated (Werley, Coggins et al., 2007). Studies finding an effect were generally of small sample size, and included both heavy-smoking Caucasians and African-Americans. In this current study of African-American light smokers, mentholated cigarette users did not have significantly higher expired CO or plasma COT levels despite our large sample, with 131 non-menthol smokers examined. Thus, cigarette mentholation did not appear to contribute to increased intensity of cigarette smoking or increased absorption of nicotine in our sample of African-American light smokers.

No differences in CPD, expired CO or plasma COT were found by age, in contrast to previous findings where older individuals had higher COT levels (Swan, Habina K et al., 1993; Patterson, Benowitz et al., 2003). In general, drug metabolism is thought to decrease by age (Wynne, 2005), and while NIC clearance rates are reduced in the elderly (age >65), this has been attributed to age-related changes in hepatic blood flow as no differences in CYP2A6 protein by age have been observed (Benowitz et al., 2009). The renal clearance of COT is also reduced in the elderly, although pharmacokinetic parameters such as area-under-the-curve and elimination half-lives are not altered (Molander, Hansson et al., 2001). Accordingly, the relationships between NIC or its metabolites with CPD or expired CO did not greatly differ among mentholated cigarette users or by age.

We did not find any gender differences in CPD, expired CO, or plasma COT. However, in the present study the proportion of variance in CPD explained by expired CO was more than tripled in females compared to males (14.4% vs. 4.4%, respectively). This was unlikely due to
differences in the type of cigarettes smoked, as there was no difference in prevalence of mentholated cigarette use by gender. A number of studies have reported gender differences in smoking topography, with males taking larger and longer puffs compared to females (Battig et al., 1982; Eissenberg et al., 1999; Melikian et al., 2007). However, we did not observe any differences in CO/cigarette or COT/cigarette by gender in this population (p > 0.10). Among African-American light smoking males, there may be more variability in the manner in which cigarettes are smoked, resulting in weaker relationships between self-reported CPD and expired CO.

One limitation of our study is that it relied on secondary analyses performed on participants that were originally recruited for a clinical trial on smoking cessation, and may not be representative of African-American light smokers in the general population. Thus, biochemical measures were collected randomly from ad libitum smoking and the time of the last cigarette may have been a significant source of variation, particularly for expired CO where the half-life is short. It is possible that this treatment-seeking sample may have attempted to stop smoking prior to the start of the trial; however, we excluded from these analyses any participants that reported smoking zero cigarettes within the past seven days prior to the collection of biochemical data. That said, we cannot exclude the possibility that there may have been a selection bias as participants were light smokers who were highly motivated to quit smoking and had difficulty quitting in the past. Thus, they may be more dependent or smoke cigarettes differently from other non-treatment seeking light smokers. In addition, participants may have underreported their cigarette consumption to meet the inclusion criteria for the clinical trial. The average plasma COT levels derived from ad libitum smoking (244 ng/ml) is similar to those found previously in a heavier smoking population of African-Americans (292 ng/ml) recruited for a clinical trial of smoking cessation at the same community health centre as the current study, with an inclusion criterion of
smoking at least 10 CPD (mean = 17 CPD) (Ahluwalia, Harris et al., 2002). It is also possible that self-reported CPD is a poor measure of average cigarette consumption among individuals at this low level of smoking, which may in part explain their weak correlations to the biomarkers. In a previous study of an African-Canadian light smoking population (median of 8 CPD), we found some discrepancies of cigarette consumption when it was reported as cigarettes per day, versus cigarettes per week (CPW), versus cigarettes per month (CPM) (Mwenifumbo JC, Tyndale RF, personal communications). For example, one individual reported consuming 6 CPD, but 18 CPW and 60 CPM. Thus, in light smokers where daily smoking is variable and smoking may occur at irregular intervals, CPD may be a poor indicator of consumption and alternative measures of self-report, such as timeline follow-back procedures (Brown et al., 1998), need to be tested. It is also notable that a large portion of the participants (45%) reported puffing or inhaling as far as the throat only. While self-reported measures of depth of inhalation may not be representative of actual smoking behaviours (Tobin Mj et al., 1982), this may be another source of variability in cigarette exposure among these light smokers.

In summary, the results from this study suggest that the commonly used biomarkers of cigarette smoke exposure, expired CO and plasma COT, are significantly but weakly correlated with self-reported CPD. Furthermore, these relationships are not greatly altered by variables that were previously reported to have an influence on these parameters, such as CYP2A6 activity, smoking mentholated cigarettes or age, although the relationships may differ slightly by gender and BMI. The proportion of variance in CPD explained by expired CO and plasma COT was generally lower than that observed in heavy Caucasian smokers even after accounting for these variables, suggesting these biomarkers are limited as indicators of cigarette smoke exposure among African-American light smokers.
Our study suggests that expired CO may be a poor indicator of smoking status as many smokers had expired CO levels below the traditionally defined cutoff level. While plasma COT may be useful in ascertaining smoking status in this population, the level is highly influenced by the rate of CYP2A6 activity, and it is also a poor indicator of the levels of smoke exposure. This suggests the rate of CYP2A6 activity needs to be considered when COT is used as a biomarker of intake in African-American populations, where there are higher proportions of individuals with reduced rates of CYP2A6 activity compared to Caucasians. A number of other biomarkers such as thiocyanate or the tobacco alkaloids anabasine and anatabine have also been proposed (Benowitz, Peyton J Iii et al., 2002); however, these also have their own set of limitations in terms of specificity, sensitivity and cost for detection. Validated biomarkers are important for ascertaining smoking status before recruitment into research studies or clinical trials for smoking cessation, or for verifying abstinence among light smokers. In addition, validated biomarkers of cigarette smoke exposure are also necessary for the proper assessment of the dose-related risk of smoking and health outcomes in epidemiological studies of African-Americans, a population that have been reported to have a disproportionately elevated risk of developing tobacco-related illnesses despite lower levels of self-reported cigarette consumption (Centers for Disease Control and Prevention, 1998; Haiman, Stram et al., 2006).

**Significance to thesis**

Biomarkers of exposure to tobacco smoke are needed due to the limitations of self-reports. Common biomarkers of tobacco smoke exposure have only been previously validated in Caucasian moderate to heavy smokers. This is the first study to examine the relationship between biomarkers and self-reported cigarette consumption, and investigate variables that may influence either tobacco smoke exposure or cotinine metabolism in a light smoking population. Validating appropriate biomarkers of tobacco smoke exposure among African-Americans is
particularly important given their low and sporadic patterns of smoking, their greater use of menthol cigarettes which may result in greater tobacco smoke exposure, and their slower rates of cotinine metabolism.

The finding that exhaled CO is a poor biomarker has important implications as it is commonly used to ascertain smoking status in clinical trials. Exhaled CO is the only available method of biochemically validating smoking abstinence in research studies that administer NRT to participants as cotinine cannot be used in these cases. Exhaled CO and cotinine were weakly correlated with self-reported cigarette consumption, and the correlations between the two biomarkers themselves were also poor. Factors previously shown to alter tobacco smoke exposure and/or cotinine metabolism (i.e. gender, menthol cigarettes, BMI, or age) did not account for additional variability in exhaled CO or cotinine levels, although CYP2A6 activity significantly altered cotinine level. Furthermore, the correlations between the biomarkers and cigarette consumption did not improve after accounting for these variables. This suggests that previously validated biomarkers of tobacco smoke exposure have limitations among African-American light smokers. Determining appropriate biomarkers to quantify tobacco exposure in this population is necessary in light of their higher risk of tobacco-related illnesses despite lower self-reported cigarette consumption. It remains to be determined whether this paradox is the result of African-Americans having greater exposure to tobacco smoke than is subjectively reported, whether they have greater biological sensitivity towards developing tobacco-related illnesses such as cancer, or whether disparities in demographic variables (e.g. socioeconomic status) may be contributing.
GENERAL DISCUSSION

SECTION 1: IDENTIFYING AND DETERMINING THE FUNCTIONAL IMPACT OF NOVEL CYP2A6 GENETIC VARIANTS

1.1  African-Americans: a population with rich genetic diversity

1.1.1  Origins of genetic variation in modern Homo sapiens

Current paleontological and genetic data support the “Recent African Origin” model that proposes anatomically modern Homo sapiens evolved in Africa approximately 200,000 years ago and dispersed globally within the past 100,000 years (Campbell et al., 2010). The size of the ancestral population leaving Africa has been estimated at 1000-1500 males and females based on autosomal microsatellite loci, mitochondrial DNA, and X- and Y- chromosome re-sequencing data (Liu et al., 2006; Garrigan et al., 2007). The geographic expansion of this small ancestral group of modern humans resulted in a large population bottleneck that greatly reduced the genetic diversity in subsequent populations. Indeed, higher levels of nucleotide and haplotype diversity have been reported among African populations (Campbell and Tishkoff, 2010), with non-African populations becoming less genetically diverse as they are found at increasing distance away from Africa (Tishkoff et al., 2009). Multiple forms of genetic variation including SNPs, microsatellite markers and large structural changes appear to be shared between various racial/ethnic populations, suggesting they existed in modern humans prior to their migration out of Africa (Redon et al., 2006; Korbel et al., 2007). Thus, there was likely one single major migration event occurring out of Africa, or multiple migrations from a single genetically homogeneous ancestral population, rather than migrations from multiple genetically distinct sources (Reed et al., 2006; Campbell et al., 2008; Campbell and Tishkoff, 2010).

