4 Nicotine Metabolism as an Intermediate Phenotype

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The prevalence of cigarette smoking has plateaued in North America and Europe, where recent estimates suggest nearly a fifth of adults are current smokers [1–4]. Smoking causes approximately 20% of deaths in these geographic regions and remains the leading preventable cause of illness and early death [5–7]. In developing nations, the prevalence of cigarette smoking is increasing, and by 2030, more than 80% of the world’s tobacco-related deaths are predicted to occur in these regions [8]. Although more than two thirds of smokers express a desire to quit [6], the process of smoking cessation is arduous and only 3% of those making unaided quit attempts remain abstinent at 6 months [9].

4.1 Neurobiology of Nicotine Addiction

Nicotine is the main psychoactive compound in cigarette smoke and is responsible for the reinforcing properties of cigarette smoking, serving to both establish and maintain dependence [5]. Following cigarette smoke inhalation into the lungs, nicotine is rapidly absorbed into the circulatory system and transported to the brain within seconds, where it binds nicotinic acetylcholine receptors (nAChRs) [10]. The activation of nAChRs results in the downstream release of dopamine in a variety of brain regions including the shell of the nucleus accumbens, which is critical in mediating the rewarding effects of cigarette smoking [10]. Smokers adjust their patterns of cigarette use to achieve stable levels of nicotine in order to attain the desired effects and to avoid withdrawal [6, 10]; periods of abstinence result in the precipitation of nicotine withdrawal and craving symptoms [11]. These symptoms are alleviated upon reinstatement of smoking and can thus predict relapse [6]. The majority of smokers require a number of quit attempts before reaching sustained abstinence [10]; hence nicotine addiction is a chronic condition with an average duration of more than 20 years [12, 13].
4.2 Genetic Influences on Smoking

A combination of genetic and environmental factors influences the onset and maintenance of smoking behaviors and the development of nicotine dependence. Twin studies reveal that genetics plays a substantial role in initiation, the development of dependence, level of dependence, amount smoked, continued smoking, and the ability to stop smoking [14–19]. There is some degree of overlap in the heritability of these phenotypes, suggesting common genetic factors, in addition to unique genetic factors, mediate these behaviors [17]. Although environmental factors play a relatively minor role in cigarette smoking behaviors in adults, these factors may have a stronger influence on the smoking behaviors of adolescents/novice smokers [20–22]. The rate of nicotine metabolism in smokers is influenced by both genetic and environmental factors and can be considered an intermediate phenotype that modulates smoking behaviors in both adolescents and adults as discussed below.

4.3 Nicotine Metabolism as an Intermediate Phenotype

A variety of genetic and environmental factors, each with small individual effect sizes, contribute to complex neuropsychiatric disorders such as addiction, and individuals must display a threshold level of clinically overt signs in order to be positively diagnosed [23, 24]. However, large interindividual variability exists regarding the clinical features of these disorders; thus intermediate phenotypes with clearer and narrower features are a powerful tool to study the causes of diseases that have complex genetic and environmental components [23, 25, 26]. Therefore, the intermediate phenotype approach, in unraveling causal links between genetic factors and disease [27], may provide greater mechanistic insight into the etiology of complex neuropsychiatric disease. Moreover, the evaluation of intermediate phenotypes may allow for the greater identification of at-risk individuals.

The majority (~80%) of nicotine is inactivated to cotinine, a reaction primarily catalyzed by cytochrome P450 2A6 (CYP2A6). CYP2A6 is responsible for more than 90% of the inactivation of nicotine to cotinine and 100% of the conversion of cotinine to 3’-hydroxycotinine [28, 29] (see figure 4.1). Thus the metabolism of nicotine accounts for the majority of nicotine removal from the body. Two surrogates of nicotine clearance, CYP2A6 genotype and the 3’-hydroxycotinine/cotinine ratio (also known as the nicotine metabolite ratio; NMR), have been utilized to study associations between nicotine metabolism rates and smoking behaviors.

The metabolism of nicotine can be considered an intermediate phenotype that mediates the relationship between genetic risk factors and the susceptibility to nicotine addiction. Additive genetic factors play a major role in mediating both total nicotine clearance (59%) and the nicotine metabolite ratio in plasma and urine (67% and
Nicotine metabolism: Approximately 80% of inhaled nicotine is inactivated to cotinine, in a two-step reaction catalyzed by cytochrome P450 2A6 (CYP2A6) and cytoplasmic aldehyde oxidase. CYP2A6 is responsible for more than 90% of the conversion of nicotine to nicotine-$\Delta^{1(5')}$-iminium ion, which is the rate-limiting step of the reaction [160]. Nicotine-$\Delta^{1(5')}$-iminium ion is then rapidly converted to cotinine by aldehyde oxidase [161]. Cotinine is subsequently metabolized to trans-3'-hydroxycotinine, in a reaction mediated exclusively by CYP2A6. The 3'-hydroxycotinine/cotinine ratio (or nicotine metabolite ratio) serves as a proxy for both CYP2A6 activity and total nicotine clearance. (Chemical structures were created using ChemDraw software, PerkinElmer, Cambridge, MA.)
47%, respectively) [30, 31]. In addition, variation in the rates of nicotine metabolism is associated with a number of smoking-related behaviors [32–35].

### 4.3.1 Nicotine Metabolism Rate Approximated by CYP2A6 Genotype

A variety of studies have investigated the impact of genetic factors on smoking in adults and have demonstrated significant associations with multiple smoking behaviors for variation in the gene encoding CYP2A6. Specifically, variation in CYP2A6 contributes to differences in current smoking status, cigarette consumption and puff volume, level of dependence, duration of smoking, likelihood of being a former versus current smoker, and likelihood of cessation in adults [33, 34, 36–40].

