Association of smoking but not HLA-DRB1*15:01, *APOE* or body mass index with brain atrophy in early multiple sclerosis

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Abstract

Background: The course of multiple sclerosis (MS) shows substantial inter-individual variability. The underlying determinants of disease severity likely involve genetic and environmental factors.

Objective: The aim of this study was to assess the impact of *APOE* and *HLA* polymorphisms as well as smoking and body mass index (BMI) in the very early MS course.

Methods: Untreated patients ($n=263$) with a recent diagnosis of relapsing-remitting (RR) MS or clinically isolated syndrome underwent standardized magnetic resonance imaging (MRI). Genotyping was performed for single-nucleotide polymorphisms (SNPs) rs3135388 tagging the HLA-DRB1*15:01 haplotype and rs7412 (E2) and rs429358 (E4) in *APOE*. Linear regression analyses were applied based on the three SNPs, smoking and BMI as exposures and MRI surrogate markers for disease severity as outcomes.

Results: Current smoking was associated with reduced gray matter fraction, lower brain parenchymal fraction and increased cerebrospinal fluid fraction in comparison to non-smoking, whereas no effect was observed on white matter fraction. BMI and the SNPs in *HLA* and *APOE* were not associated with structural MRI parameters.

Conclusions: Smoking may have an unfavorable effect on the gray matter fraction as a potential measure of MS severity already in early MS. These findings may impact patients' counseling upon initial diagnosis of MS.

Keywords: Neurodegeneration, genetics, MRI, brain atrophy, SNP, gray matter, risk factor, smoking

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Introduction

Multiple sclerosis (MS) is the most prevalent neuroinflammatory disease in young adults and commonly leads to physical and neurocognitive impairment. As a genetically complex disease, more than 200 genetic risk factors^{1–4} and several environmental factors⁵ have been identified with small to moderate effect sizes. The genetic risk factors identified thus far point primarily toward a role of the adaptive immune system in disease onset.¹ Indeed, inflammatory processes play a central role in early disease course. Subsequent

disease progression is characterized by neurodegenerative processes, reflected by whole brain atrophy as well as gray matter loss in magnetic resonance imaging (MRI).^{6,7} However, little is known about genetic or environmental factors modifying disease progression. Potentially relevant factors include smoking and body mass index (BMI), which have been shown to influence the risk of developing MS and adversely influence the progression of the disease at later disease stages.^{8–11} Furthermore, associations with

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Table 1. Demographic and clinical characteristics of 263 RRMS and CIS patients included in this study.

Characteristic	Patients (n=263)
Female, n (%)	183 (69.6)
Age in years, mean (SD) [min; max]	35.2 (10.4) [18; 66]
Disease duration (month), median (1 Qu, 3 Qu) [max]	2.1 (1.4, 3.0) [23.8]
Clinically isolated syndrome, n (%)	129 (49)
BMI ^a , n (%)	
≥26	95 (37.1)
19–26	145 (56.6)
≤19	16 (6.3)
Current smokers ^a , n (%)	77 (30.0)
<i>HLA-DRB1</i> rs3135388 genotypes (TT/CT/CC)	22/116/125
<i>APOE</i> rs429358 genotypes (CC/CT/TT)	3/56/204
<i>APOE</i> rs7412 genotypes (TT/CT/CC)	1/46/216
Lesion volume (ml), median (1 Qu, 3 Qu)	1.3 (0.5, 3.8)
SD: standard deviation, BMI: body mass index, n: number, Qu: quartile.	
^a Smoking and BMI data missing in n=6 and 7 subjects, respectively. Clinically isolated syndrome (CIS) defined as (a) fulfilling at least three Barkhof criteria or (b) fulfilling 2 of 4 Barkhof criteria and pathological visually evoked potential or oligoclonal bands in CSF; or (c) fulfilling the revised McDonald criteria 2010 for RRMS. ²²	

disease course and lesion distribution in brain imaging studies have been reported repeatedly for the MS risk haplotype *HLA-DRB1**15:01 in the *HLA* (human leukocyte antigen) locus on chromosome 6p21.^{12–14} In addition, previous studies have investigated a disease-modifying effect for the non-synonymous single-nucleotide polymorphisms (SNPs) rs7412 (E2) and rs429358 (E4) in *APOE* (encoding apolipoprotein E), the most important genetic risk factor for Alzheimer’s disease. However, results have been contradictory.^{15–20} Most studies have investigated these factors in the context of long-term disease progression.^{8,16,18} The role of any of these factors in early disease progression remains unclear.

