Elevated levels of 1-hydroxypyrene and *N′*-Nitrosonornicotine in the urine of smokers with head and neck cancer: A matched control study

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Samir Suresh Khariwala, MD

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Thesis Advisor: Dorothy Hatsukami, PhD

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is associated with tobacco use. Still, most smokers do not develop HNSCC. The mechanisms of varying susceptibility to HNSCC are poorly studied to date. Tobacco metabolite research provides insight regarding the innate metabolism and excretion of carcinogens.

Methods: Smokers with HNSCC (cases) were compared to smokers without HNSCC (controls) in a matched cohort. The tobacco metabolites studied are: 1-hydroxypyrene (1-HOP), *N'*-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

Results: In 33 subjects, mean 1-HOP was 1.82 pmol/mg creatinine vs 1.08 pmol/mg creatinine (**p=0.004**) and mean NNN was 0.10 pmol/mg creatinine vs 0.04 pmol/mg creatinine (**p=0.01**) in cases and controls, respectively. NNAL did not differ between groups.

Conclusions: Smokers with HNSCC have elevated urinary levels of 1-HOP and total NNN compared to matched controls suggesting an increased effective exposure to these carcinogens. Tobacco constituent metabolites may be useful in understanding tobacco-related carcinogenesis in HNSCC.

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Introductory Summary

Each year, 40,000 people in the US and 500,000 people worldwide are diagnosed with head and neck squamous cell carcinoma (HNSCC). Despite progress in reducing tobacco exposure in some parts of the world, HNSCC related to tobacco use remains a significant problem. The risk for HNSCC in smokers is approximately 10 times higher than that of never-smokers. When considering all new presentations of HNSCC, 80-90% are associated with tobacco and alcohol use. While SCC of some head and neck subsites is decreasing in incidence, other subsites have seen an increase.

Multiple carcinogens have been identified in tobacco-containing products. The carcinogens most commonly associated with tobacco use are tobacco-specific nitrosamines. These nitrosamines are formed from tobacco alkaloids during the curing and processing of tobacco. The most carcinogenic of these are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN). NNK is associated with the development of lung and pancreatic cancer whereas NNN is associated with esophageal cancer. Polycyclic aromatic hydrocarbons (PAH) represent a diverse group of carcinogenic compounds. 1-Hydroxypyrene (1-HOP) is a urinary metabolite of pyrene, which is always present in PAH mixtures. Techniques have been developed to quantify amounts of NNN, 1-HOP and NNAL (a surrogate measure for NNK) in urine as well as formaldehyde- and acetaldehyde –DNA adducts in leukocytes. These tobacco metabolites have been shown to readily distinguish between those who are exposed and unexposed to tobacco.

This project aimed to address the differences between tobacco users with and without carcinoma by comparing their exposure to specific tobacco carcinogens. The study subjects were patients with HNSCC and a history of tobacco use. The control group was made up of tobacco users who do not have HNSCC. Our results demonstrated higher levels of 1-HOP and NNN in those smokers who had developed a head and neck cancer. This is an important finding as this is the first study of this type to be done in patients with head and neck cancer. These findings may be applied in the clinical realm to help distinguish those smokers who are at the highest risk of developing head and neck cancer. Such data has the potential to dramatically impact future efforts at disease prevention and early detection.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is one of many cancers that are strongly associated with tobacco use[1] [2, 3]. The risk for HNSCC in smokers is approximately 10 times higher than that of never-smokers [4]. While tobacco cessation does reduce the risk of carcinoma, there are conflicting data as to whether a past smoker's risk of carcinoma ever decreases to the level of a never-smoker [5]. Alcohol enhances the risk of HNSCC in tobacco users but also acts as an individual risk factor [6]. When considering all new presentations of HNSCC, 80%-90% are associated with tobacco and alcohol use [6].

Although smoking is an important causal factor for HNSCC, only a fraction of lifelong smokers develop HNSCC over their lifetime. This is evident given that the number of smokers in the United States is estimated at approximately 46 million while there are approximately 50,000 cases of HNSCC diagnosed per year[7-9]. The factors that account for the difference in susceptibility between smokers with and without HNSCC are poorly studied to date. Some smokers may be inherently more susceptible to developing carcinoma due to patterns of tobacco use, individual intrinsic metabolism of carcinogens, or altered excretion. Additionally, variability in individual immune competence can impact susceptibility to a variety of carcinomas, including those of the head and neck[10]. Identifying those smokers at greatest risk for HNSCC would have great benefit through targeted smoking cessation efforts and enhanced surveillance in addition to a greater understanding of carcinogenic pathways.

