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A Pilot Project To Assess The Effect Of Tobacco Smoking On Multiple Sclerosis Susceptibility And Severity

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ABSTRACT

The field of multiple sclerosis (MS) is in need of a consistent method of measuring smoking to examine its role in disease risk and severity. A pilot study was conducted to examine the association between smoking and disease risk in MS cases (n=26) and controls (n=26). Disease severity within MS patients was assessed through clinical and radiological outcomes. These measures were confirmed within a larger dataset of MS patients (n=512) that additionally contained genotyping information. There were no significant differences in disease risk or severity in the pilot study. Within the larger dataset, clinical score trended toward significance (p=0.13). Stratification based on HLA DRB1*1501 status showed that MS smokers with the HLA DRB1*1501 allele had 2.86 greater odds of having more severe clinical disease than nonsmokers with the genotype (1.41, 5.80; p=0.003). To our knowledge, this is the first study to examine smoking and MS disease severity while controlling for genotype.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

INTRODUCTION

Multiple sclerosis (MS) affects over 400,000 Americans and 2.5 million people worldwide, and is the most common neurological disease in young adults.[1,2] Multiple sclerosis is characterized as an immune-mediated demyelinating disorder resulting in significant disability and decreased quality of life. While several genome-wide linkage studies have confirmed a strong genetic component of disease inheritance of MS with risk among family members up to 50 times higher than on average in the population,[3,4] MS is also thought to involve environmental factors which may be crucial to disease development.[5] For example, a study examining MS in the Australian population has attributed modifiable environmental factors in being able to prevent approximately 80% of cases.[6]

Large-scale prospective epidemiological studies of MS have been limited in number and generally lack the statistical power to detect an association due to the relatively rare status of the disease, which affects 3.6 cases per 100,000 person-years in women, and 2.0 cases per 100,000 person-years in men.[7] Despite the paucity of epidemiological research, historical and prospective cohort studies, as well as case-control designed experiments, have consistently identified three main environmental factors associated with risk of MS: vitamin D deficiency (and/or sun exposure), cigarette smoking, and infection with Epstein Barr virus.[6,8,9] Other possible agents include, but are not limited to: duration of breastfeeding,[10,11] diet,[12-15] obesity,[16] oral contraceptive use,[17,18] history of allergy,[19,20] and air pollution.[21]

These environmental studies, however, have largely been observational in nature and dependent on previously collected data, which has resulted in limited conclusive findings. Furthermore, studies examining MS risk factors have also lacked objective measures, relying primarily on subjective outcomes of physical functioning and routine assessments that are

conducted in the clinic, such as the Timed 25-Foot Walk[22] and Kurtze's Expanded Disability Status Scale (EDSS),[23] respectively. This has made it difficult to characterize the various disease subtypes that exist, both clinically and pathologically, and to follow disease progression, severity, and responses to therapy.[8]

Therefore, there exists a critical need for longitudinal data collection of environmental risk factors to investigate how these types of exposures may influence certain disease characteristics and relate to clinical, immunological, radiological, and genetic markers. This will lead to a better understanding of MS etiology, with the potential to improve treatment and eventually lead to primary preventative measures that will assist in reducing the risk of MS in the population.

The Multiple Sclerosis Center at Yale University in New Haven, CT will be undertaking a large cohort study with the intent to examine a multitude of risk factors in MS. Currently, the Yale MS Center treats over 1,000 patients annually from the greater southern Connecticut area. The Center offers state-of-the-art treatment for patients with MS and demyelinating-related diseases, and is focused on providing the best patient care available, while also being active in clinical research and public health initiatives. The multidisciplinary team is involved in many ongoing clinical trials with highly experienced personnel aiming to advance the understanding of MS and improve treatment options and quality of life for its patients.

The aim of the Yale MS Center cohort study is to build a quantitative risk assessment model by combining genetic, immunological, radiological, clinical and epidemiological measures in attempt to better assess MS disease risk, characterize disease severity, and measure disease progression over time. One important aspect of the study was to create a questionnaire that would allow for the collection of retrospective and prospective epidemiological data to

capture information on various environmental risk factors. These measures would assist in determining how these exposures relate to MS disease alone, as well as in conjunction with other covariates, such as specific genotypes and serum biomarkers.

Development of the Questionnaire

In order document patients' histories with respect to a variety of environmental exposures, the Social and Environmental Questionnaire (SEQ) was designed by the primary investigator (see Appendix A). While there have been a variety of exposures studied in relation to MS, those that were selected for the questionnaire were either previously found to be strongly associated with MS or thought to have suffered from measurement issues (i.e. inconsistent or imprecise measurement), and thus were included in an attempt to be more accurately assessed. Additionally, the questionnaire highlights areas of recent importance to the immunologists and other researchers working with the Yale MS Center in order to investigate their specific hypotheses.

