Abstract

Background: Smoking has been associated with an increased risk for multiple sclerosis, but no studies have measured levels of the nicotine metabolite cotinine in prospectively collected samples to assess exposure.

Objective: To investigate the effects of laboratory defined tobacco use on the risk for multiple sclerosis using prospectively collected biobank blood samples.

Methods: Levels of cotinine were measured in n=192 cases, and n=384 matched controls, using an immunoassay. The risk for multiple sclerosis was estimated using matched logistic regression.

Results: Elevated cotinine levels (≥10 ng/ml) were associated with a significantly increased risk for multiple sclerosis, (OR 1.5, 95% CI 1.0–2.1). This association was only present in young individuals (below median age at blood sampling, <26.4 years), (OR 2.2, 95% CI 1.3–3.8).

Conclusions: This study confirms that smoking is a risk factor for multiple sclerosis. It has the advantage of using analyses of cotinine levels in samples that were collected several years before disease onset, thus excluding any risk for recall bias and minimising the risk for reversed causation. Our results also suggest that the smoking related immunological events that contribute to the development of multiple sclerosis occur early in life.

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system with a complex aetiology.[1] Factors shown to be associated with an increased risk of MS include both certain genes[2] and environmental exposures, including low vitamin D levels,[3, 4] Epstein-Barr virus infection,[5] spring birth,[6] and smoking.[7] In the prospective studies on smoking and MS, with risk-estimates ranging from 1.3 to 1.8 for heavy smokers compared to never smokers, exposure has been assessed by the use of questionnaires.[8-11] Another study used serum levels of the nicotine metabolite cotinine to assess exposure, but most samples in that study were collected after disease onset.[12] In a large Swedish questionnaire study on newly diagnosed MS cases, the risk-estimates for MS by smoking history from the prospective studies above were confirmed, and Swedish snuff use (a common form of smokeless tobacco in Sweden) was associated with a decreased risk of MS.[13] Early smoking start has been associated with worse MS prognosis,[14] but we found no study reporting the risk for MS based on age at smoking start.

Our aim was to examine the risk for MS by levels of cotinine in prospectively collected blood samples in MS cases and controls. Secondary objectives included: 1) to estimate the risk for MS by self-reported smoking and snuff use history; 2) to compare the risk estimates for different age strata and for cotinine data including and excluding snuff users; and 3) to identify any dose-response relationship between tobacco use and the risk of MS.

Materials and Methods

Study design

This was a nested case-control study performed in the northern half of Sweden using prospectively collected biobank samples.

Study population

The procedures to find cases and controls have been detailed previously.[4] Briefly, a database of MS cases was established in 2009 by searching for MS and adjacent diagnoses by use of the International Classification of Disease (ICD) codes in different diagnostic registries. In each case, the MS diagnosis was confirmed, and the year of MS onset (index year) was appraised by medical record review. All cases fulfilled the MS diagnostic criteria as previously described.[4] The case database was then cross-linked to two local biobank cohorts (the Northern Sweden Health and Disease Study Cohort [NSHDS] containing blood samples collected during health controls since 1985, and the Northern Sweden Maternity Cohort [NSMC] containing blood samples collected during early pregnancy since 1975), and cases with prospectively collected blood samples were selected. Two controls for each case were selected, matched for sex, biobank, age at blood sampling and date of blood sampling. A total of n=192 cases and n=384 controls were identified and included in this report.[4]

Laboratory assays

Cotinine is a nicotine metabolite with a half-life of 20 hours. It is a recognised biochemical marker for recent tobacco use.[15] Cotinine levels ≥10 ng/ml has been used to discriminate active smokers from non-smokers and passive smokers.[16] Levels of cotinine were measured using the kit "Nicotine Metabolite" by Diagnostic Products Corporation (DPC), Los Angeles, California, U.S.A., analysed on the immunoassay Immulite 2000 immunoassay system, Siemens, New York, U.S.A. The method is quantitative for levels ≥10 ng/ml, <500 ng/ml.