One approach to better understand the extent of exposure to tobacco carcinogens is through the investigation of tobacco carcinogen metabolites. The inter-individual variation in risk of smoking-related HNSCC may be determined in part by individual variability in the uptake and metabolism of tobacco carcinogens. Evaluation of

tobacco-related carcinogens and their metabolites can determine exposure and extent or pattern of metabolism and perhaps ultimately characterize important differences between smokers who develop HNSCC and smokers who do not. Much of the work performed thus far on biomarkers of tobacco-related carcinogens and toxicants has addressed their role in the development of lung cancer. This work helps to inform the study in patients who develop HNSCC. There are >70 established carcinogens in cigarette smoke [11]. With regard to lung carcinoma, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons (PAH) are considered to be important causative agents. Exposure to NNK results in metabolic conversion to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), also a potent pulmonary carcinogen in rats [12]. Administration of NNK to rodents results in the development of lung and nasal tumors. Furthermore, epidemiologic data in humans directly links NNAL to the risk of developing lung cancer[12, 13]. NNAL is glucuronidated in humans to produce a mixture of glucuronides, NNAL-Glucs[14]. Although NNK itself is not detectable in human urine due to its extensive metabolism, both NNAL and NNALGlucs (the sum of which will be designated as total NNAL) can be readily quantified in human urine [15, 16].

Many polycyclic aromatic hydrocarbons (PAH) are potent locally acting carcinogens in laboratory animals[17]. The most extensively applied and reliable biomarker of PAH exposure is 1-hydroxypyrene (1-HOP). It is a urinary metabolite of pyrene, which is always present in PAH mixtures. While 1-HOP is not tobacco specific, urinary levels are generally 2-3 times higher in smokers when compared to non-smokers[16, 18, 19]. Another commonly studied member of the PAH class is benzo[*a*]pyrene (BaP). BaP causes skin tumors when applied to the skin of different strains of mice, while subcutaneous injection produces malignant tumors at the injection site. Intratracheal administration alone or mixed with particulates caused benign and

malignant tumors in hamsters. Administration of BaP in the diet to mice and rats of different strains increased tumor responses in multiple organs including the esophagus and tongue[17]. Inhalation administration of BaP to hamsters caused dose-related increases in tumors of the upper respiratory tract, including nose, larynx and trachea, and upper digestive tract including pharynx, esophagus and forestomach.

N'-Nitrosonornicotine (NNN) is thought to play a role in both esophageal and oral cancer[16]. It can be readily quantified in human urine by assaying for free, unchanged NNN plus its metabolite NNN-*N*-Gluc[20]. NNN and its metabolites are present in the urine of both smokers and smokeless tobacco users. The level of urinary NNN is significantly higher in smokeless tobacco users when compared to smokers. This finding is consistent with the relatively higher levels of tobacco-specific nitrosamines in smokeless tobacco[20].

This report describes a preliminary analysis of tobacco metabolites in patients with HNSCC. A cohort of smokers with HNSCC has been identified and enrolled in a control-matched study to determine the significance of urinary NNAL, NNN and 1-HOP in this patient population.

Materials and Methods

Study population

Approval from the University of Minnesota Institutional Review Board was obtained. Cases consisting of smokers with a new or previous diagnosis of HNSCC presenting to the Otolaryngology Clinic at the University of Minnesota from April 2009 to June 2011 were identified for enrollment. After obtaining consent, a tobacco and alcohol use questionnaire was administered. This questionnaire is comprehensive in nature and queries multiple aspects of all tobacco use (smokeless and smoked) and alcohol use including duration of use, current and previous rates of use, specific products used, attempted cessation methods and perceived obstacles to cessation. Following completion of the questionnaire, the enrollees submitted a 10 mL urine and 10 mL blood sample. The urine samples were kept in a -20°C cooler until assays were performed. The blood samples were centrifuged to allow separation of the buffy coat and then kept at -20°C for future studies.