What differs in this questionnaire compared to previous studies of MS is that investigators were interested in measuring not only the duration of environmental exposure, but also the severity or intensity of each exposure. The investigators also sought a more comprehensive measure of environmental exposures, which is often not accounted for in MS research. By looking at a number of variables in a large cohort, the group will be able to measure interaction effects between environmental risk factors, as well as in conjunction with imaging measures, immunologic biomarkers, and genetic factors.

The SEQ represents one portion of the data collection in this cohort study that will examine a multitude of risk factors through recruitment of cases from the Yale MS Center and

matched controls from the greater Connecticut area, both of which will be followed over a five-year period. Two versions of the SEQ were developed for both cases and controls: a baseline questionnaire, and a follow-up questionnaire. The baseline questionnaire includes questions relating to childhood and recent exposures, while the follow-up questionnaire focuses on the domains in relation to 'time since last visit,' with the intent to be administered at each follow-up research visit over the course of five years.

The baseline SEQ includes seven domains: 1) childhood environment; 2) sun exposure; 3) tobacco exposure (active and passive); 4) physical activity; 5) diet (childhood and current); 6) oral contraceptives; and 7) social demographics. The measures for each domain were derived from a variety of sources. In most instances, the domains were either not previously studied in MS or not well tested, and therefore the standardized questions were derived from studies examining other diseased populations.

In order to establish any areas of concern or problematic features of the questionnaire, the SEQ was pilot tested in both MS cases (N=26) and controls (N=26). Despite the sample size, the goal of the pilot study was to identify large associations and/or generate hypotheses that could be later tested and confirmed in the longitudinal study. Analysis of the cohort's baseline data will also allow investigators to verify whether results from the cross-sectional analysis are consistent with subsequent analyses of longitudinal data over time. Lastly, the data will enable the group to assess disease progression over time, matching subjective clinical measures with objective measures, such as changes measured by MRI.

Due to the small sample size of the pilot study, one domain was investigated in relation to disease risk and severity: tobacco exposure. Smoking has been cited as one of the leading modifiable environmental risk factors in MS; however, previous studies have suffered from

inconsistent measurement and inadequate adjustment of potentially important covariates. The SEQ provides researchers with a more detailed method of measuring tobacco exposure by inquiring about both first and second hand smoking, as well as allowing participants to specify during which age periods the exposure occurred.

We hypothesize that there will be significant differences between MS cases and controls with respect to smoking status. MS cases will be significantly more likely to have smoked and have been exposed to second hand smoke than control subjects. Additionally, MS cases that have smoked will have more severe clinical and radiological outcomes than MS never smokers.

Measuring Tobacco Exposure

Tobacco smoking is one of the most prominent risk factors related to a multitude of chronic diseases, especially those related to the respiratory tract and the cardiovascular system.[24] Smoking has also been associated with many immune-mediated diseases, thought to result from persistent exposure to chemicals that can indirectly cause oxidative stress and inflammation responses throughout the body, while also placing individuals at an increased risk of infection.[25]

Although the exact mechanism is still unknown, smoking and nicotine exposure are thought to act on the immune system through both pro-inflammatory and immunosuppressive mechanisms. Epidemiologic studies examining the effects of smoking and autoimmune diseases have consistently found associations with patients suffering from rheumatoid arthritis,[26-28] systemic lupus erythematous, [29,30] Graves' disease,[31,32] and primary biliary cirrhosis.[33]

Previous studies examining tobacco exposure in MS patients have used a variety of methods to assess its contribution to disease risk. Despite this heterogeneity, a recent meta-analysis found a significant association between smoking and risk of MS by combining

information from six case-control studies and three prospective cohorts (RR 1.5 [1.4-1.7]).[34] The authors stated that although the relative risk was somewhat small, consistent findings strongly argue for a causal association.

A number of studies have also investigated how smoking may influence MS disease course. Results from these studies demonstrated negative effects on various clinical measures, such as the Kurtze's Expanded Disability Status Scale (EDSS),[23] as well as T1 and T2 volume lesion load, representative of subclinical disease activity.[35,36] Additionally, smoking has been associated with faster progression to more severe types of MS (e.g. clinically-isolated syndrome to relapsing-remitting MS, and relapsing remitting MS to secondary-progressive MS), further implicating smoking as a leading environmental risk factor for disease severity in MS.[35,37,38] However, these studies were only able to identify a risk between ever smokers compared to never smokers, and did not find an association with increasing pack-years.

While first hand smoking has been well studied with respect to MS risk, there have only been three published studies examining the association between second hand smoke and disease onset. A French case-control study found an adjusted relative risk of 2.12 [95% confidence interval 1.43-3.15] in children diagnosed with MS associated with parental smoke exposure,[39] while another study found no association between maternal smoking during pregnancy and risk of MS.[40] Most recently, Hedstrom et al. (2011) looked at the effect of second hand smoking in a Swedish cohort of MS patients that identified themselves as never smokers.[41] The group found a small, yet significant association (OR 1.3 [95% confidence interval 1.1-1.6]) and demonstrated an increase in risk with increased exposure (p-value for trend = 0.008), even after controlling for a number of covariates (EBV infection, vitamin D status, ultraviolet radiation, and heredity). Furthermore, the authors found that when second hand smoke persisted for a duration of 20 years

or longer, the OR increased to 1.8 [95% confidence interval 1.2-2.6]. However, the study did not examine if certain time periods, such as childhood versus late adolescence, made individuals more susceptible to disease.