Questionnaire data

To enable exclusion of subjects with high cotinine levels due to smokeless tobacco use, data on smoking habits and smokeless tobacco consumption were collected retrospectively using a questionnaire that was mailed out to all participants (including two reminders for non-

responders). The subjects were asked eight questions: "1. Do you smoke, or have you ever smoked? 2. If yes, between which years? 3. Did you smoke cigarettes or pipe tobacco? 4. If you smoke/have smoked cigarettes, how many cigarettes per day? 5. If you smoke/have smoked pipe tobacco, how many tobacco packages per week? 6. Do you use, or have you ever used Swedish snuff? 7. If yes, between which years? 8. If yes, how many packages of Swedish snuff have you used per week?". Only tobacco-use questionnaire data from before and during the index year were used. By use of these data, subjects were categorised into three categories: 1) "ever smokers", 2) "never smokers" and 3) "ever users" of Swedish snuff. Smoke exposure and exposure to Swedish snuff before and during the index year were also quantified (number of pack-years) by use of questionnaire data. A pack-year of cigarettes was defined as consumption of one package of n=20 cigarettes per day for one year. Seventy-three per cent of cases (141/192) and 66% of controls (254/384) responded to the questionnaire and were included in the analyses where questionnaire data were used (Figure 1).

[INSERT FIGURE 1 HERE]

Statistical analyses

For statistical analyses, we used the SPSS software, version 19.0. Chi-square statistics was used to compare proportions. The Mann-Whitney U-test was used to compare median values in paired tests, and the Kruskal-Wallis test was used to compare medians across more than two strata. The risk for MS was estimated by matched logistic regression using four pre-defined stratifications of cotinine levels: 1) <10 vs. ≥10 ng/ml; 2) <10 ng/ml vs. below median and above median among those with elevated (≥10 ng/ml) levels; 3) <10 vs. ≥10 ng/ml excluding subjects who had used Swedish snuff before or during the index year; and 4) <10 ng/ml vs. below median and above median among those with elevated (≥10 ng/ml) levels excluding subjects who had used Swedish snuff before or during the index year. Non-

responders to the questionnaire were kept in the two latter stratifications. The 10 ng/ml cut-off was chosen to discriminate active smokers from passive and non-smokers.[16] For the subgroup analyses by age at blood sampling we evaluated the heterogeneity of the stratum-specific odds ratio (OR) estimates using the chi-square test of heterogeneity in the WinPepi statistical software, version 11.24. Unmatched logistic regression, adjusted for sex and age at index year, was used to examine the risk for MS from smoking history data among questionnaire responders. Self-reported quantitative tobacco use data were used to study dose-response effects by stratifying users into five groups according to number of pack-years of smoking.[13] Pearson's correlation coefficient (r) was used to test for correlations between cotinine levels and sampling year, and between cotinine levels and pack-years of smoking during the index year. Probability is denoted by *p*.

All subjects consented to donate blood samples for biobank storage. All subjects received written information about the study with the option to opt out. All subjects responding to questionnaires consented to participate in the study. All aspects of the study were approved by the local ethics committee in Umeå (Dnr 08-135M).

Results

Elevated cotinine levels (≥10 vs. <10 ng/ml) were associated with a 50% increased risk for MS (OR 1.5, 95% CI 1.0–2.1, $p = 0.045$) (Table 1). This association was only present in subjects below median age (<26.4 years) at sampling (OR 2.2, 95% CI 1.3–3.8) (Table 2), and not in older subjects (≥26.4 years), (OR 0.88, 95% CI 0.52–1.5) (p for heterogeneity = 0.02). Further sub-dividing age at sampling into 5 or 10 year intervals did not alter this finding (data not shown). When “ever users” of Swedish snuff were excluded from the analyses, the ORs for MS by cotinine levels were slightly higher (Tables 1 and 2).

[INSERT TABLES 1 AND 2 HERE]

The risk estimates based on cotinine levels agreed well with estimates based on smoking questionnaire data (Table 1). The exception was that no modification by age was observed when self-reported smoking history was used (Supplementary table). This discrepancy may relate to a higher prevalence of smoking in younger individuals, and a more pronounced risk modification by age in questionnaire non-responders (Supplementary table). For all subjects there was a fair agreement between questionnaire and cotinine data regarding tobacco use (Table 3). There was a significant, although weak, correlation in smokers (snuff users excluded) between cotinine levels and the reported number of cigarettes smoked per day during the sampling year ($r = 0.29$, $p < 0.001$; $n = 126$).

[INSERT TABLE 3 HERE]

We found no dose-response effect on the risk for MS in the analyses of cotinine levels (Table 1). However, in the smoking questionnaire data a non-significant trend for such an effect was observed (p for trend = 0.076, Table 1). The numbers of "never smokers" that had used Swedish snuff (four cases and ten controls) were too low to analyse further regarding risk for MS. There was no temporal trend for cotinine levels (cotinine level and sampling year: $r = 0.02$, $p = 0.6$; $n = 576$).

