

Cotinine Conundrum—A Step Forward but Questions Remain

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There is widespread agreement that self-reported smoking quantity, though a convenient and simple measure, is an imprecise assessment of exposure to tobacco smoke and that measuring serum cotinine levels in cigarette smokers provides a more objective estimate of current smoking behavior. Initial genome-wide association studies (1–3) suggested that single-nucleotide polymorphisms (SNPs) spanning the chromosome 15q25 region encoding the $\alpha 5$, $\alpha 3$, and $\beta 4$ nicotinic acetylcholine receptor (nAChR) subunit gene cluster, *CHRNA5-CHRNA3-CHRN4* (*CHRNA5-A3-B4*), were associated with both smoking intensity and lung cancer risk. Subsequent analyses robustly associated these SNPs with heavy smoking, nicotine dependence, craving, and related endophenotypes (4–7). The association with lung cancer, though statistically robust and initially not altered by adjustment for smoking, increasingly appears to be mediated through smoking. However, there is still uncertainty regarding a direct effect of the variants on lung cancer risk or if the risk for lung cancer is mediated solely through the genetic risk to smoking.

In this issue of the Journal, Munafò et al. (8) provide convincing evidence that genetic variation at chromosome 15q25 locus influences cotinine levels more strongly than smoking quantity (self-reported cigarettes per day). Two single-nucleotide variants in this region were studied for their association with serum cotinine level and smoking intensity—rs16969968, which has a functional effect on nicotine signaling mediated by *CHRNA5*, and rs1051730, which is strongly correlated with rs16969968. Their data from 2932 smokers replicate and extend those reported in 2009 by Keskitalo et al. (9) in a smaller sample size. Both of these studies showed a much stronger association between variants in the *CHRNA5-A3-B4* gene cluster with cotinine than with reported cigarette per day use. In an interesting and valuable application of their results to a published case-control study of cotinine levels and lung cancer risk, Munafò et al. (8) estimated that the per allele increase in cotinine level indicated a 31% increased risk of lung cancer per risk allele of rs16969968, an effect size that is very similar to the effect sizes of the GWAS odds ratios for lung cancer risk for these risk alleles. Therefore, the authors (8) conclude that the association of these variants with lung cancer risk is mediated largely, if not exclusively, through their effect on increasing tobacco exposure.

It is true that the association of the chromosome 15 region and lung cancer is not seen in nonsmokers (10). Yet, a direct association of this locus with lung cancer risk, independent of its role in nicotine dependence, is still disputed. There are compelling data that nicotine and its derived carcinogenic nitrosamines can contribute directly to lung cancer risk through binding to nAChRs,

which then activate proliferation, apoptosis, angiogenesis, and tumor invasion pathways, as well as phosphorylation of the AKT pathway (11). Lam et al. (12) reported different nAChR subunit gene expression patterns in non-small cell lung cancer from never and ever smokers and demonstrated that nicotine exposure in human bronchial epithelial cells resulted in reversible differences in nAChR subunit gene expression. These data all seem to implicate nicotinic receptor activity in bronchial carcinogenesis.

It could be argued that the decision regarding what measure of tobacco exposure to use depends on the outcome phenotype of interest. For accurate classification of current smoking intensity, serum cotinine levels might be the measurement of choice as an objective marker of recent exposure because they remain relatively stable over time in frozen serum samples. Nevertheless, variability in the measurement and the biological limitations of cotinine as a biomarker (short half-life, poorer performance of serum cotinine than urine cotinine as a dosimeter of recent smoking), as well as cost, must be factored into widespread use of this biomarker.

For nicotine dependence, there are a variety of available validated measures, including the Fagerstrom Test of Nicotine Dependence (FTND) or the Nicotine Dependence Syndrome Scale, that estimate dependence quite accurately. Chen et al. (13) have shown that none of the more comprehensive measures of smoking behaviors yielded stronger genetic associations with the chromosome 15q25 locus variants than did cigarettes per day. However, other regions of the genome may not have this same relationship between cigarettes per day and nicotine dependence measures.