It may be that smoking explains only a small component of MS risk; however, one might argue that previous measures have suffered from inexact or inconsistent measurement analysis. A recent review in the MS literature cited the difficulty in conducting meta-analyses of smoking and MS risk due to the heterogeneity in how data has been collected across studies (e.g. 'cigarettes per day,' 'pack-years,' etc.).[34] In general, previous studies have estimated smoking by 'pack-years,' a single number which is meant to represent an individuals' entire smoking history. This value, however, may not capture changes in smoking habits throughout time, nor be an accurate reflection of intensity and frequency of smoking.

In order to resolve this issue, the SEQ was designed to include a number of questions that attempt to measure smoking history in more detail. The questionnaire begins as most other smoking assessments do in current literature with the primary question: "Do you or did you ever smoke cigarettes, that means smoked *at least* 100 cigarettes in your lifetime?," with 'Yes' or 'No' as options available to respondents. Those that check "no" are asked to skip the section and move on. Those that check "yes" are prompted with the next question: "About how many packs, on average, did you smoke per day? Fill in the blanks for as many of the following as necessary":

1/2 pack a day (or less) for _____ years
1 pack a day for _____ years
1.5 packs a day for _____ years
2 or more packs a day for _____ years

By allowing respondents to answer in multiple fields, the questionnaire does not force participants to give a single approximation of smoking, which may be the source of imprecise

measurement in past literature. The creation of a more detailed method attempts to solve this issue by minimizing the potential for inaccurate measurement by providing researchers with a better estimate of smoking history.

The next question asks participants "How old were you when you first started smoking?" in order to measure any association between age at which one begins smoking and disease risk. Additionally, subjects are asked about their current smoking habits: "Are you currently smoking?; If no, how many years ago did you quit?; How many years total have you smoked, not including any time that you may have quit smoking?"

Given the recent findings that second hand smoking may have a role in disease risk, the SEQ also includes questions regarding passive exposure to tobacco. The SEQ utilizes the questionnaire used by Hedstrom et al.[41] since it provides researchers with a tool that has been validated in the MS population. This questionnaire asks participants: "Have you ever lived with one or more people that used to smoke indoors or outdoors on a daily basis?" and "At your work, have you on a daily basis spent time in rooms/spaces where people smoke?" Participants are also asked to specify the estimated duration (in years) of exposure.

In addition to the Hedstrom et al. questions mentioned above, the SEQ goes into further detail regarding the timing of exposure to environmental smoke. We asked participants to indicate not only how long the exposure occurred (years), but also for an approximate number of hours/day they were exposed to second hand smoke. Moreover, since previous studies have implicated that smoking may play more of a role during earlier stages of life, we determined that it would be important to record the periods in which exposure occurred.

If yes, please specify for each category:
a) Young Child, 0-10 years of age:
_____ hours a day for _____ years
b) Adolescent/Teen, 11-20 years of age

_____ hours a day for _____ years
c) Adult, 21+ years of age
_____ hours a day for _____ years

By including more detailed questions regarding exposure during specific periods of life, we can attempt to measure if disease risk is more susceptible during certain age periods (i.e. windows of exposure), as has been found with MS and other environmental risk factors, such as sun exposure.[42]

Important to note is that none of the aforementioned studies examining smoking and MS appropriately controlled for genotype, specifically HLA1 DRB1*1501, which has been cited in literature to be associated with MS disease susceptibility.[43-45] Individuals that carry one or more copies of this allele have been shown to have an increased number of white matter lesions and a reduction in normalized brain parenchymal volume, as well as significant cognitive impairments.[36,46] Since this allele is also associated with having a higher disease burden, both clinically and radiologically, it would be critical to control for this factor in order to better measure how smoking influences MS disease risk and severity.

To date, only one study has examined how genetics interplay with the smoking-MS relationship.[47] This group of researchers found a significant interaction between two genetic features in MS smokers, presence of HLA DRB1*15 (any allele carriage) and absence of HLA A*02. Smokers with both factors were found to have a 13.5 (95% confidence interval 8.1, 22.6) times higher odds of developing MS compared to never smokers without the two factors, while smokers without both genetic features only had 1.4 (95% confidence interval 0.9, 2.1) odds of developing MS. The study, however, only looked at disease risk and did not measure disease severity in relation to genotype and smoking status.

While these findings further support the role of smoking and risk of MS in conjunction with genetic factors, they also underline the need to explore additional gene-environment interactions, as they are crucial to understanding the etiology of MS. The SEQ, as a piece within the larger context of the five-year cohort study, will assist in establishing the role of various environmental factors in relation to more objective clinical and radiological outcomes, while also incorporating genetic susceptibility.