1.1.2  The African Diaspora: Origins of African-Americans
According to recent census data, individuals of Black-African descent comprise 2.5% of the Canadian population (Statistics Canada, 2006) and 12.4% of the American population (Grieco, 2009). Populations of Black-African descent in North America today can trace their recent origins back to Africa, with large waves of migration occurring during the Trans-Atlantic Slave Trade beginning in the 15th century. The precise estimates vary, but documentation suggests approximately 11-13 million slaves were brought over to the Americas with the majority originating from West Africa (Thomas, 1997). As a result of their complex history, African-Americans are an admixed population with an average of 80% African ancestry and 20% European ancestry (Parra et al., 1998; Halder et al., 2009; Bryc et al., 2010). The degree of admixture can vary widely between individuals, with estimates ranging from as much as 99% to less than 1% African ancestry (Halder, Yang et al., 2009; Bryc, Auton et al., 2010). Historical records and genetic evidence suggest the admixture began fairly recently within the past 20 generations (Lohmueller et al.).

As a result of their genetic ancestry and complex demographic history, African-Americans are also a population with high levels of genetic diversity (Campbell and Tishkoff, 2010). In a large sequencing study of 3873 genes, African-Americans had the highest number of SNPs found in comparison to Caucasians, Latino/Hispanics, and Asians (Guthery et al., 2007). African-Americans also had the highest percentage of rare SNPs, with 64% of the SNPs identified occurring at a minor allele frequency of less than 5% (Guthery, Salisbury et al., 2007). Moreover, 44% of all SNPs were found specifically in this population (Guthery, Salisbury et al., 2007). Similarly, African-Americans had the highest number of SNPs found in a large-scale gene sequencing project of \textit{CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4} and \textit{CYP3A5} in comparison to Caucasians or Asians (Solus Jf, 2004). As such, African-Americans are a genetically diverse population.
with a large number of unique genetic variants, each occurring at low frequencies. The functional effects for many of these genetic variants remain to be characterized.

1.1.3 Evolutionary forces driving genetic diversity

The human genome has 3.2 billion base pairs that are 99.9% identical; the 0.1% variability represents the 10 to 15 million SNPs that differ between individuals (Kruglyak et al., 2001; Venter et al., 2001). This genetic diversity is the result of various evolutionary processes including genetic drift and natural selection. Genetic drift is the change in allele frequency from one generation to the next as a result of random sampling processes. It is not driven by environmental or adaptive pressures and may result in beneficial, deleterious or neutral changes (Miller et al., 2009). The consequences of genetic drift are particularly apparent when population sizes are dramatically reduced, such as during population bottlenecks or founder effects (Miller, Vandome et al., 2009). Alternatively, genetic loci may be the target of natural selection whereby variants with favorable adaptive fitness are maintained and deleterious variants are removed (Bamshad et al., 2003). Genetic drift processes tend to influence the level of diversity across the genome (Tishkoff et al., 2003), whereas natural selection tends to act upon specific genetic loci (Tishkoff and Verrelli, 2003).

It may be difficult to distinguish between neutral and adaptive human genetic variation as both genetic drift and selection are almost always acting simultaneously within populations (Tishkoff and Verrelli, 2003). A number of methods have been developed to determine if patterns of genetic variation are the result of selective pressures (Tishkoff and Verrelli, 2003). Under neutral evolutionary processes, new genetic variants require a long time to reach high frequency in a population, and the surrounding linkage disequilibrium decays substantially as a result of recombination (Sabeti et al., 2002; Bamshad and Wooding, 2003;
In contrast, variants under positive selection will increase in allele frequency rapidly such that recombination does not substantially alter the associated haplotype relationships (Sabeti, Reich et al., 2002; Bamshad and Wooding, 2003; Nielsen, 2005; Tang, Thornton et al., 2007; Luca, Perry et al.). As such, some statistical models used to identify regions of selection test whether the allele has unusually long patterns of linkage disequilibrium compared to other regions in the genome (Sabeti, Reich et al., 2002; Bamshad and Wooding, 2003; Nielsen, 2005; Ennis, 2007; Tang, Thornton et al., 2007; Oleksyk et al.; Luca, Perry et al.). Neutral variants that are in linkage disequilibrium with the genetic variant under selection are more likely to be maintained in the population, resulting in an excess of low frequency variants (Sabeti, Reich et al., 2002; Bamshad and Wooding, 2003; Nielsen, 2005; Tang, Thornton et al., 2007; Luca, Perry et al.). Other statistical models used to identify signatures of selection examine the degree of genetic variability within the region of interest (Sabeti, Reich et al., 2002; Bamshad and Wooding, 2003; Nielsen, 2005; Tang, Thornton et al., 2007; Luca, Perry et al.). In addition, genetic variants that do not alter protein expression or activity likely reflect neutral evolutionary processes whereas functional variants are predicted to be under greater adaptive pressures. A number of statistical models have been used to compare the degree of divergence or conservation of genetic variants within and between species (Sabeti, Reich et al., 2002; Bamshad and Wooding, 2003; Nielsen, 2005; Tang, Thornton et al., 2007; Luca, Perry et al.).

1.1.4 Genetic association studies in African-Americans

1.1.4.1 Utility and limitations of self-reports of racial/ethnic ancestry

The use of self-reported race/ethnicity as a surrogate biological construct has been the topic of much debate, particularly as it may be inadvertently viewed as promoting notions of biological determinism and justification for the mistreatment of one group by another. While
it has been argued that racial/ethnic categorization is primarily a socially and culturally-defined construct, it cannot be denied that diseases affect populations differentially. Furthermore, categorization based on similarity of random genetic markers has been shown to correlate well to ancestral continent of the origin and to self-defined racial/ethnic group (Rosenberg et al., 2002; Tang et al., 2005). The sociocultural and socioeconomic factors contributing to the epidemiology and health disparities of complex diseases such as tobacco addiction are also likely similar among self-defined racial/ethnic populations (Collins, 1996; Betancourt et al., 2003). Racial/ethnic identities may also serve as a proxy for shared beliefs and acceptable behavioural norms for the use of cigarettes, alcohol and other drugs of abuse (Castro, 2004).

1.1.4.2 Importance of performing studies in different racial/ethnic groups

Genetic association studies for complex diseases have mainly been performed among individuals of Caucasian ancestry, even though much information can be gained from this type of research in other racial/ethnic populations. The prevalence of complex diseases including tobacco addiction differ widely across racial/ethnic groups, and unique genetic factors may be contributing to the disease phenotype among these different groups (Fagan, Moolchan Et et al., 2007). The effect sizes of genetic risk variants may differ across racial/ethnic populations. Furthermore, the frequency of genetic risk variants may differ between populations, or they may have different functional effects depending on their haplotype relationship with other genetic variants. Different haplotypes of the chemokine receptor gene \( CCR5 \) have been associated with altered rates of HIV-1 progression in Caucasians compared to African-Americans (Gonzalez et al., 1999). In addition, the effects of \( CYP2D6 \) genetic variants on enzyme function differ between African-Americans and Caucasians as a result of differences in haplotype relationships (Gaedigk et al., 2002). Thus,
examination of genetic factors previously associated with tobacco addiction phenotypes among Caucasians in other racial/ethnic populations is necessary to validate their generalizability and relevance, as well as account for any population-specific gene by environment interactions.

One challenge of performing genetic association studies in admixed populations such as African-Americans is the possibility of population stratification. Spurious associations (either false positives or negatives) between risk alleles and outcomes may be found if cases and controls are sampled from an admixed population where the proportion of ancestry differs in individuals, and the frequency of outcome and risk alleles examined differs between these ancestral populations (Thomas et al., 2002; Wacholder et al., 2002). Several statistical methods have been proposed to account for this potential confound. Structured Association and Genomic Control methods use ancestry informative markers that are located throughout the genome and are unlinked to the candidate locus of interest to estimate the ancestry of individuals within a sample and test whether they differ between cases and controls (Li et al.; Enoch et al., 2006; Need et al., 2009; Wang et al., 2010).

While we were not able to account for the possibility of population substructure within our sample, this highlights the importance of replication of genetic association studies. Participants recruited from a community health care center in Kansas City for our clinical trial of light smokers were self-reported as African-American or Black ethnicity. Approximately 60% of the sample reported having three or more grandparents of African heritage, while the other 40% had mixed or unknown grandparent ancestry. In spite of this, the frequency of CYP2A6 alleles that tend to be more representative of European or African ancestry did not differ according to self-reported grandparent ancestry. Similarly, the
distribution of CYP2A6 normal, intermediate and slow metabolizers did not differ by the ancestry of grandparents. Thus, there does not appear to be a disproportionate distribution of African or European ancestry among the various CYP2A6 metabolizer groups. CYP2A6 allele frequencies were also comparable between African-Americans in this study and those reported in a population of Black-African descent recruited from Canada (Appendix C), suggesting our sample of African-Americans was not likely to be distinctly admixed. Furthermore, we were able to corroborate our results using both genetic and phenotype (3HC/COT) data.

