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Smoking and two human leukocyte antigen genes interact to increase the risk for multiple sclerosis

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Both genetic and environmental factors display low or modest associations with multiple sclerosis. Hypothetically, gene–environment interactions may exert much stronger effects. In this study, we investigated potential interactions between genetic risk factors and smoking in relation to risk of developing multiple sclerosis. A population-based case–control study involving incident cases of multiple sclerosis (843 cases, 1209 controls) was performed in Sweden. Cases and controls were classified according to their smoking status and human leukocyte antigen DRB1 as well as human leukocyte antigen A genotypes. Subjects with different genotypes and smoking habits were compared with regard to incidence of multiple sclerosis, by calculating odds ratios with 95% confidence intervals employing logistic regression. The potential interaction between different genotypes, as well as between genotype and smoking, was evaluated by calculating attributable proportion due to interaction. A significant interaction between two genetic risk factors, carriage of human leukocyte antigen DRB1*15 and absence of human leukocyte antigen A*02, was observed among smokers whereas such an interaction was absent among non-smokers. There were considerable differences in odds ratios between the various groups. Compared with non-smokers with neither of the genetic risk factors, the odds ratio was 13.5 (8.1–22.6) for smokers with both genetic risk factors. The odds ratio for smokers without genetic risk was 1.4 (0.9–2.1) and the odds ratio for non-smokers with both genetic risk factors was 4.9 (3.6–6.6). Among those with both genetic risk factors, smoking increased the risk by a factor of 2.8 in comparison with a factor of 1.4 among those without the genetic risk factors. The risk of developing multiple sclerosis associated with human leukocyte antigen genotypes may be strongly influenced by smoking status. The findings are consistent with our hypothesis that priming of the immune response in the lungs may subsequently lead to multiple sclerosis in genetically susceptible people.

Keywords: multiple sclerosis; genetics; smoking; immunology
Abbreviation: HLA = human leukocyte antigen

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Introduction

Multiple sclerosis is a complex and inflammatory disease of the central nervous system causing damage to myelin and axons, and progressive neurological dysfunction often ensues. Improved therapy and prevention depend on better understanding of causes and mechanisms in multiple sclerosis. Hereby, definitions of disease-associated genes and environmental factors have begun to offer some clues. However, it is likely that studies of gene–environment interactions will provide a more complete understanding.

The familial aggregation evident in studies of twins, siblings and families proves a genetic predisposition to multiple sclerosis. The impact of discrete gene variants outside the human leukocyte antigen (HLA) complex is low (International Multiple Sclerosis Genetics Consortium, 2005, 2010) while genes of the HLA complex exert a stronger influence. The haplotype containing the HLA-DRB1*15 allele provides an increased risk with odds ratios in the order of 2–4 (Lincoln *et al.,* 2005). In addition, evidence is mounting for an additional role of HLA class I genes (Brynedal *et al.,* 2007; Burfoot *et al.,* 2008; Lorentzen *et al.,* 2009; Bergamaschi *et al.,* 2010). In particular, the HLA-A*02 allele of the HLA-A locus has been reported to have a protective role with an odds ratio of approximately 0.5 (Brynedal *et al.,* 2007; Burfoot *et al.,* 2008; Bergamaschi *et al.,* 2010). Interestingly, the combination of carriage of HLA-DRB1*15 and absence of HLA-A*02 alleles provides risk increases beyond additive effects (Brynedal *et al.,* 2007), suggesting the existence of gene–gene and/or gene–environment interaction.

Migration studies, geographical gradients and discordancy in identical twins indicate that the environment has significant influence on the development of multiple sclerosis. Accumulating data suggest particular environmental factors in the aetiology of multiple sclerosis, such as Epstein-Barr virus infection (Bagert, 2009), vitamin D deficiency (Munger *et al*., 2006) and smoking (Antonowsky *et al.,* 1965; Thorogood and Hannaford, 1998; Ghadirian *et al.,* 2001; Hernan *et al.,* 2001, 2005; Riise *et al.,* 2003; Pekmezovic *et al.,* 2006; Hedström *et al.,* 2009). In this study, we focus on the potential interaction between smoking and the HLA-DRB1*15 and HLA-A*02 alleles.