If, on the other hand, lung cancer is the phenotype of interest, neither cotinine level nor cigarettes smoked per day capture information on long-term and/or lifetime exposure that are associated with lung cancer risk in an approximately exponential fashion. Nor do they yield an accurate measure of carcinogenic exposure to the lung. This measurement inaccuracy hampers our assessment of risk of lung cancer.

LeMarchand et al. (14) demonstrated that urinary nicotine equivalents (molar sum of nicotine, cotinine, trans-3-hydroxycotinine, and their respective glucuronides) were a more accurate reflection of total nicotine exposure than self-reported cigarettes per day. Church et al. (15) showed that prediagnostic serum levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), were statistically significantly associated with lung cancer risk, even after controlling for intensity and duration of smoking. These findings suggest that adjusting for cigarettes per day and duration of smoking is unlikely to control

sufficiently for smoking dose. It is equally unlikely that cotinine is a better measurement for assessing carcinogenic exposure even though it provides some additional information. Therefore, if the goal is assessing risk for lung cancer, serially measuring urinary or serum levels of total tobacco-specific N-nitrosamines like NNAL and NNK may yield more information than that conveyed by a point prevalence estimate of cotinine level.

Incorporating genetic factors adds another level of complexity. Carriers of the risk variant of rs16969968 smoked more intensively resulting in higher exposures to both nicotine and NNK, even if they smoked equivalent numbers of cigarettes per day (14). We also need to consider the effect of the cytochrome P450 2A6 enzyme (CYP2A6), which metabolizes up to 80% of nicotine into cotinine via C-oxidation. Dependent smokers adjust their cigarette dose to maintain constant blood and brain nicotine concentrations levels and thus avoid withdrawal symptoms. Wassenaar et al. (16) showed that cigarette consumption and nicotine dependence were highest in the combined CYP2A6 normal metabolizers and carriers of *CHRNA5-A3-B4* risk variants. This combined risk group also exhibited the highest lung cancer risk, which was even higher among lighter smokers (ie, individuals smoking ≤ 20 cigarettes per day). This confirms previous data that higher risks associated with rs16969968 are evident in lighter smokers and younger (<60 years) patients (17). Unlike self-reported smoking intensity, classification based on early age at onset is not subject to misclassification and could be a surrogate for lower smoking history. Such findings argue for a role for genetic susceptibility to lung carcinogenesis irrespective of smoking dose.

We do not consider adequate statistical power to be a concern because existing consortia with smoking phenotypes and available biospecimens should yield sufficient number of samples to extend these findings. However, to convincingly demonstrate whether the chromosome 15q25 locus directly contributes to lung cancer, a large consortial study of never smoker case subjects exposed to environmental tobacco smoke and control subjects could contribute to sorting out the direct vs indirect associations posited for the chromosome 15q locus. Very light smokers (eg, <5 cigarettes per day) and remote former smokers (eg, who quit ≥ 15 years before diagnosis and who may be more like nonsmokers) should be included to examine risk gradients across smoking categories to enhance the plausibility of the findings.

In summary, the findings of this study by Munafò et al. (8) further strengthen the association between the *CHRNA5-A3-B4* gene cluster and smoking behavior, confirm that cigarettes per day is an imprecise measure of nicotine consumption, and favor the interpretation that the association with lung cancer is mediated by smoking. But the degree to which the association is mediated by smoking is yet to be determined.

Further studies that include additional biochemical assays of lung carcinogens may help tease apart the direct and indirect associations of these variants with lung cancer risk. Characterization of a comprehensive panel of nicotine dependence loci, with data on smoking behavior over time, may improve our ability to model the role of genetic vs environmental exposures. Evaluating the effects of SNPs on the expression and activity of nicotinic receptors can be explored by taking advantage of *CHRNA3*- and *CHRNA5*-knockout mouse and cellular models (17,18). Studies of cell lines and primary lung cancers can provide insights into the effects

of these variants on proliferation and apoptosis; one such study suggested a role of a proteasome gene in this region beyond the effects of nicotinic receptors (19). Emerging metabolomic markers may provide useful biomarker dosimeters of smoking damage relative to carcinogenesis. Certainly, multiple strategies need to be deployed to further tease apart these complex relationships.

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Notes

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