Controls consisted of smokers enrolled in the University of Minnesota Tobacco Research Programs for smoking intervention or biomarker studies. These smokers were also given the tobacco and alcohol use questionnaire and submitted blood and urine for analysis as above. Data on control smokers are kept in databases at the Tobacco Research Programs at the University of Minnesota. The control databases contain demographics and urinary metabolite levels at the time of enrollment in smoking intervention studies. Cases and controls were matched on gender and cigarettes per day for purposes of analysis. Alcohol consumption is known to be a risk factor for HNSCC but does not directly impact the tobacco related metabolites described in this

report. It must be noted, however, that ethanol has been shown to be an effective solvent that can increase absorption of carcinogenic substances [21]. However, this effect is measureable at ethanol concentrations much higher than that seen in the most conventional alcoholic beverages (in which the ethanol concentration is generally 15% or less). It was therefore determined that alcohol intake in our study population would have little to no measureable effect on the urinary tobacco metabolites quantified here and subjects were not matched on levels of alcohol intake.

Experimental Assays

Total NNAL, total NNN, and 1-HOP were determined as described previously. Briefly, the assay for total NNAL in urine was performed by a modification of a previously published method [15]. The method involves solid-phase extraction of urine with Chem-Elute and Oasis MCX mixed mode cation exchange cartridges followed by quantification by gas chromatography with nitrosamine selective detection. The detection limit of NNAL was 0.04 pmol/mL. The intraday precision of the assay was 10.9% relative SD (RSD) and interday precision was 13.7% RSD [15, 22]. 1-HOP was determined by HPLC with fluorescence detection, using [D_9]1-HOP as internal standard. Intraday and interday precision were less than 5% RSD[23, 24].

For the analysis of total NNN[25-27], 2-ml urine samples were mixed with [$^{13}C_6$]NNN internal standard and treated with 10N NaOH to convert NNN-*N*-Gluc to NNN [26]. After the hydrolysis, the samples were adjusted to pH 6-8 and extracted with ethyl acetate, extracted on ChemElut cartridges, and further purified by mixed mode cation exchange and normal phase extraction as previously described[27]. The samples were analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) with

selected reaction monitoring for *m/z* 178 → *m/z* 148 for NNN and *m/z* 184 → *m/z* 154 for [$^{13}C_6$]NNN.

Urinary creatinine was assayed from the same urine sample to adjust for dilutional effects. Urinary creatinine was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis) with a Kodak Ektachem 500 chemistry analyzer.

Statistical Analysis

Statistical analysis was performed with the t-test using Stata (College Station, TX) software with two-sided p values <0.05 considered significant. Given that this is an analysis of pilot data, we sought to strengthen the analysis and conclusions by performing control matching using a 3:1 ratio of controls to cases. This was performed in the analysis of NNAL and 1-HOP. 3:1 matching was performed to decrease the impact of any outlier values contained in the control dataset. However, we do not currently have sufficient controls with NNN determined to allow a 3:1 match. This was due to the nature of our control database. This database represents an ongoing collection of specimens (blood, urine) from smokers recruited for smoking cessation research. While NNAL and 1-HOP have been routinely assayed on these smokers for several years, NNN has been assayed in a consistent fashion only recently. As a result, the database contains fewer subjects with NNN values determined when compared to NNAL and 1-HOP. As a result, the analysis of NNN was done with a 1:1 matching ratio. The standard deviation in the control group for NNN was lower than that seen for 1-HOP and NNAL such that his did not significantly impact the power of this study. The number

of subjects needed to obtain power of 80% with α=0.5 to detect a 50% increase in creatinine-adjusted metabolite level ranged from 15-21 for NNN, 1-HOP and NNAL.

Results

This analysis is based on examination of the first 33 HNSCC cases enrolled in our study. The demographics and distribution of the tumor subsites in the 33 HNSCC cases is shown in Table 1. The cohort is made up of 26 males and 7 females, all of whom smoked cigarettes as their only tobacco product. The range of cigarettes per day was 4-60. All levels of urinary toxicant metabolites were adjusted for urinary creatinine level.