The highly polymorphic CYP2A6 gene consists of 9 exons and 8 introns and encodes 494 amino acids [41]. To date, 37 unique numbered CYP2A6 alleles have been characterized, many of which are single nucleotide polymorphisms (SNPs), or the result of nucleotide insertions and deletions. CYP2A6 gene duplications, conversions, and hybrid alleles have also been described (http://www.cypalleles.ki.se/cyp2a6.htm). A number of the CYP2A6 variant alleles meaningfully alter CYP2A6 enzymatic activity in vitro and in vivo [42–45] and thus are of particular importance for nicotine metabolism and smoking behaviors.

Four CYP2A6 alleles (CYP2A6*2, CYP2A6*4, CYP2A6*9, and CYP2A6*12) have received widespread attention in studies examining the association between CYP2A6 variation and a number of smoking-related outcome measures in Caucasians [33, 35, 36, 38, 46, 47]. Each of these alleles is present in Caucasian populations at relatively high frequencies (between ~1% for CYP2A6*4 to ~8% for CYP2A6*9) [33, 36, 47], and each encodes a CYP2A6 enzyme with reduced or inactive function. Specifically, CYP2A6*2 and CYP2A6*4 both code for inactive CYP2A6 enzymes. CYP2A6*2 is the consequence of a nonsynonymous SNP in the third exon of the CYP2A6 gene and encodes an inactive CYP2A6 enzyme due to the lack of incorporation of a heme molecule [48]. Similarly, individuals possessing CYP2A6*4 also do not express functional CYP2A6 enzyme as this variant results in a CYP2A6 gene deletion likely stemming from unequal crossover events within the CYP2A gene cluster [49]. Conversely, CYP2A6*9 and CYP2A6*12 are both decrease-of-function alleles. CYP2A6*9 is a SNP in the TATA box of the CYP2A6 gene, resulting in reduced promoter activity and decreased production of CYP2A6 mRNA [50]. The CYP2A6*12 variant is thought to result from an unequal crossover event between the CYP2A6 and the nonfunctional CYP2A7 pseudogene in intron 2, resulting in a decrease in both the protein and activity level of CYP2A6 as demonstrated in vitro [45] and in vivo [44].

A number of additional CYP2A6 alleles reducing CYP2A6 enzymatic function have been characterized, including CYP2A6*7, CYP2A6*10, CYP2A6*17, CYP2A6*20, CYP2A6*23, CYP2A6*25, CYP2A6*26, CYP2A6*27, and CYP2A6*35 [42, 51–54].
Expression of each of these variants is associated with reduced or inactive CYP2A6 function in vitro and/or in vivo [42, 51–54].

When investigating associations between CYP2A6 genotype and smoking behaviors, individuals are grouped into predicted CYP2A6 enzymatic activity groups based on their CYP2A6 genotype [33, 36, 46, 47]. Individuals having one copy of a reduced function variant, as determined by pharmacokinetic studies, are expected to have 75% to 80% of normal CYP2A6 activity whereas individuals expressing one copy of an inactive function variant or two copies of a reduced function variant are predicted to have approximately 50% of normal CYP2A6 enzymatic activity [44].

Large interindividual variability in the rate of nicotine metabolism exists within and between populations [42]. The frequency of CYP2A6 variants in populations varies widely by ethnicity [42]. For example, CYP2A6*17 is found at high frequency in African American subjects but rarely in Caucasian or East Asian populations [16]. In contrast, the frequencies of CYP2A6*4 and CYP2A6*9 are relatively lower in Caucasians and African Americans compared to East Asian populations [36, 42, 51]. Overall, given the variation in the prevalence of these variant alleles among different ethnic groups, it is not surprising that there is a lower prevalence of CYP2A6 reduced metabolizers (≤75% CYP2A6 activity as predicted by CYP2A6 genotype) in Caucasian populations (~10%–25%), relative to East Asian populations (~50%) [35, 42, 46, 47]. This is exemplified by overall slower rates of nicotine metabolism in East Asians compared to Caucasians [42, 55]. Since smoking rates are generally higher in East Asian countries relative to Western countries, especially among men [1, 2, 56, 57], the effect of slower nicotine metabolism on smoking behaviors is likely to have a greater relative impact in East Asian populations compared to Caucasian populations.

4.3.2 Nicotine Metabolism Rate Measured by Phenotype

Although CYP2A6 genotype is often used to predict an individual’s capacity to metabolize nicotine, it does not capture all sources of variation in CYP2A6 and nicotine metabolism rates [30]. Even among individuals classified as normal nicotine metabolizers, rates of nicotine clearance are widely variable [30]; thus it is important to also include alternative approaches that account for both genetic and environmental influences on nicotine metabolism [58]. Environmental factors including the use of certain medications may meaningfully alter nicotine metabolism rates [58]. For example, the antipsoriasis drug methoxsalen, a CYP2A6 inhibitor, is associated with reduced nicotine metabolism [59], whereas nicotine metabolism is higher in women compared to men [58], and even higher in women taking estrogen-containing oral contraceptives [60]. Moreover, dietary factors may also significantly alter nicotine metabolism rates, as broccoli consumption is associated with increased CYP2A6 enzymatic activity [61].
As previously mentioned, CYP2A6 is responsible for more than 90% of the inactivation of nicotine into cotinine and mediates 100% of the conversion of cotinine into 3′hydroxycotinine [28, 29]. The 3′hydroxycotinine/cotinine ratio (the NMR), is a phenotypic measure of the nicotine metabolism rate that has been validated as a phenotypic marker of CYP2A6 activity [62, reviewed in 63]. The utility of this phenotypic measure stems from the long elimination half-life of cotinine (approximately 13–19 hours), and the formation dependence of 3′hydroxycotinine [64]. The NMR is calculable from plasma, saliva, and urine and has strong concordance with CYP2A6 genotype [33, 54, 65]. Although using the NMR has many advantages, some of the influences on the NMR are transient (e.g., diet, drugs) and may confound the interpretation of intrinsic nicotine metabolism rates [66]. However, the NMR correlates well with total nicotine clearance and is more closely related than CYP2A6 genotype to total nicotine clearance [62, 67] (see figure 4.2).