Thus, the aim of this study was to comprehensively assess the role of smoking status, BMI, *HLA-DRB1**15:01 as well as *APOE* rs7412 and rs429358 in the very early course of MS. To this end, we analyzed 3 Tesla structural brain MRI data in a large and carefully selected dataset of untreated early MS and clinically isolated syndrome (CIS) patients from Germany.

Materials and methods

Patient cohort and clinical data

Participants for this study were selected from the German Competence Network Multiple Sclerosis (KKNMS) (<http://www.kompetenznetz-multiplesklerose.de/>) cohort, a multicenter, prospective and observational study including over 1000 MS patients. All

participants in the KKNMS cohort were at least 18 years old with CIS fulfilling at least three Barkhof criteria (for complete CIS inclusion criteria in exceptional cases see Table 1) or with relapsing-remitting (RR) MS as defined by the 2005 revised McDonald criteria.²¹ CIS was defined as a first clinical episode with neurologic dysfunction lasting more than 24 h, where the criteria to state the diagnosis of MS are not yet sufficiently fulfilled, namely dissemination in space and time. Only untreated patients whose first documented symptom had occurred not more than 6 months (with regard to CIS patients) or 3 years (with regard to RRMS patients) prior to the study were invited to participate in the KKNMS cohort. The majority of all invited patients (estimated >80%) were willing to participate. For this study, 263 patients of German Caucasian ancestry with clinical data, MRI data and genetic information available were selected. MRI data were collected until 19 November 2015 (Table 1). The study was approved by the local ethics committees; written informed consent was obtained from all study participants.

At the point of study enrollment, patients were asked to assess their current smoking habits via questionnaire. In response to the question “Do you currently smoke?” patients could select from seven categories: (0) no, (1) casually, but not on a daily basis, (2) up to 5 cigarettes daily, (3) 6–10 cigarettes daily, (4) 11–20 cigarettes daily, and (5) >20 cigarettes daily. Based on this, we dichotomized the participants into current non-smokers (categories 0 and 1) and current smokers (all other categories). Body weight and

height were physically measured on site at the same time as biomaterial collection and MRI acquisition. BMI was then calculated for this study as $\text{BMI} = \text{weight (kg)} / \text{height (m)}^2$. BMI was categorized as follows: BMI between 19 and 26 was defined as normal weight, 19 or less as underweight and 26 or greater as overweight.

MRI data analysis

MRI was performed on a 3 Tesla MRI scanner according to the KKNMS protocol. Imaging was performed using sagittal 3D T1-weighted magnetization-prepared rapid gradient-echo (MP-RAGE) sequence and sagittal 3D T2-weighted fluid attenuated inversion recovery (FLAIR) sequence. Lesion maps were generated based on T2-weighted FLAIR images. For lesion and tissue segmentation, the “statistical parameter mapping” (SPM8) software (<http://www.fil.ion.ucl.ac.uk/spm/>) with the “lesion segmentation toolbox” (LST) (<http://www.appliedstatistics.de/lst.html>) and the “VBM8” toolbox (<http://dbm.neuro.uni-jena.de/vbm/>) were used. Brain parenchymal volume, gray matter volume, white matter volume and cerebrospinal fluid volume were analyzed as global brain measures. Differences in head and brain total volume were accounted for by calculating the respective fractions. Specifically, component volumes were divided by the whole brain volume (i.e. brain parenchymal volume plus cerebrospinal fluid volume) to calculate the brain parenchymal fraction, gray matter fraction, white matter fraction and cerebrospinal fluid fraction. To account for differences due to different MRI scanners, data from each center were transformed into standard normal distributions (z-scores) for all brain fractions.