Of the 33 cases, we were able to quantify 1-HOP in 29, total NNAL in 29, and total NNN in 32. During the early stages of enrollment, some urine samples were lost or smaller than necessary such that we were not able to have all 3 metabolites assayed in some cases. For 1-HOP and total NNAL, we used a 3:1 matching ratio of controls to cases to strengthen the analysis. Given that we were able to obtain 27 matched controls for the NNN values (see statistical analysis above), our analysis of NNN is based on the 27 cases with available matched controls. Matching variables were gender and cigarettes per day. The metabolites studied here are not influenced by alcohol consumption.

The results of the statistical analysis are shown in Table 2. Levels of 1-HOP and NNN in cases were approximately 1.7-2.5 times higher than that seen in controls. In the case of 1-HOP and NNN, this difference reached statistical significance ($p=0.004$ and $p=0.01$, respectively). Levels of NNAL were not significantly different between case and control groups. Figures 1-3 are scatterplots of the total 1-HOP, NNN and total NNAL values in cases and controls.

Discussion

Tobacco is well known to be a source of multiple carcinogens. Chronic use of tobacco products is strongly linked with the development of multiple forms of carcinoma[2]. The mucosal lining of the upper aerodigestive tract receives significant exposure to tobacco smoke as it is transmitted from the lips to the lungs. As a result, it is common to see a strong history of tobacco use in patients presenting with HNSCC. Still, a relatively small proportion of smokers go on to eventually develop head and neck cancer. One explanation for this may be variability in the uptake and processing of tobacco carcinogens and toxicants based on individual genetic differences and other factors among users.

This report represents the first investigation of tobacco-related carcinogens and their metabolites in smokers with HNSCC compared to smokers without HNSCC. The results demonstrate intriguing differences in levels of carcinogen exposure between these two populations despite controlling for cigarette consumption. 1-HOP is significantly elevated in the HNSCC cases compared to controls. This is notable in that a significant difference is seen in a relatively small sample size. The results indicate that PAH-related carcinogenesis may be prominent for this disease. This suggests an exciting path for further investigation as smokers with HNSCC may have an increased susceptibility to the effects of 1-HOP and other PAH, thus resulting in their developing HNSCC while other smokers do not. The difference in susceptibility can be mediated by several mechanisms, one of which may be the previously described polymorphisms of CYP1A1, GSTM1 and GSTT1 enzymes that result in activation of PAH[28]. Therefore, the data presented here points us in the direction of investigating these polymorphisms

in tobacco induced HNSCC. Given that 1-HOP is elevated in the smokers with HNSCC in this study, investigation of other PAH compounds may be warranted.

Our data demonstrating elevated NNN in those smokers with HNSCC, compared to smokers without HNSCC, is interesting given that NNN has been shown to induce esophageal and nasal cavity tumors in rats[29]. Like the results for 1-HOP (above), NNN is present in higher levels in smokers with HNSCC compared to smokers without HNSCC when controlling for cigarette intake. This may be due to differences at the molecular level relating to activation and/or excretion of NNN such that those smokers who are less able to detoxify this carcinogen are at increased risk for HNSCC.

Given the anatomic proximity and histological similarity of the esophagus to the upper aerodigestive tract, this finding would seem to be intuitive as it is reasonable to expect a similar mechanism of carcinogenesis in tumors of the esophagus and upper aerodigestive tract. Animal studies suggest a prominent role for NNN in the development of a variety of cancers[30]. NNN given in drinking water or by subcutaneous injection induces predominantly nasal tumors in rats[12]. The tumors induced include squamous cell carcinoma, rhabdomyosarcoma, olfactory neuroblatoma as well as papillomas. Both the esophageal and nasal mucosa can metabolically activate NNN, and DNA adducts are detected in both tissues. Those rats receiving NNN in drinking water only are more likely to develop esophageal tumors than those receiving subcutaneous injection. This finding is hypothesized to be related to direct contact of NNN with the esophageal mucosa in those rats given the compound in drinking water. In smokers, urinary total NNN was found to be strongly associated with the risk of developing esophageal cancer in a prospective study based on a cohort of 18,244 Chinese men in Shanghai, China[31]. In that study, urinary total NNN was measured in samples collected before diagnosis in 77 patients with esophageal cancer and 223

individually matched controls, all current smokers. The levels of total NNN were significantly higher in cases as compared to controls. Odds ratios (95% confidence intervals) of esophageal cancer for the 2nd and 3rd tertiles of total NNN were 3.99 (1.25-12.7) and 17.0 (3.99-72.8), respectively, compared to the 1st tertile after adjustment for urinary total NNAL, total cotinine, and smoking intensity and duration (P_{trend}<0.001). These findings along with data from previous experimental studies strongly suggest a significant and unique role of NNN in esophageal carcinogenesis in humans. This is consistent with the data presented here in that total NNN is elevated in smokers with HNSCC.