4.4 Role of Nicotine Metabolism in Smoking Behaviors and Dependence

Variation in the rate of nicotine metabolism, due to its relationship with nicotine clearance, is associated with a variety of smoking-related outcomes. The following sections will describe the current state of knowledge regarding the impact of the rate of nicotine metabolism on smoking behaviors and nicotine addiction (summarized in figure 4.3). In addition, the association between nicotine metabolism, CYP2A6 genotype, and the risk for a variety of smoking-related diseases will be discussed.

4.4.1 Consumption and Nicotine Dependence

Level of Consumption

Differences in the level of cigarette consumption, as a function of predicted CYP2A6 metabolic group, have been observed in both adults and adolescents [35, 46]. In adults, these changes manifest not only in terms of the number of cigarettes smoked per day but also in terms of smoking topography, which is another quantifiable measure of smoking behavior [38]. In addition, a positive correlation exists between the rate of nicotine clearance and the total daily dose of nicotine acquired from cigarette smoking [55].

Number of Cigarettes

Smokers expressing reduced or inactive CYP2A6 variants (*2, *4, *9, and *12) smoked a mean of 5.7 fewer cigarettes daily (20.2 cigarettes) compared to smokers with normal CYP2A6 activity (25.9 cigarettes) among current controls from a lung cancer case-control study in Caucasians [35]. Moreover, CYP2A6 slow metabolizers (≤50% CYP2A6 enzymatic activity as predicted by genotype) smoked a mean of 7 fewer cigarettes per
Figure 4.2
Correlation between total nicotine clearance and 3'-hydroxycotinine/cotinine ratio: Correlation between total nicotine clearance and the plasma 3'-hydroxycotinine/cotinine ratio four hours following the administration of an oral solution of deuterium-labeled nicotine (2 mg d2 for nicotine clearance) and cotinine (10 mg d4 for the ratio) to healthy volunteers. The filled black circles represent smokers, and the empty circles represent nonsmokers. The filled gray circles represent nonsmokers homozygous for the CYP2A6*4 allele (0% CYP2A6 enzymatic activity as predicted by CYP2A6 genotype and ratio).
Nicotine metabolism is an intermediate phenotype that modulates smoking behaviors: The rate of nicotine metabolism, measured by CYP2A6 genotype and the nicotine metabolite ratio, is associated with a number of smoking behaviors on the tobacco use continuum. While the role of nicotine metabolism in smoking initiation (i.e., puffing on a cigarette for the first time) is currently unclear, a decreased rate of nicotine metabolism is associated with a number of other smoking behaviors. CPD, cigarettes smoked per day.

day compared to CYP2A6 normal metabolizers in a community-based sample of Caucasian adult smokers [36]. This finding was also replicated in a population of Caucasian treatment-seeking adults, where individuals with CYP2A6 slow activity smoked a mean of 4 fewer cigarettes per day (20 cigarettes per day) at baseline relative to CYP2A6 normal metabolizers (24 cigarettes per day) [33]. The association between CYP2A6 reduced metabolism and lower cigarette consumption also manifests in Japanese [34] and Chinese [68] smokers. In Japanese smokers, CYP2A6 slow metabolizers (≤50% CYP2A6 activity as predicted by CYP2A6 genotype) smoked 10 to 14 fewer cigarettes per day relative to CYP2A6 normal metabolizers [34], and among Chinese smokers, CYP2A6 poor metabolizers (<25% CYP2A6 activity as predicted by CYP2A6 genotype) reported lower cigarette consumption compared to CYP2A6 normal metabolizers (OR [odds ratio] = 0.49) [68].

The NMR also correlated (r = 0.33; p = 0.005) with cigarette consumption in a predominantly Caucasian population of heavy smokers [69] and was positively associ-
ated (p = 0.04) with cigarette consumption in another population of European heavy smokers that previously participated in a smoking cessation trial [67]. These findings were replicated in a pooled analysis of pretreatment data derived from three smoking cessation clinical trials [70–72] comprising over 1,000 participants, where faster nicotine metabolizers (top three NMR quartiles) smoked more cigarettes per day relative to slower nicotine metabolizers (lowest NMR quartile) [73].

In a cohort of Caucasian adolescents, there was also a trend toward a 40% to 56% decrease in cigarette consumption among CYP2A6 reduced metabolizers, relative to CYP2A6 normal metabolizers [46]. In a separate cohort of Caucasian adolescents, ~55% decreased weekly cigarette consumption (p = 0.04) was noted among CYP2A6 reduced metabolizers relative to CYP2A6 normal metabolizers [47].