In order to explore the utility of the SEQ in measuring environmental risk factors, a pilot study was conducted within a small set of patients at the Yale MS Center. We examined the association between smoking status and disease risk in MS cases and frequency age and sex matched controls. We also studied the relationship between smoking and measures of disease severity in MS cases, assessed through both clinical and radiological outcomes. These measures were then confirmed within a larger dataset of MS patients, as there will be more power to detect an effect on severity in this dataset compared to the pilot dataset. The larger dataset also contains previous genotyping information at the HLA DRB1 locus, and will additionally allow us to examine whether the effects of smoking on MS severity are modified by HLA DRB1 status.

METHODS

Participants

Pilot dataset: The primary investigator personally approached all MS patients before or after their scheduled appointments at the Yale Multiple Sclerosis Center during the fall of 2011. Eligible participants were defined as: MS patients aged 18-70 years with a confirmed diagnosis of MS by a neurologist based on the International Panel criteria;[48,49] willingness to participate; and an ability to comprehend the instructions of the questionnaire. Out of the 39 MS patients

that were approached, 26 completed the questionnaire [recruitment rate = 64%]. Control subjects consisted of healthy individuals in the greater CT area without a diagnosis of MS or other demyelinating disease, between the ages 18-70 years, frequency matched to cases based on sex and age (± 5 years). All participants signed an informed consent and the study was approved by the Yale HIC Committee (HIC#1103008246).

Confirmatory dataset: The confirmatory dataset was available to the primary investigator from Dr. Daniel Pelletier, Division Chief of the Yale Multiple Sclerosis Clinic, formally at the University of California San Francisco (UCSF) Multiple Sclerosis Center. The dataset included information collected from a five-year cohort study of MS patients (n=577) primarily from the UCSF MS Center, in which individuals aged 18-70 with a confirmed diagnosis of MS were recruited to participate.[50] The study was approved by the UCSF Committee on Human Research.

Exposure Measurement

Pilot study participants were asked to complete a Social and Environmental Questionnaire (SEQ) comprised of sixty-nine items covering the following domains: 1) Childhood Environment; 2) Sun Exposure; 3) Tobacco Exposure (Active and Passive); 4) Physical Activity; 5) Diet (Childhood and Current); 6) Oral Contraceptives; and 7) Social Demographics (see Appendix A).

First hand smoking in the pilot study was assessed by asking participants if they had smoked at least 100 cigarettes in their lifetime (ever/never), and further inquiring about the number of packs/day over time (cumulative pack-years). Second hand smoking was measured by asking participants if they have ever lived or worked with one or more people that used to

smoke indoors or outdoors on a daily basis.[41] Participants also specified the estimated hours/day and duration (in years) of exposure, as well as specific age periods during which the exposure occurred.

The confirmatory dataset included information on whether patients were never, current, or former smokers. For subsequent analyses, current and former smokers were categorized as 'ever' smokers.

Outcome Measurement

In the pilot study, the outcome of disease was determined by an individual's status as case or control participant as determined by the eligibility criteria mentioned above. Severity of MS disease was assessed by both clinic measure and magnetic resonance imaging (MRI). Clinical severity was determined by Kurtze's Expanded Disability Status Scale (EDSS)[23] scores obtained in the clinic at the patient's time of visit at which they completed the questionnaire. Clinical EDSS score was represented as a binary variable, divided by scores less than 3.0 (minimal disability) to scores equal to or greater than 3.0 (moderate to severe disability). An EDSS score of 3.0 has been consistently used in MS literature as a defined parameter in clinical trials and studies examining disease course and prognosis.[51-53] MRI scans of MS cases in the pilot study were identified at a date closest to questionnaire and EDSS assessment, preferably within a six-month period. An MS neurologist (DP) blinded to outcome and exposure variables read the brain images and recorded the number of white matter lesions as assessed by T2-weighted brain MRI scans, which were then divided into categories (0-9 = mild; 10-19 = moderate; 20+ = severe).

The confirmatory dataset included EDSS scores obtained in the clinic and MRI brain scans at the patient's baseline assessment visit. The number of gadolinium contrast-enhancing (CE) lesions (a measure of acute inflammatory MS activity) and T2-weighted lesion volume (mm^3) (a measure of overall white matter disease burden) was recorded using axial dual-echo T2/PD-weighted images.[50]

Statistical Analysis

Due to the small sample size, non-parametric tests of association were performed for the pilot dataset. Demographic differences between cases and controls in the pilot study were compared using Fisher's exact test and Mann-Whitney U test where appropriate. Differences between MS patients in the pilot study and MS patients in the confirmatory dataset were analyzed using X^2 test and independent sample t-test where appropriate.

For the pilot study, logistic regression was applied to calculate the odds ratio for the risk of having an MS diagnosis in participants who reported ever and never smoking with a 95% confidence interval. A subset analysis of only smokers was also conducted, with number of pack-years as the primary predictor of risk of MS. Pack-years was a binary variable, divided as greater/equal or less than 16 pack-years, as measured in a previous study of MS.[54] The primary predictor for each model was the smoking variable (ever/never; pack-years > or $\leq$ 16), adjusted for age.