Discussion

This study confirms earlier findings of an association between smoking and MS with a 50% increased risk for MS for subjects with elevated cotinine levels before MS onset.[7-13] The study design, which is novel in this context, includes using a biochemical marker for tobacco use (cotinine) in prospectively collected blood samples. This excludes both the risk for recall bias and the potential for misclassification due to the subjectivity of self-reported smoking history. The laboratory data were complemented by retrospective questionnaire data, and the results from these two different data sources were consistent, further strengthening that smoking is

indeed a risk factor for MS. The risk estimates in this study also confirmed those in earlier studies, which used different study designs and data sources.[8-13]

When stratifying the subjects at median age at sampling, it was evident that the effect on the risk for MS by cotinine levels was present only in younger individuals (<26.4 years old). The prevalence of smoking during the sampling year was higher in this group, compared to those with blood sampling at a higher age, and it makes sense that cotinine samples need to be drawn before a large proportion of subjects stop smoking (Supplementary table). In addition, the risk modification by age was most pronounced among questionnaire non-responders, which helps to explain why no age-related effect was seen in the questionnaire data. Altogether, this suggests that age at smoke exposure is relevant, and that younger individuals might be more vulnerable to the negative effects of smoking regarding MS risk than older subjects. This supports the hypothesis that the fundamental biological changes that may lead to MS probably occur during adolescence/young adulthood, a notion which emanates from several observations: 1) migration studies showing that an individual's risk for the disease is largely established before the age of 20;[17] 2) data showing that the protective effect associated with vitamin D status regarding MS risk is more pronounced in the young;[3] and 3) data showing that infectious mononucleosis, which mainly strikes during adolescence in the western world, is associated with an increased MS risk.[18]

Several mechanisms by which smoking could increase the risk for MS have been suggested, e.g. cyanide neurotoxicity,[19] nicotine related immune modulation,[20] nicotine related increased blood-brain barrier permeability,[21] post-translational protein modification in the lungs,[22, 23] and other mechanisms.[13] Swedish snuff use has been associated with a decreased risk for MS,[13] and an interaction between smoking and HLA-DRB1*15 and HLA A*02 carriage (two genes encoding antigen presenting molecules) regarding MS risk has been shown.[23] These two findings argue that it is not the nicotine in itself, but rather some other constituent(s) in

cigarette smoke that is (are) responsible for the association between MS and smoking. The event(s) that increase MS risk may take place in the alveoli of the lung where immune modulation or post-translational protein modification may occur.[23]

This study was designed as a prospective nested case-control study, and as only samples drawn before disease onset were used, the risk for reversed causation is minimised. The cases and controls were well matched as previously described,[4] however residual confounding by other factors that were not controlled for cannot be excluded. Except for a non-significant trend in the questionnaire data, no clear dose-response effect was seen for smoking as a risk factor for MS. This is likely to be a spurious finding, as many well-designed studies before us have shown such an effect.[7, 8, 10, 12, 13] As for cotinine measurements, a relative lack of correlation between the number of cigarettes smoked per day and serum levels has been described,[15] and the same pattern was apparent in the present study. It is obvious that one single measurement of this short-lived metabolite does not reflect the number of years of exposure integrated in the "pack-year" concept, and such a dose-response effect is better studied in questionnaire studies. That significance was not reached for the dose-response trend in the questionnaire analysis might be explained by low power, inexact reporting of quantities by the respondents, or the fact that this study had few heavy smokers. An additional weakness of this study is the lack of data on HLA DRB1*15 and HLA A*02 carriage.[23]

In conclusion, this study confirms that smoking is a risk factor for MS with an increased risk of 50% among subjects with elevated cotinine levels in prospectively collected blood samples. Since the elevated cotinine levels were associated with MS risk only in young individuals (<26.4 years old), this study points towards adolescence/young adulthood as being a key period in MS aetiology. Further endeavours in this field should be focused on preventing MS by improving public awareness, and on elucidating the molecular mechanisms behind the association between smoking and MS.

Conflict of interest statement

Dr. Salzer has received financial support for this study from Biogen Idec, Merck Serono and The Swedish Association of Neurologically Disabled, received honoraria from Merck Serono for a lecture, and received support to travel to scientific meetings from Biogen Idec and Merck Serono.

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Table 1 Odds ratios of Multiple Sclerosis by levels of the nicotine metabolite cotinine in prospectively collected blood samples (n=192 cases and n=384 matched controls), and by retrospective tobacco use questionnaire data (n=141 cases and n=254 controls).