Despite the observed association between reduced nicotine metabolism and lower cigarette consumption in a variety of adult and adolescent populations, these findings are not replicated in all light-smoking (≤10 cigarettes per day) populations [54, 74]. For example, African American light smokers did not alter total cigarette consumption to compensate for differences in the rate of nicotine metabolism, as measured by either CYP2A6 genotype or the NMR [54]. In addition, the NMR was not correlated with cigarette consumption in adolescent light smokers [74]. Among light smokers, cigarettes per day may be a relatively weaker indicator of smoking, and individuals with slower nicotine metabolism may acquire lower nicotine doses through reducing puff volume [38, 75], described in the following section.

In general, there is a need for utilizing alternative biomarkers of consumption to more accurately assess the total nicotine dose achieved by smokers. Only modest associations are observed between cigarette consumption and biomarkers of cigarette smoke exposure [54, 76, 77]. For example, in an African American light-smoking population, exhaled CO and plasma cotinine levels were only weakly correlated (r ~ 0.31–0.37) with self-reported cigarette consumption [77]. Overall, urinary total nicotine equivalents (TNE), which is the molar sum of nicotine and its major metabolites, may be a more optimal biomarker for approximating total cigarette smoke exposure in smokers [76, 78, 79].

**Topography**

Both CYP2A6 genotype and the NMR are associated with alterations in smoking topography, whereby different nicotine doses can be acquired from an equivalent number of cigarettes through changes in puff volume [38, 75]. In a sample of treatment-seeking smokers who smoked more than 20 cigarettes per day, CYP2A6 slow metabolizers (≤50% CYP2A6 activity as predicted by genotype) puffed significantly less deeply on cigarettes relative to CYP2A6 normal metabolizers, despite there being no significant difference in the number of puffs taken [38]. Similarly, heavy smokers in the slowest (first) NMR quartile had a significantly reduced total puff volume relative to heavy smokers in the third and fourth NMR quartiles [75].
Nicotine Dependence

Acquisition and Progression
Up to 90% of adult smokers surveyed report initiating smoking by the end of their teenage years [80]; thus adolescence is a critical period for the acquisition of smoking behaviors. To date, the association between CYP2A6 variation and risk for acquisition of and progression in nicotine dependence has been studied prospectively in two adolescent cohorts [46, 47]. An investigation of the risk of tobacco dependence among adolescent ever-inhalers in the Nicotine Dependence in Teens (NDIT) cohort revealed the hazard ratio for conversion to International Classification of Diseases (10th rev.; ICD-10) tobacco dependence was 2.8 for slowest (one to two copies of *2 or *4) metabolizers compared to normal metabolizers [46]. Having symptoms of depression was also associated with conversion to tobacco dependence among CYP2A6 normal metabolizers; however the association was more modest among CYP2A6 slowest metabolizers [81].

Although CYP2A6 slowest metabolizers were more likely to convert to ICD-10 [46] or modified Fagerström Tolerance Questionnaire (mFTQ) [82] nicotine dependence compared to CYP2A6 normal metabolizers, data from the Georgetown Adolescent Tobacco Research (GATOR) study suggested reduced metabolizers progress in mFTQ dependence levels more slowly than normal metabolizers [47]. Although having a positive initial smoking experience predicts the future development of nicotine dependence [83, 84], positive initial smoking experiences did not appear to be responsible for mediating the association between CYP2A6 genotype and risk for, or progression in, nicotine dependence [46, 47]. Nevertheless, the findings from NDIT and GATOR provide the framework for a novel, integrated hypothesis regarding the relationship between CYP2A6 and nicotine dependence: while CYP2A6 reduced metabolizers acquire nicotine dependence before CYP2A6 normal metabolizers, they stabilize in the course of onset sooner, at lower levels of nicotine dependence and smoking quantities [82].

In late adolescence (age 18), CYP2A6 reduced metabolism is also associated with a greater likelihood of being a current smoker (OR = 2.23 for current vs. ex-smoker) [85], consistent with the findings from NDIT [46]. However, their slower progression in nicotine dependence relative to normal CYP2A6 metabolizers [47] may translate into increased quit rates in later adolescence and early adulthood, resulting in the lower observed prevalence of adult smokers (both Diagnostic and Statistical Manual of Mental Disorders [4th ed.] dependent and nondependent) with reduced CYP2A6 metabolism [36] (see below).

The NMR also positively correlates with self-described level of addiction in adolescent light smokers (consuming ≤6 cigarettes per day), such that individuals with faster nicotine metabolism report greater levels of self-described nicotine addiction [74].
However, the NMR was not significantly correlated with nicotine dependence as measured by the Hooked on Nicotine Checklist or mFTQ [74].

Nicotine Dependence Scores in Adults
Among Caucasian heavy smokers, FTND (Fagerström Test of Nicotine Dependence) scores were significantly higher in CYP2A6 normal metabolizers (5.1) relative to CYP2A6 reduced metabolizers (4.2) [35]. However, the NMR did not correlate with FTND scores in a community-based sample of heavy smokers [69].

Several clinical studies examining potential associations between the rate of nicotine metabolism and level of dependence have also found no significant relationship [33, 54, 67, 69, 86]. In a clinical trial investigating the efficacy of transdermal nicotine for smoking cessation, neither NMR [86] nor CYP2A6 genotype [33] were significantly associated with FTND scores. However, one aspect of the FTND, cigarettes consumed per day, was significantly associated with CYP2A6 genotype [33]. In addition, neither pretreatment NMR nor CYP2A6 genotype was associated with FTND scores in a clinical trial involving nicotine gum [54].