Disease severity outcomes in the pilot dataset of MS patients included EDSS score ($\geq$ or < 3) as the clinical dependent variable and number of T2 lesions (mild vs. moderate/severe) as the MRI dependent variable, using separate logistic regression models. The primary predictor of

each model was smoking status (ever/never), adjusted for age and disease duration. Due to the small sample size, all data for the pilot study were determined significant if $p \leq 0.05$.

In the confirmatory dataset, MS severity outcomes included EDSS score ($\geq$ or < 3) as the clinical dependent variable using a logistic regression model, and T2-weighted lesion volume (mm^3) by conducting a linear regression model. T2-weighted lesion volume was log-transformed in order to reflect a more normal distribution.[35] Number of gadolinium contrast-enhancing lesions also served as an MRI outcome and was analyzed using Poisson log-linear regression approach, as previously done in MS literature.[36,55] The primary predictor of each model was smoking status (ever/never), adjusted for age, sex, disease duration, and HLA DRB1 status (positive or negative for the *15:01 allele; at least one copy was considered positive).[46] Interaction variables were also created to test the multiplicative effect of smoking and HLA DRB1 status on disease severity outcomes in this dataset. All data for the confirmatory dataset analyses were determined significant if $p \leq 0.01$.

RESULTS

Demographics

Table 1 describes demographic and disease characteristics of pilot and confirmatory dataset participants. The pilot study included 26 MS cases and 26 frequency age and sex matched controls. The mean age of MS cases in the pilot study was 47.1 $\pm$ 9.4 years, and 48.5 $\pm$ 9.9 year for controls (p=0.28). There were an equal number of females (n=22) and males (n=4) in each group (p=1.00).

The confirmatory dataset was compromised of 577 MS patients. Of these, 512 had both smoking and genotype baseline information on file. Among the 512 MS patients, 68.75% were female (n=352) and 31.25% were male (n=160).

Compared to the confirmatory dataset, MS patients in the pilot study were older (p=0.03) and had a higher ratio of men to women (p=0.09). MS patients in the pilot study also had a shorter disease duration (6.1 $\pm$ 6.4 vs. 9.2 $\pm$ 9.0; p=0.09), higher EDSS (2.6 $\pm$ 2.0 vs. 2.0 $\pm$ 1.6; p=0.07), and were also more likely to have smoked (p=0.03) compared to patients in the confirmatory dataset. However, these values were not significant at alpha = 0.01 level.

MS Susceptibility

A slightly higher percentage of MS patients in the pilot study were smokers compared to controls (65.4% vs. 57.7%), but results were not significant (p=0.78). MS patients that identified themselves as ever smokers also had a higher mean number of average pack-years compared to controls (13.4 $\pm$ 12.7 vs. 9.8 $\pm$ 7.5), but these results were not significant (p=0.63). Compared to control participants, MS cases were not more likely to have lived with a smoker during their lifetime (p=0.76), or during specified age periods (childhood, p=0.40; teenage years, p=0.39) (Table 1).

Ever smokers had increased odds of having a diagnosis of MS compared to never smokers, but this was not significant (OR 1.38 [95% confidence interval 0.45, 4.25]; p=0.57). After adjusting for age, smokers had 1.21 higher odds of developing MS (95% confidence interval 0.38, 3.90) compared to never smokers, but results were not significant (p=0.75). In a sub-analysis of only smokers (n=32), those with 16 or more pack-years had 1.50 odds of having an MS diagnosis, but this was not significant (95% confidence interval 0.33, 6.83; p=0.60).

When adjusted for age, the odds increased to 1.65, but this was also not significant (95% confidence interval 0.32, 7.66; p=0.58) (Table 2).

MS Severity

There were no significant differences between MS ever and MS never smokers with respect to age and disease duration in the pilot study (n=26; p=0.17 and p=0.95, respectively). There were also no significant differences between MS ever smokers and MS never smokers with respect to T2 lesion category (n=21; OR 3.71 [95% confidence interval 0.42,33.1]; p=0.24) and EDSS score (n=24; OR 0.16 [95% confidence interval 0.02, 1.53]; p=0.11) (Table 3). Two of the 26 MS patients did not have an EDSS score reported within six-months of questionnaire assessment, and 5 of the 26 MS patients did not have MRIs within the specified time period.

Within the confirmatory dataset of MS patients, there were no differences between ever and never smokers with respect to age (p=0.25), sex (p=0.18), disease duration (p=0.48), and genotype status (p=0.94). Unadjusted analyses reflected no significant differences in outcome measures between ever and never MS smokers (p=0.56 for number of CE lesions; p=0.74 for T2 lesion volume; p=0.06 for clinical EDSS score). In the adjusted analysis, there were no significant differences in MRI measures between ever and never MS smokers (p=0.40 for number of CE lesions; p=0.58 for T2 lesion volume). MS ever smokers had 1.39 odds of having an EDSS of 3.0 or greater, however this was not significant (95% confidence interval 0.90, 2.13; p=0.13) (Table 4).