In this study, total NNAL did not differ between cases and controls. This suggests a less significant role for NNK (precursor of NNAL) in the carcinogenic pathway of HNSCC. On the other hand, total NNAL is associated with the risk of developing lung cancer. A recent study by Church et al prospectively examined serum levels of total NNAL in smokers with and without lung cancer [32]. Prior to adjustment for confounders, the variables of age, duration of smoking and serum total NNAL were statistically significantly associated with the risk of lung cancer. After logistic regression analysis, only age and total NNAL level remained statistically significant. A second recent study examined pre-diagnostic levels of tobacco metabolites and the risk of lung cancer in two prospective cohorts of cigarette smokers [13]. Patients with total urinary NNAL in the second and third tertiles had an increased risk of developing lung cancer (RR:1.43 and 2.11 respectively). When considering levels of cotinine in combination with total NNAL, an interesting result was observed. Those subjects who were in the highest tertile of total NNAL and total cotinine had an 8.5-fold risk of developing lung cancer compared to smokers with a comparable smoking history but total cotinine and NNAL levels in the

lowest tertile. Similar more extensive studies need to be conducted among individuals with HNSCC.

We are encouraged by the potential implications of the data presented here as it supports the identification of tobacco-related carcinogens that are most important in the development of HNSCC. This information will be useful in targeting the metabolism of those specific carcinogens that are most responsible for HNSCC. In this way, the genetic polymorphisms that account for varying metabolism, and therefore varying degrees of risk for tobacco-induced HNSCC, may be identified. Ultimately, this information has the potential to identify those who are at highest risk for developing tobacco-induced HNSCC. It is our hope that, with further study of the metabolites investigated in this report, as well as additional metabolites and markers, we can eventually describe tobacco constituent metabolite profiles that indicate “standard risk” or "highest risk” for developing HNSCC. Given that the assays performed in this study are relatively inexpensive, it is conceivable that they may eventually be utilized in screening for those smokers who are most likely to develop HNSCC. Lastly, our data suggests an important difference between the carcinogenic pathways of HNSCC and lung carcinoma in that NNAL levels have been shown to indicate risk for lung carcinoma but were not elevated in our subjects with HNSCC. This is consistent with carcinogenicity studies of orally administered NNK and NNAL in rats.

The main limitation of our study is that it is that it is preliminary in nature and thus contains a small cohort. However, we feel that the potential implications of the data are nonetheless significant as discussed above. An additional limitation is that, due to the small sample size, we were unable to perform an analysis based on tumor sub-site within the upper aerodigestive tract. We anticipate performing this analysis as our cohort increases in size.

In summary, we have presented the first report of urinary tobacco-related carcinogen metabolites in patients with head and neck cancer. This preliminary analysis has identified statistically significant differences between smokers with HNSCC and smokers without HNSCC. We now plan to study these metabolites in a larger group of subjects. We anticipate that further study along this line will improve our understanding of tobacco associated carcinogenesis in head and neck cancer and provide a potential opportunity for pre-diagnosis risk assessment and prevention.

Tables and Figures

Table 1	N (%)
Male	26/33 (79)
Female	7/33 (21)
Tumor Subsite	
Nasopharyx	1/33 (3)
Oropharynx	11/33 (33)
Larynx	11/33 (33)
Hypopharynx	3/33 (9)
Oral cavity	7/33 (21)

Table 2	**Case**	**Control**	**p value**
n (1-HOP)	30	100	
1-HOP (pmol/mg creatinine) **(+/- SE)**	1.82 (+/-.25)	1.08 (+/-.12)	**0.004**
n (NNN)	27	27	
NNN (pmol/mg creatinine)	0.10 (+/-.02)	0.04 (+/-.005)	**0.014**
n (NNAL)	29	100	
NNAL (pmol/mg creatinine) **(+/- SE)**	1.51(+/-.19)	1.68 (+/- .11)	0.48