While the FTND captures daily cigarette consumption and time to first cigarette, both of which are associated with CYP2A6 [33], the FTND also includes variables less likely to be altered by nicotine metabolism, such as the ability to refrain from smoking in places where it is forbidden [87]. Conversely, the rate of nicotine metabolism can modulate withdrawal and craving scores [32, 74]; however these elements are not captured by the FTND. Future studies assessing nicotine dependence using measures in addition to the FTND scale may reveal significant associations.

4.4.2 Cessation
Variation in the rate of nicotine metabolism also predicts patient response to smoking cessation pharmacotherapy in clinical trials. In addition, altered nicotine metabolism is associated with spontaneous quit rates in observational studies. These findings are discussed in detail below.

Clinical Trials
Clinical trials involving smoking cessation pharmacotherapies demonstrate significant associations between variation in nicotine metabolism and therapeutic response to the nicotine patch [32, 86]. In a predominantly Caucasian population, pretreatment NMR, among individuals receiving 21 mg transdermal nicotine, was significantly associated with treatment response at both end of treatment and follow-up. At end of treatment, 46% of smokers in the lowest NMR quartile achieved abstinence, compared to only 28% of smokers in the fastest NMR quartile. Interestingly, this difference persisted at six months follow-up, where 30% of individuals in the slowest NMR quartile successfully quit relative to only 11% in the fastest NMR quartile [32]. Lerman et al.
also examined whether an association exists between the rate of nicotine metabolism and efficacy of nicotine nasal spray but found no significant association between NMR quartile and treatment response [32]. However, the rate of nicotine metabolism was associated with the degree of nicotine nasal spray usage, such that CYP2A6 normal metabolizers used nearly six more doses per day relative to CYP2A6 slow metabolizers (p < 0.02) [33].

A follow-up investigation involving a larger, predominantly Caucasian study population reexamined whether pretreatment NMR predicts therapeutic response to nicotine patch treatment [86]. All patients received 21 mg nicotine patch treatment for eight weeks, and end-of-treatment quit rates were significantly associated with pretreatment NMR [86]. Forty-two percent of individuals in the slowest NMR quartile achieved abstinence, compared to only 28% of individuals in the fastest NMR quartile, validating the previous findings of Lerman et al. [32]. The quit rate observed among slow nicotine metabolizers is on par with that achieved by the newest smoking cessation drug, varenicline [86]. Thus, variation in nicotine metabolism rates as assessed by pretreatment NMR serves as a predictor of successful smoking abstinence achieved with transdermal nicotine therapy.

In a smoking cessation trial in African American light smokers randomized to receive either nicotine gum or placebo, both CYP2A6 genotype and pretreatment NMR predicted overall cessation rates [54]. While there was no overall effect of nicotine gum treatment on cessation outcome [88], individuals in the slowest NMR quartile were more likely to quit at the end of follow-up relative to individuals in faster NMR quartiles, and there was a nonsignificant trend toward increased quit rates at both end of treatment and follow-up among CYP2A6 slow metabolizers relative to CYP2A6 normal and intermediate metabolizers [54]. In addition, a significant twofold increase in abstinence rates on nicotine gum was observed among CYP2A6 slow metabolizers relative to CYP2A6 normal and intermediate metabolizers [54].

In a placebo-controlled smoking cessation clinical trial involving bupropion, individuals with faster pretreatment NMR that received bupropion had higher end-of-treatment quit rates compared to those that received placebo (34% vs. 10%, respectively) [89]. In addition, the relationship among fastest nicotine metabolizers remained significant at six-month follow-up, when 27% of individuals that received bupropion were abstinent relative to only 8% of individuals who received placebo [89]. Of note, the NMR effect was primarily in the placebo arm, whereby 32% of individuals in the slowest NMR quartile quit smoking at end of treatment relative to only 10% of individuals in the fastest NMR quartile, with little difference in bupropion response (32% vs. 34%) [89]. As a result, treatment with bupropion did not significantly enhance end-of-treatment cessation rates among slow metabolizers: individuals in the slowest NMR quartile had equal quit rates on placebo and bupropion (32%) [89]. Overall, the effect of NMR on quitting smoking while on placebo...
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pill [89] is similar to that while on placebo gum [54], suggesting slow metabolizers have better quit rates in general.

**Observational Studies**
Observational studies have also found significant associations between CYP2A6 genotype and smoking cessation. Adults expressing the inactive CYP2A6*2 allele experienced a shorter duration of smoking and increased probability of quitting relative to individuals homozygous for the wild type CYP2A6*1 allele [40], and CYP2A6 reduced metabolizers were less likely to be current smokers (OR = 0.52) [36]. However, these findings were not replicated in an epidemiological study of Southern Chinese [68]. A recent study in Caucasian adolescent smokers also demonstrated a significantly increased likelihood of smoking cessation among CYP2A6 slow metabolizers (~≤50% CYP2A6 activity) relative to normal metabolizers (~100% CYP2A6 activity; OR = 2.3), suggesting CYP2A6 variation influences smoking cessation even in novice smokers [90].

**Withdrawal and Cravings**
The rate of nicotine metabolism is also associated with the severity of both withdrawal and craving symptoms in smokers. Adults with higher CYP2A6 activity (CYP2A6*1/*1, *1/*9, *1/*4, *9/*9) reported more severe withdrawal symptoms compared to adults with lower CYP2A6 activity (CYP2A6*4/*9, *4/*4) [91], consistent with the lower cessation rates observed for faster nicotine metabolizers compared to slower nicotine metabolizers [32]. Rubinstein et al. [74] examined the effect of NMR on withdrawal symptoms in adolescent smokers. Individuals in the fastest NMR quartile reported greater withdrawal symptoms after 24-hour abstinence compared to individuals in slower NMR quartiles, even after adjusting for cigarette consumption [74].