Analysis did not show an interaction effect between genotype and smoking in relation to number of CE Lesions (p=0.70) or T2 lesion volume (p=0.46). There was, however, a significant interaction effect between genotype and smoking status with respect to clinical EDSS

categorization (p=0.01), thus the data were stratified by HLA DRB1*1501 status. Upon stratification by genotype, MS smokers carrying at least one copy of the HLA DRB1*1501 allele were significantly 2.86 times more likely to have an EDSS score $\geq$ 3 than never smokers with the allele (95% confidence interval 1.46, 5.80; p=0.003). There was no significant difference in EDSS score between MS ever and MS never smokers without the HLA DRB1 allele (OR 0.89 [95% confidence interval 0.50, 1.56]; p=0.68) (Table 5).

DISCUSSION

The purpose of this pilot study was to explore the relationship between smoking and MS disease risk, as well as disease severity. While previous studies have shown an association with ever/never smoking, the relationship with respect to number of pack-years smoked is less clear. By allowing patients to more accurately specify the number of packs-per-day throughout their smoking history, the SEQ attempted to capture information that may not have historically been recorded in previous studies. Furthermore, the pilot study utilized clinical (EDSS) as well as radiologic (MRI) outcomes to measure disease severity. In addition to first hand smoking, the SEQ captures information on second hand smoke exposure throughout various periods of one's life, which also may be important in determining disease risk and severity.

Compared to previous studies examining smoking and MS disease susceptibility, our pilot study did not find an association between ever smoking and risk of MS. Though we found that slightly more MS patients were smokers compared to controls, these results were not significant. In a subgroup analysis of only smokers, pack-years seemed to better predict disease risk in the adjusted model, though results were also not significant. MS cases were not more likely to have ever lived with a smoker, nor more likely to have been exposed to second hand

smoke during childhood or teenage years. There were also no significant differences between MS ever smokers and MS never smokers with respect to categorical T2 lesion count and EDSS score.

The lack of significant findings in relation to disease susceptibility is most likely due to low power. A power analysis indicated that in order to detect an association with 80% power at an alpha level of 0.05, at least 310 individuals per case and control group would be needed, assuming an effect size of 1.5.[34] An ongoing cohort study based out of the Yale MS Center with a larger sample size will be able to better determine whether our measure of pack-years allows for a more specific and sensitive assessment of MS disease risk.

Results from the pilot study were strengthened by the ability to compare findings of disease severity within a larger confirmatory dataset of MS patients. This second dataset made it possible for us to control for genetic factors that may play a role in influencing disease severity. While we did not find a significant association between smoking and MRI measures in this dataset, we did find an association that trended toward significance with respect to clinical EDSS score after controlling for important covariates. Detection of an interaction effect between smoking and genotype led us to stratify analyses based on HLA DRB1*1501 status. These results showed a striking difference for disease severity between MS patients carrying at least one copy of the allele and MS patients that did not. MS ever smokers positive for HLA DRB1*1501 were 2.86 times more likely to have an EDSS score of 3 or higher than MS patients with the allele that never smoked. Among MS patients without the allele, there was no difference between ever smokers and never smokers in relation to clinical disease severity.

To our knowledge, this is the first study to examine the influence of smoking on MS disease severity while also controlling for genetic factors. A previous study found an association

between smoking and HLA DRB1 status and risk for MS, but did not look at how these factors affected clinical or radiological outcomes.[47] It is interesting that although we detected an association between smoking and clinical measure (EDSS) based on genotype, this was not true for MRI measures. This is contrary to a previous study that found associations between smoking and MS severity as measured by contrast enhancing lesions, as well as T1 and T2 volume load; however, this study did not control for genetic factors.[36] Our results suggest that there are differential effects of smoking on MS severity by genotype as measured by EDSS, and while we did not find this to be true for MRI measures, future studies should incorporate genotype status in their analyses as it may modify results. Repeated studies adjusting for genotype will be able to determine if our findings are valid in other populations.

There are several hypotheses as to why we may not have seen an association between MRI outcomes and smoking status. EDSS measures are largely based on functional and walking ability, which may reflect lesions in the spinal cord rather than the brain. Therefore, a correlation between MRI measures of the brain and smoking would not be detectable using our methods, but would be measurable using EDSS scores. Future studies should consider examining both brain and spinal cord lesions as an outcome measure in relation to smoking, and measure how this compares to measurement using EDSS scores. Additionally, our MRI analyses used only measures of white matter lesions (T2-weighted scans), and not gray matter lesions, which may result in different findings.