Genotyping

The SNPs rs429358 and rs7412 in *APOE*, *HLA-DRB1* rs3135388, and the Y chromosome marker rs2032598 (for sampling and bioprobe handling control) were analyzed using allelic discrimination assays based on Taqman chemistry according to the manufacturer’s protocol (Applied Biosystems, Inc.). Genotyping was performed on 96-well plates with approximately 5% controls run in duplicates across plates. Genotyping efficiency was $\geq 99.6\%$ for all SNPs.

Deviation of the genotypes from Hardy-Weinberg equilibrium (HWE) as a potential marker for genotyping quality was assessed using Pearson’s chi square test. The genotype distribution of rs429358, rs7412, and rs3135388 did not deviate from HWE ($\alpha = 0.05$).

Statistical analysis

Statistical analyses were performed using *R* (version 3.2.3). Data analysis is exploratory. Hence, no adjustments for multiple testing were performed. *P* values are given for descriptive purposes only. Due to the large number of tests, *p* values should be interpreted with caution and assessed in the context of the corresponding effect estimates. Association analyses of smoking, BMI, *HLA-DRB1* rs3135388 as well as *APOE* rs429358 and rs7412 as exposures and the structural MRI parameters brain parenchymal fraction and gray matter fraction as outcome variables were performed using linear regression. Analyses were adjusted for age and sex. BMI was treated as a three-level variable in one single regression model. For the brain fractions, z-scores were calculated center-wise and then combined. Due to limited numbers of individuals with homozygous genotypes for the minor alleles, genetic analyses were performed using a dominant model.

Results

Across the 263 patients included in this study (Table 1), current smoking was associated with lower gray matter fraction (β (95% confidence interval (CI)) = -0.296 (-0.515 , -0.078), $p = 0.008$) compared to non-smoking (Table 2). Smoking also showed an association with lower brain parenchymal fraction ($\beta = -0.289$ (-0.537 , -0.041), $p = 0.022$) (Table 2).

Underweight and overweight did not show an association with the gray matter fraction or the brain parenchymal fraction ($p > 0.05$, Table 2). Furthermore, the polymorphisms *HLA-DRB1* rs3135388 as well as *APOE* rs429358 and rs7412 were not associated with these two structural brain imaging parameters either ($p > 0.05$, Table 2).

For smoking, we also assessed its impact on other structural brain imaging parameters, that is, on the cerebrospinal fluid fraction and on the white matter fraction. As expected, we observed an association of smoking with the cerebrospinal fluid fraction compared to non-smoking ($\beta = 0.289$ (0.041, 0.536), $p = 0.022$) as a reflection of its effect on the gray matter fraction and the brain parenchymal fraction. In contrast, smoking showed no effect on the white matter fraction ($\beta = 0.051$ (0.210, 0.312), $p = 0.700$).

Discussion

In MS, environmental and genetic factors have been reported to be associated with disease severity. In this study, we assessed the putative role of smoking, BMI

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Table 2. Association analyses of *APOE* and *HLA* polymorphisms, smoking and body mass index with brain parenchymal and gray matter fraction. Carriers are defined as being heterozygous or homozygous for the minor allele. Linear regression analyses were adjusted for age and sex. For the brain fractions, z-scores were calculated center-wise and then combined.