Among abstinent adults receiving nicotine patch treatment for one week, craving intensity increased linearly with increasing quartile of nicotine patch–derived NMR [32]. This suggests that faster nicotine metabolizers experienced more intense cravings compared to slower nicotine metabolizers even while abstinent and on patch, which is an important consideration for smoking cessation interventions as craving associated with abstinence can predict relapse in adults [92].

Differences in smoking cue-evoked brain activity in faster versus slower nicotine metabolizers, measured by CYP2A6 genotype or NMR, have also been documented [93] and may contribute to smoking relapse. Among young adult smokers, faster metabolizers demonstrated increased responding to visual smoking cues (versus control cues) in several brain reward regions, relative to slower metabolizers, as measured using functional magnetic resonance imaging (fMRI) [93]. This increase in cue-evoked responding may contribute to the relatively higher rate of smoking relapse observed among faster metabolizers relative to slower metabolizers.
4.4.3 Gene–Gene Interactions

**CYP2A6 and CYP2B6**

Cytochrome P450 2B6 (CYP2B6) may contribute to nicotine metabolism but possesses a lower affinity for nicotine and metabolizes nicotine at a slower rate than CYP2A6 in human liver microsomes [94]. Some studies have implicated a significant role for CYP2B6 in altering NMR [67] and nicotine clearance [95]. Interestingly, Ring et al. [95] observed a larger effect of CYP2B6 variation on nicotine clearance in reduced CYP2A6 metabolizers compared to normal CYP2A6 metabolizers. However, when controlling for CYP2A6 genotype, virtually no effect of CYP2B6 variation on baseline NMR [96] and in vitro nicotine C-oxidation activity [97] was observed. In addition, the CYP2B6*6 variant was associated with poorer abstinence rates in the placebo arm of a bupropion smoking cessation trial [98]; however, it is not clear whether this effect was mediated by alterations in nicotine metabolism and/or smoking behaviors.

The apparent effects of CYP2B6 genetic variation on nicotine metabolism and/or smoking behaviors may occur via coregulation with CYP2A6, as the two genes may share a 5’ regulatory region due to their close proximity and opposed transcriptional start sites [97]. In support of this notion, correlations between CYP2B6 and CYP2A6 mRNA [99] and protein [97, 100] levels have been observed. Alternatively, CYP2B6 be associated with nicotine metabolism through genetic linkage disequilibrium (LD) with CYP2A6. This is plausible since CYP2A6 and CYP2B6 are within close proximity to one another (<150 kb apart) in a common gene cluster on chromosome 19 [101]. While no statistically significant effect of LD was observed on in vivo nicotine clearance [95] or on in vitro nicotine metabolism in human liver microsomes [97], a large sequencing study found some evidence of LD between several variants in CYP2B6 and the CYP2A6*12 allele [102]. In addition, evidence of strong LD between CYP2B6*6 and CYP2A6*9 has been reported [67]. Thus, the role of CYP2B6 genetic variation in altered nicotine metabolism, and resulting smoking behaviors, requires further clarification.

**CYP2A6 and CHRNA5–CHRNA3–CHRNB4 Cluster**

Several genome-wide association studies identifying susceptibility loci associated with smoking amount, nicotine dependence, lung cancer risk, and response to smoking cessation therapy have found significant associations with variation in a region of chromosome 15 containing the α5, α3, and β4 subunit genes of the nAChR [103–107]. A recent meta-analysis investigating the impact of variation in the CHRNA5–CHRNA3–CHRNB4 cluster found significant associations for level of cigarette consumption and lung cancer risk [108]. Because variation in CYP2A6 is also associated with smoking behaviors [33, 34, 36, 38] and lung cancer risk [109–112], it is logical to investigate...
the combined effect of variation in nicotine and nitrosamine pharmacokinetic genes (e.g., CYP2A6) and nicotine and nitrosamine pharmacodynamic genes (e.g., CHRNA5–CHRNA3–CHRNB4) on these outcome measures. In a case–control lung cancer study in adult smokers [35], CYP2A6 appeared to play a relatively larger role in modulating smoking behaviors and nicotine dependence while CHRNA5–CHRNA3–CHRNB4 was more important in influencing lung cancer risk. Normal CYP2A6 metabolizers smoked about six more cigarettes per day and had significantly higher FTND scores than CYP2A6 reduced metabolizers while those with the CHRNA5–CHRNA3–CHRNB4 rs1051730 AA genotype smoked about one more cigarette per day (not significant) and did not have significantly higher FTND scores relative to those with the CHRNA5–CHRNA3–CHRNB4 rs1051730 GG/GA genotype [35]. Although CHRNA5–CHRNA3–CHRNB4 played only a small role in modulating smoking behaviors, CHRNA5–CHRNA3–CHRNB4 AA individuals were at significantly greater risk for lung cancer relative to CHRNA5–CHRNA3–CHRNB4 GG/GA individuals (OR = 1.57). In contrast, there was a modest, nonsignificant increase in lung cancer risk among CYP2A6 normal metabolizers relative to CYP2A6 reduced metabolizers (OR = 1.26), except in those smoking less than or equal to 20 cigarettes per day [35]. In examining the effects of CYP2A6 and CHRNA5–CHRNA3–CHRNB4 in combination, individuals in the high-risk genotype group (CYP2A6 normal metabolism and CHRNA5–CHRNA3–CHRNB4 AA) smoked a mean of 7.1 more cigarettes per day (27.9 vs. 20.8) and had greater lung cancer risk (OR = 2.03) relative to individuals with neither risk genotype [35]. This suggests that genetic variation in CYP2A6 and CHRNA5–CHRNA3–CHRNB4 independently and additively combines to modulate both cigarette consumption and lung cancer risk [35]. A follow-up investigation in the same study population revealed a potential role for CYP2B6 variation as a risk factor for lung cancer, independently of CYP2A6 and CHRNA5–CHRNA3–CHRNB4, where the CYP2B6*1/*1 + CYP2B6*1/*6 genotype group displayed a trend toward increased risk for lung cancer (OR = 1.25; vs. CYP2B6*6/*6 genotype group) [113]. Variation in CYP2B6 is thought to modulate lung cancer risk through the differential activation of tobacco-specific nitrosamines, rather than via alterations in cigarette consumption [113].