Though there is substantial evidence to suggest a role for both first and second hand smoke in MS disease risk, the biological mechanism through which this occurs is still unclear.[56,57] It may be the chemical components of cigarette smoke and not tobacco itself, as a population-based study of MS examining tobacco snuff use did *not* detect an association.[58]

There are over 4500 potential chemical factors in cigarette smoke that could be responsible for mediating disease activity.[27] Two current candidates of interest include cyanide and nitric oxide. Increasing doses of these chemicals have been associated with demyelination in the CNS of animals[59,60] and found to induce axonal loss.[61] Previous studies exposing smoke to animal models of MS (experimental autoimmune encephalomyelitis mice) suggest a mechanism via the immune system, specifically with B- and T-lymphocytes and natural killer cells.[24,62,63]

Cigarette smoke has been associated with a number of autoimmune diseases, such as rheumatoid arthritis,[26-28] systemic lupus erythematous, [29,30] Graves' disease,[31,32] and primary biliary cirrhosis.[33] Furthermore, it has been shown that HLA genes interact with smoking status to increase the risk of rheumatoid arthritis,[64] as well as MS.[47] Therefore, it is hypothesized that these genes, which have a role in presenting peptides to T cells, trigger post-translational modification of peptides cross-reactive with CNS antigens, thus stimulating autoimmunity against the central nervous system.[47]

Other hypotheses linking smoking to MS risk include the possibility of increased risk of infection (e.g. Epstein-Barr virus or other respiratory infection) or influence on estrogen levels, thus disrupting the immune system balance via inflammation.[65,66] Previous research has also indicated that nicotine can have a direct effect on blood-brain barrier functioning, which serves to protect the brain from passage of substances from blood into the central nervous system.[67] This site has been shown to be particularly susceptible in MS patients, as it is thought that one of the first events in MS pathogenesis is the passage of autoreactive T-cells across the blood-brain barrier.[68,69] It will be important for future studies to examine how these and other environmental risk factors interact in predicting MS disease risk. For example, smokers have been found to have lower serum concentrations of 25(OH) vitamin D and 1,25 (OH) vitamin D than non-

smokers,[70] alluding to a possible link between these two well-known risk factors in MS patients. While a smoking-vitamin D concentration interaction has been found in studies examining rheumatoid arthritis patients,[71] this hypothesis has yet to be explored in the MS population.[57,72]

There were several limitations to the pilot study. Firstly, the sample size was not large enough for us to detect an association with enough power. Due to the small sample size, we were also not able to control for disease subtype. Future studies should examine whether there are differential effects of smoking on disease risk and severity depending on one's subtype of MS. We also did not have genetic information in our pilot sample, which our confirmatory analyses showed to be an important covariate to include in statistical models examining smoking in MS. Furthermore, we did not have information on EBV infection or vitamin D status, both of which may serve as mediators or moderators to disease risk and severity.

There also is the possibility that other potential confounders that were not measured in the study track with smoking and may result in detection of an increased risk for disease (i.e. health behaviors such as diet, alcohol consumption, and low exposure to sunlight). We cannot rule out the possibility that recall bias and/or selection bias may have influenced results in both cases and controls. Participants also may have misreported smoking behaviors (social desirability bias); however, since participants answered questions in relation to a number of environmental factors and were not aware of the specific nature of this study (i.e. tobacco smoking), we believe that there was not a differential misreporting of smoking history in cases more than controls. We also did not consider changes in cigarette manufacturing over time, such as tar content and filters. Lastly, we were unable to assess second hand smoking in never smokers in our pilot study due to the small sample size. Unfortunately, second hand smoking information was not collected in the larger confirmatory dataset. Future studies should examine

the extent to which second hand smoke during certain age periods influences disease risk and severity while controlling for susceptible genotype. This information would aid in identifying individuals who may be at increased risk not only through first hand, but also second hand smoking.

While results from the pilot study were not significant, they will assist in the creation of a more specific measure of environmental exposures in MS that will serve to improve epidemiological data collection in a future cohort study examining disease risk, severity, and progression. Results from the confirmatory dataset demonstrated an association between MS disease severity as measured by EDSS and smoking status when stratified by genotype. These results will aid in formulating new hypotheses to be tested in the future with regards to genetic predisposition and risk of developing MS, as well as disease severity in relation to smoking status and other environmental risk factors.

This study has large public health relevance due to its epidemiological scope of which the field of MS is currently in critical need. The Yale MS Center's cohort study will be the first to appropriately examine environmental risk factors in conjunction with genetic, immunologic, clinical and radiologic measures to determine how they influence disease risk, severity, and progression over time. It will also assist in determining how smoking may affect responses to treatment and various immunomodulatory therapies.[69] The information gained from this study adds to literature by suggesting that gene-environment interactions may be associated with MS, and contributes to an improved understanding of the disease's etiology and progression.