Exposure		Median (1 Qu, 3 Qu)		Linear Regression Analysis			
		BPF	GMF	β_{BPF} (95%-CI)	p_{BPF}	β_{GMF} (95%-CI)	p_{GMF}
<i>HLA</i> rs3135388 carriers vs non-carriers	carriers	0.837 (0.821, 0.853)	0.463 (0.453, 0.482)	−0.118 [−0.347, 0.112]	0.313	−0.018 [−0.222, 0.185]	0.859
	homozygous major (ref)	0.838 (0.820, 0.850)	0.462 (0.439, 0.481)				
<i>APOE</i> rs429358 carriers vs non-carriers	carriers	0.834 (0.821, 0.849)	0.461 (0.445, 0.470)	−0.117 [−0.392, 0.157]	0.401	−0.002 [−0.245, 0.242]	0.989
	homozygous major (ref)	0.838 (0.819, 0.852)	0.463 (0.447, 0.483)				
<i>APOE</i> rs7412 carriers vs non-carriers	carriers	0.835 (0.819, 0.856)	0.455 (0.443, 0.481)	0.042 [−0.261, 0.346]	0.783	−0.132 [−0.400, 0.137]	0.335
	homozygous major (ref)	0.838 (0.821, 0.851)	0.463 (0.448, 0.481)				
Current smoking vs non-smoking	smoking	0.834 (0.812, 0.850)	0.458 (0.439, 0.482)	−0.289 [−0.537, −0.041]	0.022	−0.296 [−0.515, −0.078]	0.008
	non-smoking (ref)	0.841 (0.824, 0.852)	0.464 (0.452, 0.480)				
BMI ≤ 19 vs BMI 19–26	BMI ≤ 19	0.837 (0.825, 0.850)	0.465 (0.455, 0.473)	−0.249 [−0.731, 0.233]	0.309	−0.315 [−0.740, 0.110]	0.146
	BMI 19–26 (ref)	0.841 (0.819, 0.854)	0.464 (0.453, 0.485)				
BMI ≥ 26 vs BMI 19–26	BMI ≥ 26	0.834 (0.820, 0.846)	0.459 (0.438, 0.475)	−0.080 [−0.324, 0.163]	0.515	−0.11 [−0.322, 0.108]	0.327
	BMI 19–26 (ref)	0.841 (0.819, 0.854)	0.464 (0.453, 0.485)				

Ref: group used as reference in the linear regression analysis, Qu: quartile, BPF: brain parenchymal fraction, GMF: gray matter fraction, β : estimate/effect, CI: confidence interval, p : p value.

and polymorphisms in *HLA* and *APOE* as disease-modifying parameters in CIS and early RRMS patients. The brain parenchymal fraction and gray matter fraction were used as MRI-derived surrogate markers for MS severity. Smoking was associated with a lower gray matter fraction in our study. It should be noted that its role in healthy aging and on brain atrophy has been discussed as well.^{23,24} In non-MS subjects, multiple studies reported dose-dependent

effects for smoking on gray matter integrity in the insula,²⁵ parts of the prefrontal cortex^{26,27} and cerebellum.²⁸ These widespread dose-dependent negative associations between cortical thickness and pack-years have been confirmed in a recent study of a large sample of older adults.²⁹ Another study in general population applying voxel-based morphometry (VBM) reported associations between smoking and regional gray matter volume loss, while white matter volumes

were not significantly reduced in smokers.³⁰ In contrast, Yu *et al.*³¹ reported regional white matter volume increases in the cingulate gyrus and the putamen in heavy smokers. The structural alterations in the gray matter associated with smoking are reflected by results of studies assessing neurocognition that identified visuospatial and memory deficits among smokers relative to non-smokers.³² Furthermore, smokers performed worse than nonsmokers on neurocognitive measures of fine motor skills and postural stability. Interestingly, distinct brain regions, for example, those affected in Alzheimer's disease, seem to be susceptible to smoking.³³ However, in order to assess surrogates for MS that are straight-forward to measure, we focused on whole brain measures. Furthermore, we adjusted our analyses for age. In our study, the effect on the brain parenchymal fraction was less pronounced, which likely reflects its impact on the gray matter but not the white matter fraction. This showcases the additional value of a separate analysis of brain tissue compartments (i.e. the analysis of gray matter or white matter fractions as applicable instead of only the investigation of the brain parenchymal fraction). In contrast, we did not observe an association of BMI or of polymorphisms in *APOE* and the *HLA* locus with these brain volume measurements.