1.2 Origins of CYP2A6 polymorphisms

1.2.1 Types of genetic polymorphisms

Within the past five years, the number of identified CYP2A6 alleles has increased from 22 to 37 (http://www.cypalleles.ki.se/cyp2a6.htm). CYP2A6 is located on chromosome 19q13.2, a region with a high rate of recombination (Wang et al., 2009). Many of the known CYP2A6 genetic variants are single nucleotide conversions, truncations, duplications, or chimeras resulting from mispairing or unequal crossovers between CYP2A6 and the nearby CYP2A7 locus during meiotic recombination (Hoffman and Hu, 2007). This suggests high rates of recombination exist even between tandem pairs of paralogs (i.e. CYP2A6 and CYP2A7) within the CYP2ABFGST gene cluster. In contrast, allelic variants of the polymorphic CYP2A13, which does not have a nearby paralog, are mainly single nucleotide base substitutions (Hoffman and Hu, 2007).

Copy number variants (CNVs), whereby DNA segments ranging in size from thousands to several million base pairs exist in variable number of copies, have also been the recent focus of much research attention. CNVs were initially thought to be rare events. However,
currently annotated CNVs cover approximately 29% of the genome and more than 5600 CNV loci have been identified (http://projects.tcag.ca/variation) (Iafrate et al., 2004; Mcelroy et al., 2009). As such, CNVs are a major source of human diversity with a potentially large impact on function. Seven versions of the $CYP2A6$ deletion allele and two versions of the $CYP2A6$ duplication allele, each with a different crossover junction, have been identified to date. Genetic polymorphisms in other CYP enzymes that result in variable copies of the gene have also been identified. For example, individuals can have anywhere from 0 to 13 functional copies of the $CYP2D6$ gene (Ingelman-Sundberg et al., 2007).

While the functional characterization of genetic variation has primarily focused on nonsynonymous SNPs encoding amino acid changes, cis-acting genetic polymorphisms that influence gene expression may also be functionally significant. Synonymous SNPs may contribute to differences in gene expression by altering mRNA splicing or mRNA turnover rates (Johnson et al., 2005). Evidence for allelic expression imbalance, whereby expression of the paternal and maternal copies of the gene differs due to the presence of genetic polymorphism(s) on one of the alleles, have been reported for $CYP3A4$ (Hirota et al., 2004), $CYP1A2$ (Ghotbi et al., 2009), $CYP2D6$ (Johnson et al., 2008), $MAO-A$ (Pinsonneault et al., 2006), $TPH2$ (Lim et al., 2006; Johnson, Zhang et al., 2008), $OPRM1$ (Zhang et al., 2005; Johnson, Zhang et al., 2008) and $DRD2$ receptors (Zhang et al., 2007; Johnson, Zhang et al., 2008).

1.2.2 Discovery of novel $CYP2A6$ alleles

African-Americans are an admixed population with contributions from both European and African ancestry. Therefore, unique allele frequencies and patterns of linkage disequilibrium are present in their genomes. The high levels of genetic diversity among populations of
Black-African descent suggest there are likely unidentified sources of variations with functional consequences. Within the past five years, seven new numbered \textit{CYP2A6} genetic alleles consisting of SNPs have been identified (\textit{CYP2A6*23, CYP2A6*24, CYP2A6*25, CYP2A6*26, CYP2A6*27, CYP2A6*28, and CYP2A6*35}) (Appendix C), in addition to several new deletion alleles and a novel duplication allele, among populations of Black-African descent (Ho, Mwenifumbo Jc et al., 2008; Mwenifumbo, Al Koudsi N et al., 2008; Al Koudsi, Ahluwalia Js et al., 2009; Mwenifumbo, Zhou Q et al., 2010). Many of these have substantial impact on reducing enzyme function and appear to be found specifically in this population, which may help explain the slower rates of nicotine and cotinine clearance observed between African-Americans and Caucasians. Large sequencing projects have likely identified the more common genetic variants in this population and full gene sequencing of phenotypic outliers will result in the discovery of additional rarer alleles. The development of a copy number variation assay for this gene will also help identify large structural variants such as gene deletions and duplications with crossover junctions that are not detected using currently available genotyping assays.

1.2.3 Evolution of CYPs and their genetic variants

Cytochromes P450 were thought to have emerged in an ancestral prokaryotic species as early as 1.5 billion years ago (Lewis et al., 1998). These enzymes are found in virtually every living species and have undergone extensive expansion and modification (Ingelman-Sundberg, 2005). The number of \textit{CYP2} genes quickly expanded approximately 400 million years ago when animals first became land-bound and presumably began consuming terrestrial plant forms (Nebert, 1997). Considerable inter-individual and inter-ethnic differences in CYP enzyme expression and function are observed among humans. The domestication of plants and animals approximately 10 000 years ago led to a rapid increase
in human population size, with these new food sources becoming available at the same time as the development of new cultural environments and the spread of new infectious diseases (Sabeti, Reich et al., 2002). Many important genetic adaptations in humans were thought to have arisen at this time (Sabeti, Reich et al., 2002).

Genetic variability in CYP enzymes is likely the result of both random genetic drift due to migration of populations, as well as adaptive responses due to selection. Because of their roles in the detoxification of xenobiotics, including dietary plant toxins, CYP enzymes are likely targets of natural selection (Bamshad and Wooding, 2003). Genetic variation in CYP2D6, an enzyme that metabolizes a number of plant alkaloids but does not appear to be readily inducible, may have been a result of selection (Ingelman-Sundberg, 2005; Ingelman-Sundberg, 2005). Genetic variants resulting in multiple copies of active CYP2D6 genes are prevalent among individuals in North-East Africa, suggesting selection may have played a role in increasing the variety of food sources that can be consumed, particularly under detrimental environmental conditions (Ingelman-Sundberg, 2005; Ingelman-Sundberg, 2005). The pattern of SNPs found in a 3.7 kb sequence in the 5’-regulatory region of the CYP1A2 gene is also indicative of recent positive selection (Wooding et al., 2002).

It is plausible that CYP2A6 was the target of natural selection. The gene deletion allele is highly prevalent among Asian populations, with approximately 4% of Chinese and approximately 12% of Japanese individuals completely lacking functional copies of CYP2A6 (Mwenifumbo and Tyndale, 2007). However, these individuals do not appear to suffer from any deleterious effects, and deletion of the homologous gene Cyp2a5 in mice does not result in lethality or developmental deficits (Tomizawa and Casida, 2003). In addition, the gene duplication alleles are not commonly found, occurring at less than 2% allele frequency across
various racial/ethnic populations (Schoedel, Hoffmann Eb et al., 2004; Nakajima, Fukami et al., 2006; Fukami, Nakajima et al., 2007). Endogenous substrates of CYP2A6 have not been identified, although Cyp2a5 in mice was found to contribute to the metabolism of bilirubin under conditions of oxidative stress induced by cadmium (Abu-Bakar et al., 2005). A combination of neutral genetic drift processes and adaptive selection likely contributed to the large variability in CYP2A6 allele frequencies observed between different racial/ethnic groups. Future studies investigating potential evidence of positive selection will help clarify its role in shaping the variability observed in CYP2A6.

1.3 Comparison of CYP2A6 genetic variability between racial/ethnic groups

Racial/ethnic differences in the frequencies of CYP2A6 alleles and rates of nicotine and cotinine metabolism have been observed (Mwenifumbo and Tyndale, 2007). CYP2A6 genetic variants that are known to reduce enzyme function occur at low frequencies in Caucasians but are found in a much larger proportion of African-Americans and Asians. Accordingly, nicotine and cotinine clearance are fastest among Caucasian individuals and reduced by 20 to 25% in African-Americans and Chinese-Americans (Benowitz, Perez-Stable et al., 1999; Benowitz, Perez-Stable et al., 2002). Interestingly, the profile of CYP2A6 variants varies widely between Asians and African-Americans. Approximately 50 to 60% of Asians have CYP2A6 genetic variants known to reduce enzyme function. This is primarily due to the high frequency of the gene deletion allele CYP2A6*4 and the loss-of-function SNP CYP2A6*7. In contrast, 35 to 40% of African-Americans have CYP2A6 variants known to reduce enzyme function, although many of these occur at less than 2% allele frequency (e.g. CYP2A6*23, CYP2A6*24, CYP2A6*25, CYP2A6*26, CYP2A6*27, CYP2A6*28, and CYP2A6*35). This suggests that different evolutionary processes may have been responsible for the distribution of CYP2A6 genetic variants across various racial/ethnic populations.
1.4 Functional consequences of *CYP2A6* genetic variants

Determining the functional consequences of genetic variants will provide a biological rationale for how the gene may be associated with disease states. Bioinformatic programs can be used to predict the effect of an amino acid change based on its physicochemical properties, and phylogenetic analyses can determine its degree of evolutionary conservation. SNPs can alter enzyme function through various mechanisms. These include alterations in the affinity of the enzyme for substrate binding or product release, a shift in the orientation of substrate with the heme moiety, or ability of the enzyme to interact with required co-enzymes and co-factors to facilitate the electron transport chain during the CYP catalytic cycle. SNPs can also affect enzyme stability or heme incorporation, alter rates of transcription, or disrupt mRNA processing.