An independent and combined effect of variation in CYP2A6 and CHRNA5–CHRNA3–CHRNB4 on smoking behavior has also been demonstrated in adolescents [114]. CYP2A6 reduced metabolizers with two copies of the nonsynonymous CHRNA5 SNP rs16969968 (AA) were at an increased risk of regular smoking relative to CHRNA5 GG/GA individuals expressing CYP2A6*1 (OR > 4) [114].

Thus, it is important to investigate the individual and combined impact of variation in nicotine metabolism genes and nicotine receptor genes on smoking behaviors and the risk for tobacco-related diseases, as both appear to contribute singly and additively to modulate smoking behaviors and disease risk.
4.5 Impact of Smoking and Nicotine Metabolism on Disease

4.5.1 Cancer
Cigarette smoking increases the risk of developing several different types of cancers including, but not limited to, those of the oral cavity, lung, bladder, cervix, stomach, and kidney [115]. A variety of epidemiological studies have shown cigarette smoke exposure to be the main causal factor in the etiology of lung cancer, and lung cancer accounts for the highest rate of cancer-associated mortality worldwide [115]. Up to 90% of lung cancer cases are attributable to cigarette smoking [115, 116]. In addition, lung cancer risk increases with increasing levels of cigarette consumption and duration of smoking [117]. Exposure to secondhand smoke as well as the use of smokeless tobacco products such as chewing tobacco also increase cancer risk, with the latter causing mostly oral cancer [115].

More than 60 carcinogens have been identified in cigarette smoke, including polycyclic aromatic hydrocarbons, aromatic amines, and tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNK) and N'-nitrosonornicotine (NNN) [115, 118]. These tobacco-specific nitrosamines are present at considerable levels in both cigarette smoke and a variety of smokeless tobacco products [118]. CYP2A6 is involved in the metabolic activation of both NNN and NNK [119, 120]. Thus, in addition to its role as the principal enzyme mediating nicotine inactivation, CYP2A6 contributes to the activation of procarcinogens. Importantly, altered CYP2A6 metabolism may influence the risk for lung cancer directly, as well as indirectly through the modulation of smoking behaviors [35]. Interestingly, polymorphisms in CYP2A6 resulting in reduced CYP2A6 enzymatic activity are associated with reduced risk of lung cancer [35, 109–112]. Conversely, normal CYP2A6 activity is associated with greater lung cancer risk, even after adjusting for cigarette consumption [110] and cigarette pack years [35], a measure of total active cigarette smoke exposure. This suggests CYP2A6 may directly contribute to lung cancer risk independently of consumption, perhaps via procarcinogen activation [35]. Moreover, the relative lung cancer risk associated with CYP2A6 may be magnified in people smoking 20 or fewer cigarettes per day, suggesting greater relative genetic risk for lung cancer at lower levels of cigarette consumption [35].

In addition to significant protective effects of reduced CYP2A6 metabolism on lung cancer risk, reduced CYP2A6 metabolism also protects against head and neck squamous cell carcinoma (HNSCC) [121], colorectal cancer [122], oral cancer [123], and upper aerodigestive tract cancer [124].

4.5.2 Cardiovascular Disease
Cigarette smoking is also associated with a number of adverse cardiovascular outcomes including peripheral vascular disease, aortic aneurysm, stroke, coronary heart disease,
and myocardial infarction [125–127]. Although smoking indirectly influences cardiovascular disease risk by modulating levels of other risk factors, there is also a direct association between smoking and cardiovascular disease [125]. After adjusting for differences in other risk factors between smokers and nonsmokers, an independent association between smoking and risk for cardiovascular disease remains [125].

A variety of studies have implicated a role for cigarette smoke in vascular endothelial cell dysfunction and accelerated atherosclerosis [128–131], which are major antecedents of coronary heart disease [132]. Importantly, the risk for cardiovascular disease attributable to smoking is positively associated with heaviness of smoking and smoking duration [125, 133–135]. Even a low level of smoking (one to four cigarettes per day) increases the risk for coronary heart disease [136] and death from ischemic heart disease [135]. In addition, passive cigarette smoke exposure is associated with a greater risk of coronary heart disease among nonsmokers, and the risk increases with increasing level and duration of exposure [137].

To date, only one study has examined a potential role for altered nicotine metabolism/CYP2A6 genotype as a modulator of cardiovascular disease risk in smokers [138]. Consistent with the positive association between cigarette smoke exposure and risk for cardiovascular disease, heavier smokers (more than 15 cigarettes per day) had an increased risk of hypertension relative to lighter smokers (15 or fewer cigarettes per day) (OR = 1.59), and this relationship was moderated by CYP2A6 [138]. Specifically, the risk for hypertension was highest among heavier smokers with normal CYP2A6 metabolism, relative to lighter smokers with slower CYP2A6 metabolism (≤50% CYP2A6 enzymatic activity as predicted by genotype) (OR = 2.74) [138].