To our knowledge, this is the first study to investigate MRI parameters of disease severity in untreated patients in the early phase of MS in relation to environmental factors and genetic markers. Of note, the percentage of current smokers (30%) in our investigated cohort was comparable to the percentage of current smokers (27% in 2014) in the microdata of the German Socio-Economic Panel, a representative survey of the German population.³⁴ Two previous studies on a similar study population reported that smoking was associated with an increase in the Expanded Disability Status Scale (EDSS) as a clinical measure of disease severity.^{8,35} Furthermore, the group reported an increased number of gadolinium-enhancing lesions and less brain parenchymal fraction in MS patients.^{8,35} Notably, the mean disease duration in these two studies was 12 (± 9)⁸ and 15 (± 10) years.³⁵ A Swedish study investigated the conversion to secondary progressive (SP)MS for smokers at initial diagnosis who quit smoking completely and smokers at initial diagnosis who smoked continuously. Each additional year of smoking after diagnosis accelerated the time to conversion to SPMS by 4.7%.¹⁰ Our data suggest a negative effect on gray matter brain volume already in the very early disease process. The Swedish study,¹⁰ suggests that quitting of smoking directly after diagnosis of MS has substantial prognostic benefits for the disease course. Although we did not look at smoking

cessation directly, the results of our study are in line with this observation. If validated in independent studies, this would need to be considered in the patients' counseling immediately after diagnosis of MS.

While we did not observe an effect of the *HLA*-DRB1*15:01 haplotype (tagged by rs3135388) on brain volume, a large recent study found an effect of a *HLA*-derived genetic burden score on disease course in MS patients with at least 6 years of disease duration, which was mainly driven by *HLA*-DRB1*15:01.¹⁴ One explanation of these discrepant results may lie in the length of disease duration of the investigated patients. It appears possible that genetic variation in *HLA* exerts substantial effects predominately later in the course of MS as the disease process may need to be active for some years before genotype-dependent differences in disease progression become relevant. However, it is worth noting that the association of the *HLA* genetic burden score reported by Isobe *et al.*¹⁴ was also not replicated in our larger German genetics cohort.³⁶ Regarding the role of *APOE* in MS course, genetic association results have overall been largely contradictory,^{15–20} suggesting that the functional *APOE* variants rs429358 and rs7412 do not play a major role in defining MS severity. Along these lines, our current study may support this notion. However, similar to the interpretation of the *HLA* results in our study, we cannot exclude an impact of *APOE* variants after longer disease exposure. To investigate such effects, long-term follow-up data are required, which are currently being generated via the KKNMS cohort.

Concerning BMI, we grouped patients in categories of under-, normal- and overweight, following the pathophysiologic thought that either extreme may contribute to a worse outcome, as described previously.³⁵ We observed an effect size pointing to smaller brain parenchyma for both under- and overweight subjects, but these results were not statistically relevant. The previous study, which investigated the effect of BMI on MRI parameters predominately in late disease progression, showed no effect of obesity on brain volume parameters, but it reported an association of obesity with an increased burden of T1 lesions.³⁵ In that study, obesity was correlated with brain volume loss only when combined with smoking and hypertension.³⁵

Some limitations of our study should be noted. Although our study is based on a large study population, we cannot exclude more modest effects of BMI or polymorphisms on brain volume in early MS. Furthermore, from the current data we cannot infer causality regarding the association of smoking with brain

atrophy. The association with smoking may reflect other smoking-correlated factors such as nutrition, physical activity or socioeconomic status,³⁷ which may have an impact on the course of MS that is not mediated by smoking. Moreover, additional data are needed to determine whether the effect of smoking or the magnitude of effect observed in our study is specific to MS or may reflect an effect of smoking in the general population. It has also to be taken into account that especially self-reported smoking behavior may be affected by measurement error and certain types of bias, for example, selective reporting. The questionnaire used to assess smoking habits did not allow for a distinction of ex-smokers from non-smokers. Furthermore, the questionnaire did not assess the smoking duration.

Regardless of the potential presence or absence of differential effects in MS patients versus controls, our finding suggests a substantial impact of smoking on gray matter in early MS and could have an impact on patients' counseling upon initial diagnosis of MS. Furthermore, potential smoking effects should be considered in the analyses and interpretation of data from observational or interventional studies investigating brain imaging parameters or disease progression.

In conclusion, in this study, we show that smoking may have an impact on brain volume and especially on gray matter already early in the course of MS, while neither BMI, HLA-DRB1*15:01 nor functional polymorphisms in *APOE* show an influence on brain atrophy.

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