Interestingly, *CYP2A6*\(^\text{*23}^\) (2161C>T, Arg203Cys) was found to reduce enzyme activity whereas *CYP2A6*\(^\text{*16}^\) (2161 C>A, Arg203Ser) did not alter function. While the precise mechanism to explain this observation remains to be elucidated, molecular modeling suggests Arg203 is located on the outer surface of the enzyme (Kiyotani, Fujieda M et al., Oct 23-27, 2005). The side chains of Ser (*CYP2A6*\(^\text{*16}^\)) and Cys (*CYP2A6*\(^\text{*23}^\)) are both substantially smaller than the wildtype Arg residue. It is possible that the reactive Cys residue in *CYP2A6*\(^\text{*23}^\) may be hindering activity by forming Cys-Cys disulfide bonds with other proteins or other Cys residues in CYP2A6. Cys203 could potentially disrupt the critical interactions between CYP2A6 with NADPH-CYP reductase (POR) or cytochrome b5, and evidence for altered interactions between POR and other CYP enzymes have been reported. The Cys98 residue in CYP3A4 is critical for enzyme stability, and substitutions with Trp or
Phe altered protein conformation and decreased the binding affinity to POR, resulting in reduced coupling efficiency towards NAPDH oxidation (Wen et al., 2006).

Similar to genetic variation in other CYP enzymes (Mo et al., 2009; Watanabe et al., 2010), the functional effects of *CYP2A6* genetic variants may differ depending on the substrate examined. *CYP2A6*17 encodes an enzyme with reduced catalytic activity towards nicotine, but it appeared to have normal activity towards coumarin *in vitro* (Fukami, Nakajima et al., 2004). *CYP2A6*7 and *CYP2A6*18 also differentially altered the metabolism of nicotine compared to coumarin (Ariyoshi et al., 2001; Fukami et al., 2005). Thus, the functional consequences of *CYP2A6* genetic variants towards other substrates such as tobacco-related nitrosamines (NNK and NNN) should also be tested directly given the impact of these variants may differ from those reported towards nicotine.

1.5 Other biological sources of variability in CYP2A6 activity

Genetic variability in *POR*, the required co-enzyme for CYP function, may potentially affect the activity of all CYP enzymes. Genetic variability in the transcription factors responsible for *CYP2A6* expression may also contribute to the large variability observed in CYP2A6 activity. Furthermore, gene expression is more complex than initially thought, with various additional layers of regulation identified in recent years including epigenetic and miRNA modulations (Ingelman-Sundberg, Sim et al., 2007). These alternative sources of variation can potentially contribute to the large differences observed in CYP2A6 expression and activity between individuals.

1.5.1 Polymorphisms in *POR*
The CYP catalytic cycle involves cycles of oxidation and reduction of the heme iron in conjunction with substrate binding and oxygen activation, with POR supporting the electron transport chain that supplies two electrons from NADPH (Gibson et al., 2001). Genetic polymorphisms in POR and their contribution to variability in xenobiotic metabolism have been gaining attention. POR knockout mice die during embryonic development (Shen et al., 2002) and liver-specific deletion of POR results in greatly disrupted hepatic drug metabolism (Gu et al., 2003; Henderson et al., 2003). POR deficiency has been associated with altered steroidogenesis, congenital malformations and syndromes involving altered skeletal development in humans (Scott et al., 2008; Miller et al., 2009). The effects of 35 known POR genetic variants was recently tested in vitro, including some variants that had been previously associated with disease states (Agrawal et al., 2008). Thirteen of the POR genetic variants examined had no drug metabolizing capabilities when reconstituted with CYP1A2 or CYP2C19, and many other variants had substantially lowered levels of activity (Agrawal, Huang et al., 2008). Although POR genetic variants with complete loss-of-function are rare, the frequency of POR genetic variants with less severe functional effects and their impact on xenobiotic metabolizing enzyme activity remains to be determined.

1.5.2 Polymorphisms in transcription factors

Inter-individual variability in expression or activity of xenobiotic metabolizing enzymes is under the transcriptional control of nuclear hormone receptors. In an analysis of human livers, the mRNA levels of various xenobiotic metabolizing enzymes including CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, MRP2, OATP2, and UGT1A1 were found to be significantly correlated (Wortham et al., 2007). The mRNA levels of the transcription factors CAR, HNF4α and steroid/xenobiotic receptor (SXR) were also significantly correlated with mRNA levels of these xenobiotic metabolizing enzymes, suggesting they are co-regulated at
the transcriptional level (Wortham, Czerwinski M et al., 2007). A number of SNPs and alternative splice variants in CAR and PXR have been recently identified (Wang et al., 2007; Lamba, 2008; Wang et al., 2008). However the implications of these genetic variations on xenobiotic metabolism in vivo, particularly in combination with genetic polymorphisms in xenobiotic metabolizing enzymes, remain to be explored. A recent study found two haplotypes in the 3’-UTR of PXR was associated with altered oral clearance rates of midazolam in vivo, with greatest effects observed in African-Americans (Oleson et al.).

1.5.3 Epigenetics

Epigenetic regulation of gene expression involves heritable or acquired modifications of DNA (e.g. methylation) or its associated proteins (e.g. histone acetylation) (Renthal et al., 2008). Expression of CYPs can be altered by epigenetic modifications. For example, the methylation status of CYP1A1, CYP1A2, CYP1B1 and CYP2W1 has been associated with altered expression in tumors (Hammons et al., 2001; Tokizane et al., 2005; Karlgren et al., 2006; Okino et al., 2006; Gomez et al., 2007; Ghotbi, Gomez et al., 2009; Habano et al., 2009). Environmental factors can also influence epigenetic processes. Smoking alters the methylation status of CYP1A1, with lowest levels found in heavy smokers and highest levels found in non-smokers (Anttila et al., 2003).

Evidence for epigenetic modulation has been reported for the CYP2A subfamily; CYP2A13 expression in a human lung cancer cell line was increased following treatment with a demethylating agent and a histone deacetylase inhibitor (Ling et al., 2007). DNA methylation status at two potential CpG sites in CYP2A6, one within a DR4-like element at -5.5 bp and another within intron 2 to exon 3 at 1.6 to 1.9 kb, did not differ among human liver bank samples with high versus low levels of CYP2A6 expression and activity (Al
Koudsi, Hoffmann et al., 2010). While this study did not conclusively provide evidence for epigenetic regulation of CYP2A6 expression, only two probable sites were examined. There are many other CpG-dense sites within CYP2A6 that can possibly serve as targets of methylation, and other types of epigenetic regulation, such as histone acetylation, may also be of importance.

1.5.4 mRNA silencing (microRNA)

MicroRNAs (miRNA) are small non-coding RNA molecules (approximately 21 to 25 nucleotides in length) that bind to complementary sequences in the 3′-UTR of target mRNA transcripts, thereby inhibiting gene expression by degrading mRNA or suppressing translation (Meltzer, 2005). MicroRNAs regulate genes involved in various cell processes including proliferation, morphogenesis, apoptosis and differentiation (Ingelman-Sundberg, Sim et al., 2007). Genetic polymorphisms occurring at miRNA binding sites on the mRNA, or within miRNAs themselves, may also contribute to variable expression of xenobiotic metabolizing enzymes. CYP1B1 has an extremely long 3′-UTR (approximately 3kb), containing regions of high levels of conservation, and its expression can be modulated by miR-27b (Tsuchiya et al., 2006). Although the length of the 3′-UTR of CYP2A6 mRNA is only 257 bp (Gilmore et al., 2001), the miRNA Registry Release 16.0 has identified a number of putative miRNAs that may interact with this region via complementary binding (Griffithsâ€ Jones, 2004). It is notable that the 3′-UTR of CYP2A6 can also affect the stability of mRNA through other mechanisms (Ross, 1995). For example, the CYP2A6*1B allele, consisting of a 58 bp gene conversion in the 3′-UTR with CYP2A7, has been associated with increased mRNA stability (Ariyoshi et al., 2000; Wang et al., 2006). While the mechanism for this is unclear, a number of nuclear proteins such as the heterogeneous
nuclear ribonucleoprotein A1 (hnRNP A1) can interact with the 3’-UTR of CYP2A6 and increase its stability (Gilmore, Rotondo et al., 2001; Christian et al., 2004).

1.6 Utility of genotype versus phenotype measures of CYP2A6 activity

Currently, both genotype and phenotype measures are available as proxy measures of CYP2A6 activity, although each has its own set of advantages and limitations. Advances in DNA sequencing and genotyping technologies have greatly improved our ability to detect CYP2A6 genetic variants in recent years. However, several issues continue to limit the utility of CYP2A6 genotype (Altman, 2009; Schickedanz et al., 2009). The low frequencies of genetic variants in some racial/ethnic populations may reduce the statistical power of studies. Secondly, some discordance in the genotype-phenotype relationship remains as a result of incomplete functional characterization of known genetic variants and the existence of unidentified variants. Similar challenges have been reported for other xenobiotic metabolizing enzymes. CYP2D6 has been extensively studied, with over 80 alleles identified to date, yet development of a consistent and unified framework for the categorization of individuals according to predicted phenotype has proven difficult (Kirchheiner, 2008). Although currently identified CYP2D6 genotypes greatly alter enzyme activity, they account for only a fraction of the observed variability in phenotype (approximately 60%) despite the fact that this enzyme is not regulated by any known environmental or endogenous compound (Ingelman-Sundberg, 2005; Frank et al., 2007; Kirchheiner, 2008).