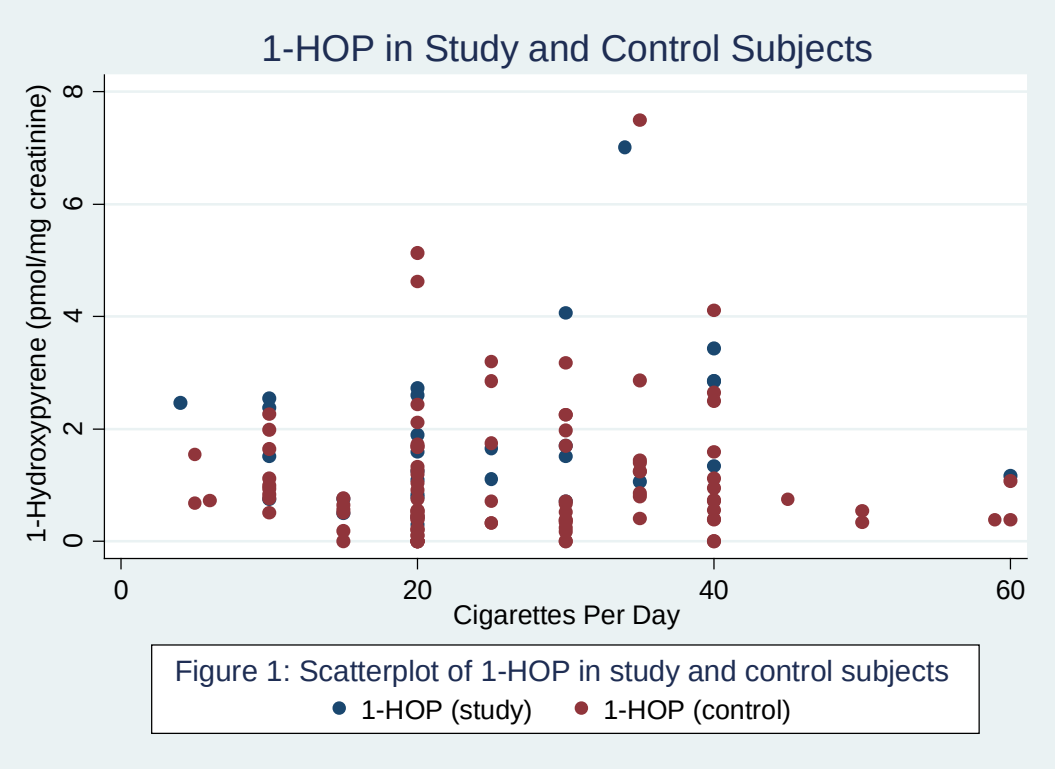

Figure 1: Scatterplot of 1-Hydroxypyrene in cases and controls

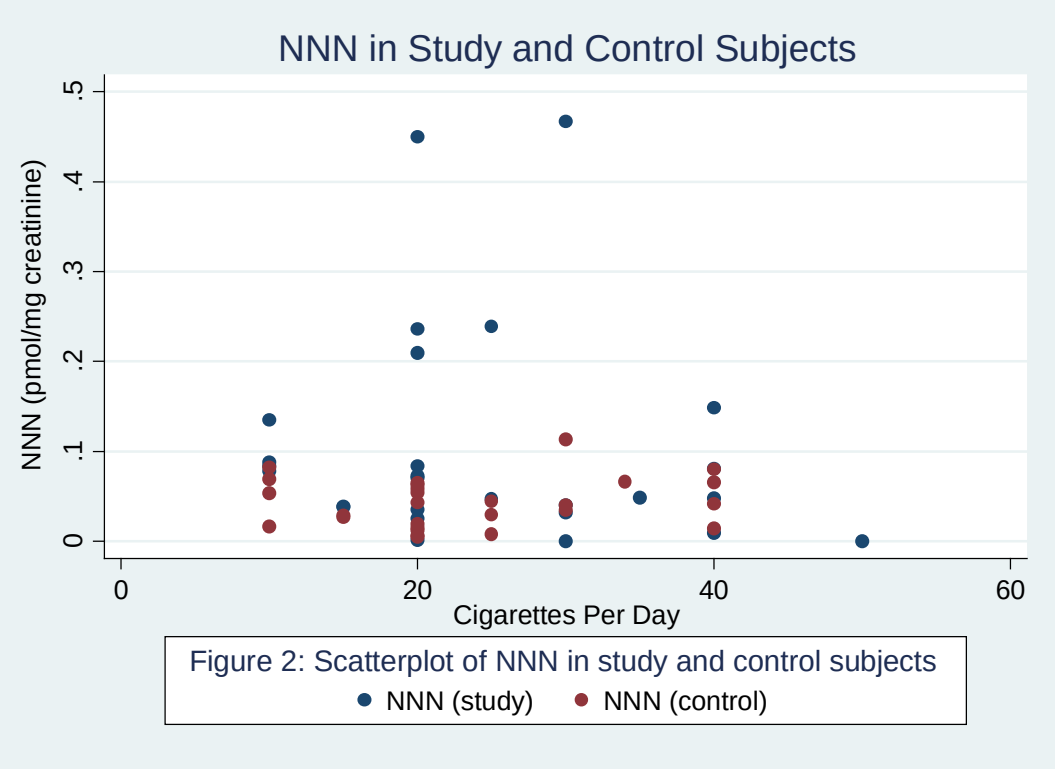

Figure 2: Scatterplot of *N*'-Nitrosonornicotine in cases and controls

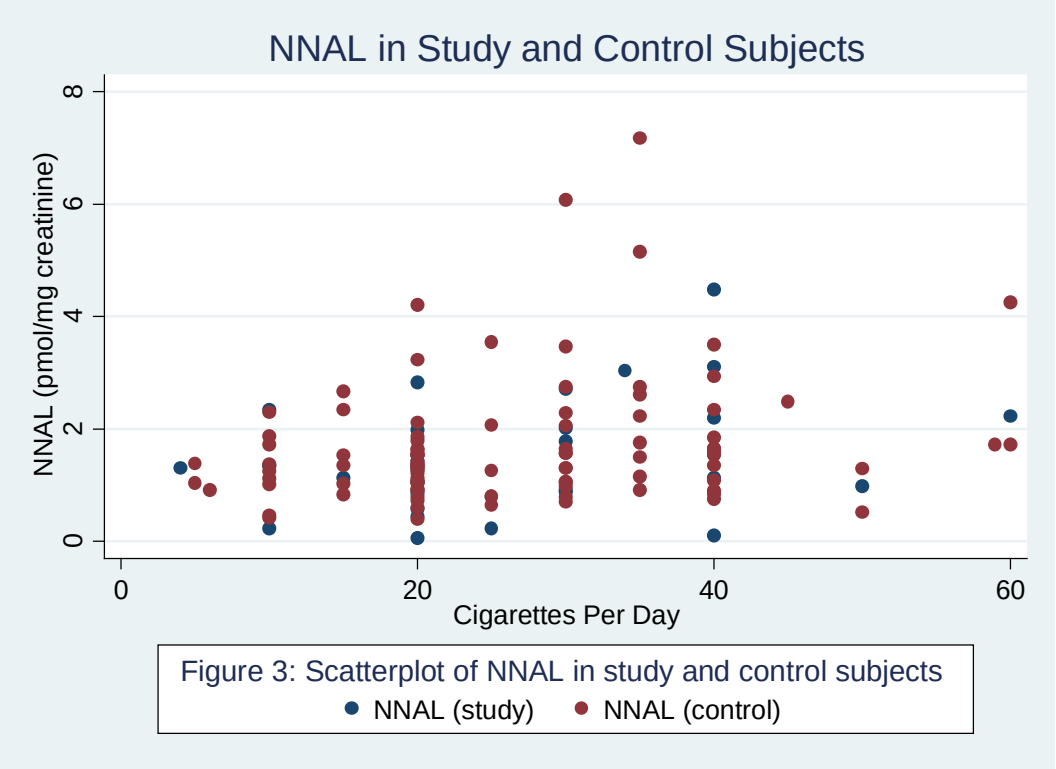

Figure 3: Scatterplot of NNAL in cases and controls

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