Table 1. Demographic and disease characteristics of pilot and confirmatory study participants[a]

Characteristic	MS – Pilot (N = 26)	Controls – Pilot (N = 26)	P[b]	MS – Confirmatory (N=512)	P[c]
Age (years)	47.1 ± 9.4	48.5 ± 9.9	0.28	42.5 ± 9.8	0.03
Sex			1.00		0.09
Female	22 (84.6)	22 (84.6)		352 (68.8)	
Male	4 (15.4)	4 (15.4)		160 (31.2)	
Disease Duration	6.1 ± 6.4	--	--	9.2 ± 9.0	0.09
EDSS	2.6 ± 2.0	--	--	2.00 ± 1.6	0.07
Smoker			0.78		0.03
Never	9 (34.6)	11 (42.3)		291 (56.8)	
Ever	17 (65.4)	15 (57.7)		221 (43.2)	
Pack-Years (smokers, n=32)	13.4 ± 12.7	9.8 ± 7.5	0.63	--	--
Live with Smoker (n=50)			0.76	--	--
Yes	16 (64.0)	18 (72.0)			
No	9 (36.0)	7 (28.0)			
Live with Smoker, Child			0.40	--	--
Yes	10 (40.0)	14 (56.0)			
No	15 (60.0)	11 (44.0)			
Live with Smoker, Teen			0.39	--	--
Yes	8 (32.0)	12 (48.0)			
No	17 (68.0)	13 (52.0)			

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.

[b] P-value is for Mann-Whitney U test (continuous variables) or Fisher's exact test (categorical variables) between MS vs. Controls, Pilot Study

[c] P-value is for t-test (continuous variables) or χ^2 test (categorical variables) between MS Pilot Study vs. MS Confirmatory Dataset

Table 2. Bivariate associations between smoking and MS diagnosis, pilot study (N=52)

Characteristic	N	% MS	Unadjusted OR (95% CI)	p-value	Adjusted OR* (95% CI)	p-value
Smoking status				0.57		0.75
Never	20	45%	1.00		1.00	
Ever	32	53%	1.38 (0.45, 4.25)		1.21 (0.38, 3.90)	
Pack-years (smokers)				0.60		0.58
< 16	22	50%	1.00		1.00	
≥ 16	10	60%	1.50 (0.33, 6.83)		1.56 (0.32, 7.66)	

*Adjusted for age

Table 3. Demographic and disease characteristics of MS patients by smoking status, pilot study (n=26)[a]

Characteristic	Never (N = 9)	Ever (N = 17)	Unadjusted P[b]	Adjusted P[c]
Age (years)	49.8 ± 10.0	45.1 ± 7.9	0.17	--
Disease Duration	5.5 ± 5.1	6.4 ± 7.1	0.95	--
T2 Lesion Load (n=21)			0.36	0.24
Mild	5	6		
Moderate / Severe	2	8		
Clinical Score (n=24)			0.17	0.11
EDSS < 3	4	13		
EDSS ≥ 3	4	3		

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.
[b] P-value is for Mann Whitney U-test (continuous variables) or Fisher's exact (categorical variables), Unadjusted
[c] P-value is for Mann Whitney U-test (continuous variables) or Fisher's exact test (categorical variables), Adjusted for age and disease duration

Table 4. Demographic and disease characteristics of MS patients by smoking status, confirmatory dataset (n=512)[a]

Characteristic	Never (N = 291)	Ever (N = 221)	P[b]	P[c]
Age (years)	42.0 ± 9.2	43.1 ± 10.5	0.25	--
Sex			0.18	--
Female	207 (71.1)	145 (65.6)		
Male	84 (28.9)	76 (34.4)		
Disease Duration	8.9 ± 8.5	9.5 ± 9.5	0.48	--
HLA DRB1 1501			0.94	--
0	159 (54.6)	120 (54.3)		
1-2	132 (45.4)	101 (45.7)		
Number CE Lesions	0.32 ± 1.05	0.38 ± 1.11	0.56	0.40
T2 Lesion Volume (mm^3)	7556.1 ± 12,286.4	7922.9 ± 14,858.7	0.74	0.58
Clinical Score			0.06	0.13
EDSS < 3	222 (76.3)	152 (68.8)		
EDSS ≥ 3	69 (23.7)	69 (31.2)		

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.
[b] P-value is for t-test (continuous variables) or χ^2 test (categorical variables); Unadjusted
[c] P-value is for t-test (continuous variables) or χ^2 test (categorical variables); Adjusted for age, sex, disease duration, and genotype

Table 5. Bivariate associations between smoking and EDSS ≥ 3, confirmatory dataset (N=512)

Characteristic	N	% EDSS ≥ 3	Adjusted OR (95% CI)	p-value
Smoking status*				0.13
Never	291	23.7	1.00	
Ever	221	31.2	1.39 (0.90, 2.13)	
HLA DRB1 1501 (0)	279			
Smoking status‡				0.68
Never	159	29	1.00	
Ever	120	30	0.89 (0.50, 1.56)	
HLA DRB1 1501 (1-2)	233			
Smoking status‡				**0.003**§
Never	132	17.4	1.00	
Ever	101	32.7	2.86 (1.41, 5.80)	

*Adjusted for age, sex, disease duration, and genotype

‡Adjusted for age, sex, and disease duration

§ Significant at alpha = 0.01

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