The 3HC/COT phenotype measure has the advantage of being able to account for both genetic and environmental sources of CYP2A6 variability. While this ratio can be derived from metabolites present in smokers as a result of ad libitum smoking, administration of a probe drug is required to determine the rate of enzyme activity among those who are not
exposed to tobacco smoke such as former or never smokers. Furthermore, the 3HC/COT ratio is technically a measure of cotinine metabolism. The 3HC/COT ratio is highly correlated with rates of nicotine clearance and is generally in good concordance with CYP2A6 genotypes (Dempsey, Tutka et al., 2004; Benowitz, Swan et al., 2006; Johnstone, Benowitz et al., 2006; Malaiyandi, Goodz et al., 2006; Malaiyandi, Lerman et al., 2006; Mwenifumbo et al., 2007; Mwenifumbo, Al Koudsi et al., 2008). As such, the 3HC/COT ratio appears to be a good indicator of CYP2A6 activity towards nicotine for the majority of individuals. However, it remains to be determined whether this ratio is a good indicator of activity towards other CYP2A6 substrates such as tobacco-specific nitrosamines or clinically used pharmaceuticals such as tegafur and letrozole (Ikeda, Yoshisue et al., 2000; Tomizawa and Casida, 2003). In addition, phenotype measures can be limited if the environmental stimulus altering enzyme function is only transient, such as temporary exposure to inducers or inhibitors. In contrast, genetic information is stable throughout the lifetime of an individual. Finally, the greater cost and time associated with detecting metabolite levels in biological matrices may limit the use of phenotype measures in large-scale population studies.

As such, the choice of which measure to use ultimately depends on several factors, such as the purpose of study, sample population of interest, and budget and/or time constraints. Collection of both genetic and phenotype information in our clinical trial of African-American light smokers, and a recent smoking cessation trial in Caucasians (Lerman, Jepson et al., 2010), has the additional benefit of accounting for limitations in either measure, thus further aiding interpretation of the results.
SECTION 2: IMPACT OF CYP2A6 GENETIC VARIATION ON SMOKING BEHAVIOURS IN AFRICAN-AMERICAN LIGHT SMOKERS

2.1. Smoking initiation

2.1.1. Factors associated with smoking initiation in African-Americans

The majority of smoking initiation occurs during adolescence; however, African-Americans consistently report later ages of smoking initiation compared to Caucasians, with a large proportion of individuals reporting onset at ages 18 to 20 instead of the early teen years (Kandel et al., 2004; Trinidad, Gilpin et al., 2004; Trinidad, Gilpin et al., 2004). Furthermore, only 10% of African-American high school students reported smoking at least one cigarette in the past month whereas 23% of Caucasian students reported doing so (Centers for Disease Control and Prevention, 2010). Potential reasons for this delayed onset of smoking have been explored. Even though peer influence is often a strong predictor of smoking initiation among Caucasians, this was not associated with current and lifetime smoking among African-American youths (Landrine et al., 1994; Griesler and Kandel, 1998; Gritz et al., 1998; Mermelstein, 1999). Parental smoking, weaker parent-child relationships and inconsistent discipline are also known to contribute to problem behaviours such as smoking during adolescence (Skinner et al., 2009), although the influence of parental smoking on smoking has not been as consistently found among African-American adolescents as with Caucasian adolescents (Hu et al., 1995; Griesler and Kandel, 1998; Gritz, Prokhorov et al., 1998; Mermelstein, 1999). African-American youths may be protected from smoking initiation as they appear to receive stronger anti-smoking messages from parents compared to Caucasians, with stricter parental guidelines set and clearly stated consequences for smoking (Mermelstein, 1999; Skinner, Haggerty et al., 2009). As such, African-Americans may have more protective factors against the development of smoking initiation during adolescence that may in part explain the observed delay in onset.
Interestingly, African-American adolescents also have lower rates of alcohol and illicit drug use compared to Caucasians, suggesting that these protective effects may also influence initiation of other drugs of abuse (Szapocznik et al., 2007).

2.2. Cigarette consumption

2.2.1. Light smoking behaviours among African-Americans

A higher proportion of African-American smokers consume 10 or fewer CPD compared to Caucasians although reasons for this are not well known. African-Americans experience substantial socioeconomic disparities; they have the lowest education attainment, highest poverty rates, and lowest median income compared to all other racial/ethnic groups (Us Bureau of the Census et al., 2004; Us Bureau of the Census et al., 2005). Socioeconomic status is an important determinant of smoking behaviours (Novotny, Warner et al., 1988; Townsend et al., 1994; Graham et al., 1999). African-Americans were more likely than Caucasians to report that cigarette tax increases had some impact on their smoking behaviours, resulting in smoking reduction and increased quit attempts (Frieden et al., 2005). In addition, African-Americans are more likely to illegally purchase single cigarettes which may contribute to their lower levels of daily consumption (Klonoff et al., 1994). However, differences in socioeconomic status are unlikely to fully account for the unique smoking patterns observed among African-Americans. Light smoking is also highly prevalent among individuals of Black-African descent in Canada, who are biologically similar to African-Americans, yet income and employment status were not associated with smoking status or the amount of cigarettes smoked per day (Mwenifumbo, Sellers et al., 2008).

It is possible that African-Americans are biologically more sensitive to the effects of nicotine. It has been hypothesized that the slower rates of nicotine metabolism among
African-Americans contribute to their lower levels of cigarette consumption, although differences in the function of nAChRs or modulation of the brain reward pathway by nicotine may also play a role. In a focus group of adult African-American light smokers who have smoked an average of 7 CPD for 22 years, many individuals reported they had great difficulty smoking a whole cigarette at a time and that smoking a cigarette too fast or too many consecutively made them feel dizzy and light-headed (Okuyemi et al., 2003). Many also reported smoking while in the presence of food or alcohol, and that smoking is often dependent on cigarette availability (Okuyemi, Scheibmeir et al., 2003). Furthermore, a recent study examined the acoustic startle reflex, a reliable index of how emotional stimuli are processed, in response to images of smoking-related cues or images designed to trigger positive or negative affect among African-Americans and Caucasians (Lam et al., 2008). Following acute nicotine administration, African-Americans had increased positive affect when exposed to images of smoking-related cues while Caucasians had decreased negative affect when exposed to unpleasant images (Lam, Robinson et al., 2008). Thus, nicotine may differentially alter emotional processing between the two racial/ethnic groups, suggesting relapse may be more likely to occur in response to smoking-related cues for African-Americans, whereas Caucasians may be more susceptible to relapse under situations that invoke negative affect (Lam, Robinson et al., 2008).

There is evidence to suggest that the lower cigarette consumption among African-Americans compared to Caucasians is apparent even during the initial learning stages of smoking. In a longitudinal study of males aged 10 to 25, African-Americans had lower prevalence rates of cigarette use and reported smoking fewer cigarettes at virtually every age compared to Caucasians (White et al., 2004). For Caucasians, the quantity of cigarettes smoked increased most rapidly from ages 13 to 15 and peaked at ages 20 to 22 at approximately 20 CPD. In
contrast, among African-Americans, cigarette consumption increased most rapidly between ages 17 to 18, with use also peaking at age 20 but at approximately 10 CPD (White, Nagin et al., 2004). As such, African-Americans appear to increase their cigarette consumption levels at a later age, and do not reach the high levels of use that are observed among Caucasians.

2.2.2. Comparisons between African-American light and moderate/heavy smokers

A limited number of studies have drawn comparisons between African-American light (10 or fewer CPD) and moderate (11 to 19 CPD) to heavy (20 or more CPD) smokers to better understand the factors contributing to light smoking patterns in this racial/ethnic group. African-American light smokers were more likely to be females, initiated smoking at a later age, and smoked for a shorter duration compared to African-American moderate to heavy smokers (Okuyemi, Ahluwalia et al., 2001; Okuyemi et al., 2004). African-American light smokers also had greater positive affect, less depressive symptoms according to the CES-D scale, and lower scores on the Perceived Stress Scale compared to African-American moderate to heavy smokers (Businelle et al., 2009).

In terms of smoking patterns, African-American light smokers were less likely to smoke their first cigarette of the day within 30 minutes of waking and had lower dependence scores compared to African-American moderate to heavy smokers (Choi et al., 2004; Businelle, Kendzor et al., 2009). These light smokers were equally willing to participate in a formal smoking cessation program, were more motivated to quit, and had greater confidence in their ability to quit compared to moderate to heavy smokers (Okuyemi, Ahluwalla et al., 2004; Businelle, Kendzor et al., 2009). In spite of this, African-American light smokers had just as much difficulty quitting as those with higher levels of consumption. African-American light smokers remained abstinent for similar durations as moderate to heavy smokers prior to
relapsing according to retrospective reports of their most recent quit attempts (Choi, Okuyemi et al., 2004). Furthermore, the low quit rates in our clinical trial of African-American light smokers were comparable to the quit rates observed in a clinical trial of African-American moderate to heavy smokers (Ahluwalia, Harris et al., 2002; Ahluwalia, Okuyemi et al., 2006).