Variables	Categories	Number of		Logistic regression analysis		
		Cases	Controls	OR	95% CI	*P*
Cotinine levels (ng/ml)[a]	<10	109	251	1.0	–	
	≥10	83	133	1.5	1.0–2.1	0.045
	<10[b]	109	251	1.0	–	
	≥10, <227[b]	44	66	1.6	0.99–2.5	
	≥227[b]	39	67	1.3	0.86–2.1	
Cotinine levels (ng/ml), excluding users of smokeless tobacco[c]	<10	103	243	1.0	–	
	≥10	73	114	1.6	1.1–2.3	0.024
	<10[b]	103	243	1.0	–	
	≥10, <223[b]	37	57	1.7	1.0–2.8	0.044
	≥223[b]	36	57	1.5	0.9–2.4	
Ever smoker[d]	No	57	129	1.0	–	
	Yes	84	125	1.5	0.98–2.3	
No. of pack–years[d]	0	60	135	1.0	–	
	>0, <10	62	90	1.5	0.97–2.4	
	≥10	19	29	1.5	0.77–2.9	

[a]Matched logistic regression was used to estimate ORs and CIs.
[b]Cotinine levels categorised into undetectable (<10 ng/ml), and below and above median among those with higher levels. Subjects with unquantifiable high levels (>500 ng/ml) were assigned a value of 501 ng/ml.
[c]Analysis by cotinine levels excluding the 43 ever users of smokeless tobacco (n=14 snuff only, n=29 mixed users) before or during the index year according to the questionnaire. Matched logistic regression was used to estimate ORs and CIs.
[d]According to questionnaire. Smoking was considered before and during the index year within each set. All pipe smokers (n=4) were also cigarette smokers, and pipe smoking did not affect pack-year classification. Adjustment for smokeless tobacco use, or excluding ever users of smokeless tobacco only slightly altered the ORs and CIs (data not shown). Unmatched logistic regression, adjusted for sex and age at index year, was used to estimate ORs and CIs.
Abbreviations: OR = odds ratio. CI = confidence interval.

Table 2 Odds ratios of Multiple Sclerosis by levels of the nicotine metabolite cotinine in prospectively collected blood samples from young (<26.4 years; median) individuals (n=96 cases and n=192 matched controls).

Variables	Categories	Number of		Logistic regression analysis		
		Cases	Controls	OR	95% CI	*P*
Cotinine levels (ng/ml)[a]	<10	47	130	1.0	–	
	≥10	49	62	2.2	1.3–3.8	0.004
	<10[b]	47	130	1.0	–	
	10–195[b]	28	31	2.3	1.3–4.3	0.007
	≥195[b]	21	31	2.0	1.0–4.1	0.048
Cotinine levels (ng/ml), except users of smokeless tobacco[c]	<10	44	127	1.0	–	
	≥10	41	56	2.4	1.3–4.3	0.004
	<10[b]	44	127	1.0	–	
	10–195[b]	24	28	2.5	1.3–5.0	0.009
	≥195[b]	17	28	2.2	0.99–4.7	

[a]Matched logistic regression was used to estimate ORs and CIs.
[b]Cotinine levels categorised into undetectable (<10 ng/ml), and below and above median among those with higher levels. Subjects with unquantifiable high levels (>500 ng/ml) were assigned a value of 501 ng/ml.
[c]Analysis by cotinine level excluding the 20 ever user of smokeless tobacco (n=4 snuff only, n=16 mixed users) before or during the index year according to the questionnaire.
Abbreviations: OR = odds ratio. CI = confidence interval.

Table 3 Cotinine levels by reported tobacco use during the sampling year.

Tobacco use[a]	N	Cotinine		
		Median (range) ng/ml[b]	≥10 ng/ml N (%)	<10 ng/ml N (%)
Smoker	130	125 (0–501)	92 (71)	38 (29)
Snuff user	15	180 (0–501)	9 (60)	6 (40)
Combined user	4	222 (64–501)	4 (100)	0 (0)
No tobacco use	246	0 (0–501)	37 (15)	209 (85)

[a]Tobacco use during the sampling year in the 395 subjects who provided complete answers to the questionnaire.

[b]Cotinine concentration among subjects with undetectable levels (<10 ng/ml) counted as 0 ng/ml. In 17 subjects with levels >500 ng/ml, a level of 501 ng/ml was assigned. All tobacco users had significantly higher medians compared to non-users ($p < 0.001$, Mann-Whitney U-test)