4.5.3 Obesity

Cigarette smoking is negatively associated with total body weight as smokers tend to present with lower body mass index (BMI) than nonsmokers [139, 140]. The rate of nicotine metabolism, as measured by NMR, is also negatively associated with BMI among smokers [31, 54, 141]; however the mechanisms governing this relationship remain to be clarified. Differences in BMI may change the volume of distribution, metabolism, or renal clearance of nicotine’s metabolites, or it may alter the regulation of the CYP2A6 enzyme [77].

Smokers gain body weight upon quitting smoking [142]. In a smoking cessation trial, individuals who were continuously abstinent for one year had gained an average of 5.90 kg while individuals who had continuously smoked for one year had gained an average of only 1.09 kg [142]. The ability of nicotine to reduce food intake has been demonstrated in animal models, and cigarette smoking is associated with appetite suppression in humans [143, 144]. Nicotinic drugs decrease food intake in mice via β4 subunit-containing nicotinic acetylcholine receptors, in a process that involves activation of pro-opiomelanocortin neurons [144]. Activation of pro-opiomelanocortin
neurons in the arcuate nucleus is thought to decrease food intake as well as increase energy expenditure [145], and disruption of pro-opiomelanocortin neurons promotes obesity in both humans and animals [146, 147].

While smoking appears to be associated with lower total body weight, it is associated with increased waist circumference and waist-to-hip ratio [148, 149]. A prospective investigation in five birth cohorts of Finnish twins revealed a significant positive association between smoking in adolescence and future development of abdominal adiposity [150]. After adjusting for confounders, smoking at least ten cigarettes per day between the ages of 16 and 18 was associated with an approximately 30% increased risk of developing abdominal obesity in adulthood [150]. In addition, the level of cigarette consumption appears to alter risk for abdominal obesity, such that heavier smokers (more than 15 cigarettes per day) are at increased risk of abdominal obesity compared to lighter smokers (15 or fewer cigarettes per day) (OR = 1.57) [151]. Importantly, \textit{CYP2A6} has been shown to moderate the association between heavy smoking and abdominal obesity [151]. Among individuals with poor \textit{CYP2A6} metabolism (less than 25% of the activity of normal metabolizers [68]), heavy smokers were at increased risk of abdominal obesity compared to lighter smokers (OR = 3.90) [151].

These findings hold important implications for public health as abdominal obesity is a major risk factor for type 2 diabetes (T2DM) and a number of adverse cardiovascular outcomes, including congestive heart failure, myocardial infarction, and stroke [152, 153]. Together smoking and abdominal obesity promote insulin resistance and altered secretion of proinflammatory cytokines, the latter of which are thought to cause endothelial dysfunction and the subsequent development of cardiovascular disease [153].

4.5.4 Diabetes

Cigarette smoking is associated with the development of T2DM [154], consistent with the increased level of insulin resistance observed among smokers relative to non-smokers [155]. A recent meta-analysis comprising over one million subjects found a pooled adjusted relative risk of 1.44 for developing T2DM among current smokers relative to nonsmokers [154]. In addition, heavy smoking (more than 20 cigarettes per day) increases the risk of T2DM relative to light smoking (10 or fewer cigarettes per day), with an odds ratio of 1.75 [156]. Interestingly, \textit{CYP2A6} genotype modulated the interaction between heavy smoking and risk for T2DM, such that heavier-smoking individuals with poor \textit{CYP2A6} metabolism ($\leq$25% \textit{CYP2A6} enzymatic activity as predicted by genotype) were at greater risk of having T2DM compared to lighter-smoking individuals with normal \textit{CYP2A6} metabolism (OR = 8.54) [156]. This increased risk for T2DM among heavy-smoking individuals with poor \textit{CYP2A6} metabolism may be
due to greater exposure of the pancreas to nicotine as a result of higher circulating nicotine levels among heavy-smoking poor metabolizers [156]. Nicotine exposure was previously shown to cause impaired function and apoptosis of beta cells in rodent models [157, 158], and a reduction in beta cell mass is a hallmark of T2DM in humans [159].

4.6 Conclusions and Significance

Nicotine addiction is a complex, multifactorial neuropsychiatric disorder with numerous genetic and environmental risk factors contributing to its etiology. The utility of an intermediate phenotype approach to aid our understanding of the causal pathways underpinning genetic susceptibility to nicotine addiction is evident. As an intermediate phenotype, the rate of nicotine metabolism has a strong genetic component and varies widely between individuals, influencing a number of smoking-related behaviors including cigarette consumption, dependence, and the ability to stop smoking. Moreover, variation in nicotine metabolism contributes to the risk among smokers for a variety of illnesses, such as lung cancer, cardiovascular disease, abdominal obesity, and diabetes.

Future studies investigating the individual and combined impact of variation in CYP2A6 and genes encoding other putative targets involved in nicotine pharmacokinetics and pharmacodynamics may reveal additional pharmacologically relevant intermediate phenotypes and gene interactions that may offer new insights into the complexities of nicotine addiction. Advancements in this field will enhance our current understanding of the mechanisms governing the progression from experimental smoking to nicotine addiction, interindividual variation in the success of smoking cessation pharmacotherapies, and differences among people in their ability to stop smoking unaided. This in turn may inform novel tobacco control programs and improve personalized treatment interventions, with the eventual goal of reducing the high morbidity, mortality, and societal burden associated with cigarette smoking.

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Conflict of Interest

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References


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