2.3. Tobacco dependence

2.3.1. Dependence in African-American light smokers

The Fagerström Test for Nicotine Dependence (FTND), the most commonly used scale to assess tobacco dependence, was developed primarily as a measure of physical dependence that focuses on smoking as a way to acquire nicotine in order to relieve or avoid withdrawal symptoms. However, dependence is a multi-dimensional construct and newer measures, such as the Nicotine Dependence Syndrome Scale and Wisconsin Inventory of Smoking Dependence Motives, have been developed to better capture the many other aspects of dependence. For example, these measures also examine the withdrawal symptoms and cravings present during abstinence, the contexts under which smoking occurs (e.g. in response to mood or under certain social or environmental situations), and the importance of the sensory experience of smoking (Piper, Piasecki et al., 2004; Shiffman et al., 2004). These other components are likely to be important in the manifestation of tobacco dependence, particularly among light smokers. Indeed, in a sample of light smokers (average 12 CPD) from Switzerland, the FTND appeared to be measuring no more than the number of cigarettes smoked per day (Etter et al., 1999). When three dependence scales (FTND, ICD-10 and DSM-IV) were administered to a sample of Black-African descent, individuals were not consistently defined as dependent using these various measures, suggesting they may be capturing distinct sub-populations of smokers and/or different dimensions of dependence.
(personal communications). Tobacco dependence has not been well defined among African-Americans and further research is needed to identify the factors motivating smoking behaviours in this population.

2.3.2. Models of tobacco dependence

A number of models have been proposed to explain tobacco dependence; some of these were initially derived from observations of alcohol, stimulant or opiate dependence (Shadel, Shiffman et al., 2000; Glautier, 2004; Tiffany St et al., 2004). The dependence models that have been proposed are not mutually exclusive and can be present in different individuals in various degrees. The traditional negative reinforcement model of tobacco dependence, whereby use is mainly driven by physical dependence and avoidance of withdrawal effects, has limitations in explaining light smoking behaviours. Some of the other theories that have been proposed may help explain how individuals can sustain low levels of consumption, yet appear to have great difficulty stopping use.

2.3.2.1. Reinforcement theories

Negative reinforcement models suggest tobacco dependence is driven by smokers attempting to avoid or relieve the aversive withdrawal symptoms that develop during abstinence (Eissenberg, 2004). In contrast, positive reinforcement models propose that the immediate rewarding effects of smoking lead to further repetition of the behaviour (Shadel, Shiffman et al., 2000). The role of positive rather than negative reinforcing properties of smoking may be more important in maintaining the behaviour among light smokers. The low and sporadic smoking patterns observed in light smokers likely result in plasma nicotine levels that fluctuate widely over the course of the day due to the short half-life of nicotine (Benowitz and Jacob P 3rd, 1994). Cigarette consumption did not differ according to the rate of
nicotine metabolism among African-American light smokers, in contrast to observations in Caucasian moderate to heavy smokers (Rao, Hoffmann et al., 2000; Schoedel, Hoffmann Eb et al., 2004). This suggests that strict regulation of nicotine plasma levels, presumably to avoid the development of withdrawal symptoms, is not a significant determinant of smoking behaviours in this population.

2.3.2.2. Classical conditioning theories

Drug dependence is an associative learning process involving classical Pavlovian conditioning (Shadel, Shiffman et al., 2000; Glautier, 2004; Tiffany St, Conklin Ca et al., 2004). Drugs with physiological and subjective effects that are repeatedly administered within the same context will result in the context itself being able to elicit effects in the absence of the drug (Caggiula et al., 2002; Chaudhri, Caggiula et al., 2006). Although nicotine is thought to be the primary reinforcer present in tobacco smoke, it has relatively weak reinforcing properties when administered alone (Caggiula, Donny et al., 2002; Chaudhri, Caggiula et al., 2006). As such, it has been proposed that nicotine can enhance the reinforcing properties of non-pharmacological stimuli associated with the drug (Caggiula, Donny et al., 2002; Chaudhri, Caggiula et al., 2006). These non-nicotine components include social reinforcement, emotional reinforcement, and reinforcement derived from sensory modalities such as the sight, taste, and smell associated with cigarette smoking (Chaudhri, Caggiula et al., 2006). Exposure to these smoking related cues can strongly enhance the urge to smoke and is often a cause of relapse (Van Gucht et al.; Perkins et al., 1994).

Smoking denicotinized cigarettes can relieve craving and withdrawal symptoms, presumably because of the reinforcing properties of the non-nicotine components of smoking, although
individuals will prefer cigarettes that contain nicotine if given a choice (Shahan et al., 1999; Rose et al., 2004; Chaudhri, Caggiula et al., 2006). Studies that test whether denicotinized cigarettes can satisfy cravings, or if they are preferred to a similar degree as cigarettes containing nicotine, in African-American light smokers will be useful in determining the role of nicotine in maintaining smoking behaviours. In addition, ecological momentary assessments that ask smokers to report their smoking experiences in real-time over the course of the day on a hand-held computer will be useful in determining the mood states and environmental contexts associated with smoking in African-American light smokers. Such studies will help identify the cues that are important motivators of smoking behaviours within a naturalistic setting.

The ability of nicotine to enhance the reinforcing properties of non-nicotine stimuli has important implications as smoking is a complex behaviour involving more than just the acquisition and maintenance of nicotine levels. For intervention methods to successfully aid smoking cessation, they need to account for these other behavioural components that may be particularly salient among light smokers.

2.3.2.3. Importance of social and cultural contexts

The expression of tobacco dependence is contingent upon numerous sociocultural factors. Societal factors can influence drug dependence by limiting access through pricing, taxes and legality of use (Shadel, Shiffman et al., 2000). Cultural perceptions of smoking, such as smoking to facilitate social interactions or smoking as a sign of social distinction, are also important determinants of use (Shadel, Shiffman et al., 2000). Acculturation, the process by which individuals from foreign countries adopt the cultural and social norms of their host country, can also be a strong predictor of smoking behaviours. African-Americans born in the
Caribbean or Africa are less likely to be ever or current smokers compared to those born in the United States (Taylor et al., 1997; King et al., 1999; Bennett et al., 2008). Individuals who identify more strongly with African-American culture, such as having similar family values and practices, childhood experiences or growing up in predominantly African-American communities, were more likely to be smokers (Klonoff et al., 1996; Klonoff et al., 1999). African-Americans were also more likely to smoke menthol cigarettes if they believed most African-American smokers preferred this type of cigarettes (Allen et al., 2007). The tobacco industry has also targeted marketing strategies at African-Americans, with menthol cigarette brands such as Kool and Newport heavily advertised in magazines oriented towards these readers (King Iii et al., 2000; Landrine et al., 2005).

Variability in the rate of nicotine metabolism may contribute to differences in smoking patterns between racial/ethnic groups. However, sociocultural contexts are also of considerable importance, and the influence of \textit{CYP2A6} on smoking behaviours is likely highly contingent on the environmental context. The lower daily cigarette consumption observed among African-Americans would be predicted given their slower rates of nicotine metabolism compared to Caucasians (Benowitz, Perez-Stable et al., 1999; Benowitz, Perez-Stable et al., 2002; Trinidad, Perez-Stable et al., 2009). In contrast, Hispanics have similar rates of nicotine metabolism as Caucasians, yet they report lower daily cigarette consumption (Trinidad, Perez-Stable et al., 2009). Smoking behaviours among Hispanics is strongly associated with the degree of acculturation, with those who identify more strongly with mainstream culture in the United States being more likely to smoke and consuming greater number of cigarettes daily (Wilkinson et al., 2005). As another example, Asians living in their home countries (e.g. China and Japan) tend to have high levels of cigarette consumption, despite their slow rates of nicotine metabolism, as smoking is socially and culturally accepted and even encouraged for
males in these countries (Yang et al., 1999; World Health Organization, 2002). It is notable that among these Asian moderate to heavy smokers, \textit{CYP2A6} slow metabolizers reported smoking fewer cigarettes per day compared to normal metabolizers. Further investigations are needed to determine whether \textit{CYP2A6} is associated with cigarette consumption among Asian-Americans, who tend to be light smokers unlike their counterparts back in their native home countries.

2.4. Smoking cessation

Numerous studies have found that African-Americans were less likely to quit smoking compared to Caucasians (Novotny, Warner et al., 1988; King, Polednak et al., 2004; Agrawal et al., 2008; Fu et al., 2008; Piper et al., 2010). Few studies have explored the perceptions about smoking, motivations for smoking cessation or experiences with previous quit attempts specifically among African-American light smokers. In a focus group of African-American light smokers, the main reasons listed for wanting to quit smoking included restrictions at home and concerns of exposing children to secondhand smoke, as well as general health concerns such as fear of developing cancer, wanting to stay physically fit and having fresher breath (Okuyemi, Scheibmeir et al., 2003). Concerns about future quit attempts were expressed due to the failures of past attempts, with many reporting they were able to abstain from smoking for only a few days before relapsing as a result of withdrawal symptoms such as anxiety and irritability (Okuyemi, Scheibmeir et al., 2003).

The African-American light smokers enrolled in our clinical trial had made an average of three previous quit attempts in the past year despite strong motivation and confidence to quit smoking (Okuyemi et al., 2007; Okuyemi et al., 2007). Variables that have been associated with more successful treatment outcomes in Caucasian moderate to heavy smokers, such as
being male and older age (Sherman et al., 1996; Wetter et al., 1999; Ferguson et al., 2003; Grandes et al., 2003), were also predictors of higher abstinence rates in African-American light smokers (Nollen, Mayo Ms et al., 2006). Furthermore, post-cessation weight gain, which is often a cause of relapse during quit attempts among Caucasian moderate to heavy smokers, may also influence the ability to achieve abstinence in African-American light smokers (Parsons et al., 2009). Individuals with concerns of weight changes from smoking cessation were less confident in their ability to refrain from smoking (Thomas et al., 2008).

Moreover, individuals with higher BMI were more likely to quit smoking (Nollen, Mayo Ms et al., 2006), which may be due in part to their fewer smoking-related weight control expectancies or slower rates of nicotine metabolism (Thomas, Pulvers et al., 2008). Low socioeconomic status, another predictor of poorer smoking cessation outcomes among Caucasian moderate to heavy smokers, was also associated with abstinence in African-American light smokers (Nides et al., 1995; Gilman et al., 2003; Broms et al., 2004). African-American light smokers with income of less than $1800 per month were significantly less likely to quit smoking compared to those with higher income (Nollen, Mayo Ms et al., 2006).

2.4.1. Impact of CYP2A6 on smoking cessation

Based on studies in moderate to heavy smoking Caucasians, the higher smoking cessation rates among CYP2A6 slow metabolizers was originally thought to result from their lower number of cigarettes smoked daily. Fewer repetitions of smoking over the course of the day should allow for easier extinction of the behaviour. While lower levels of cigarette consumption is generally associated with increased likelihood of smoking cessation (Hellman et al., 1991; Hymowitz et al., 1997), it is notable that in previous clinical trials of smoking cessation among Caucasian moderate to heavy smokers, the higher quit rates among
CYP2A6 slow metabolizers remained even after accounting for amount of cigarettes smoked at baseline (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Lerman, Jepson et al., 2010). It is possible that the higher quit rates among CYP2A6 slow metabolizers may be a reflection of how dependence is manifested in these individuals. Previous work has found that CYP2A6 slow metabolizers report fewer cravings (Piper, Mccarthy et al., 2008), experience less severe withdrawal symptoms (Kubota, Nakajima-Taniguchi et al., 2006) and are less likely to smoke within five minutes of waking (Kubota, Nakajima-Taniguchi et al., 2006; Malaiyandi, Lerman et al., 2006).

Interestingly, among African-American light smokers, slow CYP2A6 activity was associated with an increased likelihood of smoking cessation even though cigarette consumption did not differ according to the rates of nicotine metabolism. While the mechanisms underlying these observations remain to be elucidated, it is possible that even among African-American light smokers, CYP2A6 slow metabolizers have less severe withdrawal symptoms and cravings compared to normal metabolizers, allowing them to maintain abstinence better. Further studies in laboratory and naturalistic settings are needed to elucidate how CYP2A6 activity influences smoking cessation in both light and moderate to heavy smokers. For example, tests of cognitive function and attentional biases, as well as responses to smoking-related cues during smoking abstinence, may provide further insight into the processes occurring during cessation attempts that contribute to cravings and subsequent relapse.

Tobacco addiction results from a series of neuroadaptative changes leading to alterations in the function of the brain reward system (Kreek et al., 1998; Mao and Mcgehee, 2010). Our findings suggest that although smoking behaviours among these African-American light-smoking adults do not appear to be primarily driven by the maintenance of nicotine levels,
variability in the levels of nicotine may have been an important determinant in how the brain reward system was modulated during the early learning stages of smoking. There is evidence to support a role for *CYP2A6* in the acquisition of tobacco dependence among Caucasian adolescents (O'loughlin, Paradis et al., 2004; Audrain-McGovern, Koudsi et al., 2007); it would be of interest to determine how this gene contributes to the acquisition of tobacco dependence in African-Americans.

2.4.2. Gender differences in smoking cessation outcomes in African-Americans

It has been proposed that males are more likely to smoke for pharmacological reinforcement mediated by nicotine, while females are more likely to smoke for psychological reinforcement gained through social interactions and stress alleviation (Perkins, 1996; Perkins, 2001). Gender differences in smoking cessation have been reported, and there is some evidence to suggest females are less successful at quitting compared to males (Perkins, 2001; Schnoll et al., 2009). While conflicting data exist to support gender differences in withdrawal symptoms, hormonal changes during the menstrual cycle may mimic or exacerbate symptoms such as irritability and moodiness, increasing the chance of relapse (Carpenter et al., 2006).

Unique biological predispositions for tobacco dependence may also contribute to gender differences in smoking behaviours. Several examples of gene by gender effects with smoking cessation outcomes have been reported. The *COMT* Val105/158Met allele has been associated with smoking abstinence among Caucasian females but not males in two independent samples including a clinical trial for NRT and a case-control study of former and current smokers (Colilla S et al., 2005). COMT can contribute to estrogen metabolism by inactivating 2-hydroxy-estrogens via methylation (Creveling, 2003), and estrogen was found
to regulate the expression of *COMT* such that lower levels of the enzyme were found in females compared to males (Harrison et al., 2007). In addition, the AA genotype of the *OPRM1* 118 A>G SNP (N40E) was associated with higher smoking abstinence rates among Caucasian males whereas females with this genotype had the lowest quit rates (Munafo et al., 2007). A haplotype by gender effect in a longitudinal study of African-American smokers was reported where the GTG haplotype within the dopamine D2 receptor gene (*DRD2/ANKK1*) was associated with greater smoking cessation among males but not females (David et al.). CYP2A6 activity was associated with smoking cessation outcomes in our sample of African-American females, although these effects were not significantly different among the males. CYP2A6 can be induced by estrogens and females have higher rates of nicotine clearance (Benowitz, Lessov-Schlaggar Cn et al., 2006; Higashi, Fukami et al., 2007). As such, the impact of CYP2A6 on smoking behaviours may be more pronounced among females compared to males. However, the effect of CYP2A6 on smoking cessation did not differ by gender in studies of Caucasian moderate to heavy smokers (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Lerman, Jepson et al., 2010). While this gene by gender effect may be a specific finding among African-Americans, it is notable that there was an over-representation of females in the study, and the analyses in males may have been underpowered. Therefore, further replication of these findings among African-Americans is necessary.

2.4.3. Improving treatment outcomes for smoking cessation

Our clinical trial of African-American light smokers is the first to examine the efficacy of pharmacotherapy and two forms of behavioural counseling for smoking cessation in this group. Treatment adherence is a strong predictor of smoking cessation outcome (Stapleton et al., 1995; Shiffman, Rolf et al., 2002) and the World Health Organization reports adherence
rates for smoking cessation treatments vary from 5 to 96% (World Health Organization, 2003). Approximately 72% of the participants in our study were adherent to the counseling program by attending at least 5 of the 6 counseling sessions (Ahluwalia, Okuyemi et al., 2006; Okuyemi, Zheng et al.). In contrast, only 36% of the sample reported adherence to gum (nicotine or placebo), defined as using 75% or more of the total prescribed gum over the 8 week treatment period (Okuyemi, Zheng et al.). The poor adherence to the 2 mg nicotine gum may have contributed to its lack of efficacy (Okuyemi, Zheng et al.). A low adherence rate to gum treatment is consistent with previous reports in other racial/ethnic groups and may be reflective of improper usage of the gum leading to insufficient nicotine replacement, and undesirable effects of the gum such as dislike of its taste (Shiffman, Rolf et al., 2002; Schneider, Olmstead et al., 2004; Lam, Abdullah et al., 2005; Schneider, Terrace et al., 2005). Factors associated with greater adherence to gum use among African-American light smokers included higher BMI, more quit attempts in the past year, higher baseline exhaled CO, and higher perceived stress (Okuyemi, Zheng et al., 2010). The lack of efficacy of the 2 mg nicotine gum in aiding smoking cessation among African-American light smokers cannot be generalized to higher doses of gum or other formulations of NRT, and further investigations are needed to determine their efficacy in this population. Additional studies are also needed to determine if other forms of pharmacotherapies (i.e. bupropion or varenicline) are beneficial in aiding smoking cessation for this population.

African-American light smokers are likely a heterogeneous group, representing individuals who were once heavier smokers, those who are still progressing to heavier smoking, and those who have maintained low levels of consumption throughout their smoking careers. In an analysis of the African-American light smokers in our clinical trial, 18% of the sample quit smoking while 41% reduced their cigarette consumption by an average of 5.8 CPD by
Predictors of cessation compared to reduction included enrolment into the health education counseling, older age, being male, lower baseline cigarettes smoked, lower baseline cotinine levels, higher nicotine dependence scores, higher BMI, lower levels of stress and higher scores on negative social impression (Berg, Thomas et al.). Smoking reduction is an important step towards successful cessation (Hyland et al., 2005; Broms et al., 2008), and gradual reduction of cigarettes smoked prior to target quit date, as opposed to quitting abruptly, may be an alternative approach for encouraging smokers to quit (Lindson et al., 2010). The observation that a large proportion of African-American light smokers were able to reduce their cigarette consumption suggests such methods may be useful in helping these individuals quit.

The high level of consent for genetic testing (83%) suggests that this population is receptive to the idea of using genetic research to advance our understanding of how individual differences contribute to smoking behaviours and how such knowledge may be eventually used to improve treatment outcomes (Cox et al., 2007). Based on clinical trials in moderate to heavy Caucasian smokers, it appears that CYP2A6 slow metabolizers would respond well to nicotine patch therapy in conjunction with counseling, whereas alternative treatment such as bupropion should be considered for CYP2A6 faster metabolizers (Lerman, Tyndale et al., 2006; Patterson, Schnoll et al., 2008; Schnoll, Patterson et al., 2009; Lerman, Jepson et al., 2010). The efficacy of non-NRT pharmacotherapies, such as bupropion and varenicline, remains to be tested in African-American light smokers. Further studies into the biological and environmental factors that are predictive of smoking in this population will lead to the development of more efficacious intervention methods.

2.5 Utility of biomarkers of tobacco exposure among African-Americans
Cigarette smoking is a complex behaviour with large variability observed in smoking topography. As such, self-reports of consumption levels are subjective and can be biased indicators of tobacco smoke exposure. A number of biomarkers have been proposed for a variety of purposes. For instance, biomarkers are often used to verify smoking abstinence in clinical trials of smoking cessation or for research studies, and in large-scale population health surveys. Biomarkers are also useful for studying the effects of environmental tobacco smoke exposure on health outcomes and validating claims of reduced harm for new tobacco products.

Biochemical verification is often used to control for the tendency to over-report smoking abstinence in clinical trials for smoking cessation. In our clinical trial of African-American light smokers, exhaled CO was collected at end-of-treatment (week 8), and both exhaled CO and plasma cotinine were collected at follow-up (week 26) to verify smoking abstinence. At week 26, 26% of the sample reported abstinence, 21% of the sample were abstinent as verified by exhaled CO and only 13% of the sample were abstinent as verified by plasma cotinine (Ahluwalia, Okuyemi et al., 2006). This suggests that many individuals may have been incorrectly classified as abstinent when using exhaled CO to validate smoking status. As such, cotinine may be a more useful biomarker for verifying smoking abstinence compared to exhaled CO in African-American light smokers due to its short half-life and contributions from environmental sources (Scherer, 2006). However, exhaled CO is often used as the sole method for biochemical verification of smoking status in clinical trials given its detection is much simpler and cheaper compared to cotinine (Benowitz, Peyton J Iii et al., 2002; Jatlow et al., 2008). Our data suggest cotinine should be used to verify smoking abstinence in light smokers whenever possible, and among individuals exposed to NRT,
other biomarkers such as the nicotine-related alkaloids anabasine and anatabasine may have greater utility (Benowitz, Peyton J Iii et al., 2002).

Biomarkers of tobacco smoke exposure are needed among African-American light smokers given their higher incidences of tobacco-related illnesses in spite of lower self-reported cigarette consumption (Ries, Harkins D et al., 2006; American Cancer Society, 2008; Kirkpatrick and Dransfield Mt, 2009). Higher cotinine plasma levels, and cotinine per cigarette, have been observed among African-American smokers (Caraballo, Giovino et al., 1998; Benowitz, Perez-Stable et al., 1999; Moolchan and Franken Fh, 2006; Signorello, Cai et al., 2009). One interpretation is that African-Americans have greater exposure to tobacco smoke despite lower self-reported consumption. Alternatively, our study suggests rates of CYP2A6 activity can also significantly alter cotinine levels in this population, with slow metabolizers having significantly higher cotinine levels. In contrast, among Caucasian moderate to heavy smokers where only a small proportion of individuals are CYP2A6 slow metabolizers, cotinine levels are reflective of the number of cigarettes smoked per day, and CYP2A6 slow metabolizers have significantly lower cotinine levels compared to normal metabolizers (Rao, Hoffmann et al., 2000).

It has been proposed that the higher prevalence of menthol cigarette use by African-Americans contributes to the disproportionately higher rates of tobacco-related illnesses among this group, although findings have not been uniformly supported across studies (Sidney et al., 1995; Werley, Coggins et al., 2007; Heck, 2010). The cooling effect of menthol is thought to result in deeper inhalation patterns, and there is some evidence to support that menthol cigarette smokers have higher exhaled CO and cotinine levels (Clark, Gautam et al., 1996). However, these findings have also been mixed (Werley, Coggins et al.,
2007; Heck, 2009; Muscat, Chen et al., 2009; Heck, 2010), and exhaled CO or cotinine levels were similar between menthol cigarette smokers and those who smoke regular cigarettes in our large study of African-American light smokers. A recent study reported that other biomarkers of tobacco exposure such as NNAL and thiocyanate did not differ between menthol and regular cigarette smokers (Muscat, Chen et al., 2009). However, the ratio of NNAL-glucuronide to NNAL was lower among menthol smokers, suggesting they may have reduced detoxification of this carcinogen (Muscat, Chen et al., 2009). The addition of menthol to cigarettes has been controversial as it may potentially increase the risk of adverse health effects. Clarification of the effect of menthol on tobacco smoke exposure is essential particularly since the Food and Drug Administration now has some authority in the regulation of tobacco products, including the ability to ban this additive from cigarettes.

CONCLUSIONS

Individuals of Black-African descent have high levels of genetic diversity as a result of their complex demographic history, and their genomes contain a high number of unique genetic variants, with each occurring at a low frequency in the population. Until recently, the CYP2A6 gene has not been extensively studied in populations of Black-African descent. We have identified many new CYP2A6 genetic variants in the past five years that help account for the slower rates of nicotine and cotinine metabolism observed in this population. Our study is also the first to examine the impact of CYP2A6 on smoking behaviours in African-American light smokers. The results found herein suggest CYP2A6 activity has a unique influence on smoking behaviours in this population. For example, African-American light smokers do not appear to alter their cigarette consumption to maintain constant plasma nicotine levels, and nicotine gum was not successful in aiding smoking cessation. Thus, the
factors that are salient in motivating smoking behaviours in this population remain to be determined. Elucidation of the mechanisms underlying the greater ability of CYP2A6 slow metabolizers to quit smoking will also provide a better understanding of how tobacco dependence is manifested in African-American light smokers. In addition, biomarkers previously validated in Caucasian moderate to heavy smokers have a number of limitations in African-American light smokers. Given the higher risk of tobacco-related illnesses among African-Americans despite their lower self-reported cigarette consumption, it is of utmost importance to assess the actual exposure level of this population to tobacco smoke and the harmful compounds within.
REFERENCES


Centers for Disease Control and Prevention (1998). Tobacco use among us racial/ethnic minority groups: A report of the surgeon general. Office on Smoking and Health and National Center for Chronic Disease Prevention and Health Promotion.


Coffman, et al. (1998). "The glucuronidation of opioids, other xenobiotics, and androgens by human ugt2b7y(268) and ugt2b7h(268)." Drug Metabolism And Disposition 26(1): 73-77.


Cooper, et al. (1994). "Evidence that the acute behavioral and electrophysiological effects of bupropion (wellbutrin) are mediated by a noradrenergic mechanism." Neuropsychopharmacology 11(2): 133-41.


Hadidi, et al. (1997). "A single amino acid substitution (Leu160His) in cytochrome P450 CYP2A6 causes switching from 7-hydroxylation to 3-hydroxylation of coumarin." Food and Chemical Toxicology 35(9): 903-907.


Itoh, et al. (2006). "Induction of human cyp2a6 is mediated by the pregnane x receptor with peroxisome proliferator-activated receptor-{gamma} coactivator 1 {alpha}." *J Pharmacol Exp Ther*: jpet.106.107573.

Iwahashi, et al. (2004). "Whole deletion of cyp2a6 gene (cyp2a6*4c) and smoking behavior." *Neuropsychobiology* 49(101-104).


Lindson, et al. (2010). "Reduction versus abrupt cessation in smokers who want to quit." Cochrane database of systematic reviews (Online) 3.


National Center for Chronic Disease Prevention and Health Promotion (1998). Tobacco use among us racial/ethnic minority groups—afican americans, american indians and alaska natives, asian americans and pacific islanders, and hispanics: A report of the surgeon general. Office of Smoking and Health, Atlanta, GA.

National Survey on Drug Use and Health (2006). "Past month cigarette use among racial and ethnic groups. The nsduh report;." 


Parsons, et al. (2009). "Interventions for preventing weight gain after smoking cessation." Cochrane Database of Systematic Reviews(1).


Picciotto, et al. (2008). "It is not "Either/or": Activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood." *Progress in Neurobiology* 84(4): 329.


Tiffany St, et al. (2004). "What can dependence theories tell us about assessing the emergence of tobacco dependence?" *Addiction* 99(Suppl 1): 78-86.


Wacholder, et al. (2002). "Counterpoint: Bias from population stratification is not a major threat to the validity of conclusions from epidemiological studies of common polymorphisms and cancer." Cancer Epidemiology Biomarkers & Prevention 11(6): 513-520.


APPENDICES


Appendix B: Ho MK, Tyndale RF. Role of CYP2A6 genetic variation on smoking behaviors and clinical implications. *ASCO Education Book 2008*. Reprinted with permission. © 2010 American Society of Clinical Oncology. All rights reserved.