Materials and methods

Study design and subjects

This study is based on the ongoing project Epidemiological Investigation of Multiple Sclerosis (EIMS), which is a population-based case–control study, comprising the general population aged 16–70 years in defined areas of Sweden. The present report analysed incident cases and controls included between April 2005 and August 2009. The general structure of the study has been previously reported (Hedström *et al.,* 2009).

Newly diagnosed cases with multiple sclerosis were recruited via 32 neurology units. All cases were diagnosed by a neurologist located at the unit where the case was entered. Cases that did not fulfil the McDonald criteria (Polman *et al*., 2005) at the time of this report were excluded.

For each case, two controls were randomly selected from the national population register, frequency matched for the case's age in 5-year age strata, gender and residential area.

Data collection and definition of smoking habits

Information regarding lifestyle factors—including smoking—was collected using a standardized questionnaire given to the cases shortly after receipt of diagnosis and sent by post to the controls. Completed questionnaires were obtained from 1121 cases and 2347 controls; the response proportion was 93% for the cases and 73% for the controls. Questions regarding smoking defined the current and previous smoking habits over the life span of the subject.

For each case, the year of the initial appearance of multiple sclerosis symptoms was defined as the index year. Smoking was considered prior to and during the index year in the cases and during the same period in the controls. Subjects who smoked regularly during the index year were defined as smokers whereas those who had never smoked before or during the index year were defined as non-smokers. We have previously demonstrated that the increased risk of developing multiple sclerosis associated with smoking only remains up to 5 years after stopping smoking (Hedström *et al.,* 2009). Therefore, subjects who had stopped smoking within 5 years prior to index were excluded from this report. Subjects who had stopped smoking more than 5 years prior to index were defined as non-smokers.

We received blood samples from 98% of the cases who answered the questionnaire and from 58% of the controls. Unfortunately, a fraction of the samples submitted was lost due to failures in the DNA preparation at the central Biobank at the Karolinska Institutet.

In total, 867 cases and 1227 controls were genotyped for HLA-A and HLA-DRB1 alleles. For reasons stated above, subjects who had stopped smoking within 5 years prior to index were excluded from this report (24 cases, 18 controls). The study thus comprised 843 cases and 1209 controls and was approved by the Regional Ethical Review Board at Karolinska Institutet.

Genotyping and definition of genetic risk factors

HLA-A and DRB1 genotypes were determined with sequence-specific primers using OLERUP SSP™ HLA Kits (Olerup and Zetterquist, 1992). Compared with HLA-DRB1*15 negative subjects, both HLA-DRB1*15 homozygotes and heterozygotes have an increased risk of developing multiple sclerosis. Consequently, cases and controls were classified according to the carriage of any HLA-DRB1*15 allele versus no carriage. Compared with HLA-A*02 negative subjects, both HLA-A*02 homozygotes and heterozygotes have a reduced risk of developing multiple sclerosis. In the main analysis, cases and controls were classified according to carriage of any HLA-A*02 allele versus no carriage. Absence of HLA-A*02 is thus a risk factor, but since other genetic models other than a dominant one for protection by A*02 may be of relevance we also performed the analysis with absence of A*02 as the risk factor.

Statistical analysis

In order to distinguish specific HLA genotypes contributing to multiple sclerosis susceptibility, we applied unconditional logistic regression, using a model with all HLA-DRB1 and HLA-A alleles with frequencies $>10\%$ among controls. Alleles with a frequency of $<10\%$ among controls were grouped together into DRB1X and AX, respectively.

Table 1 Frequencies of HLA-DRB1 and HLA-A alleles among cases and controls together with adjusted odds ratios and 95% confidence intervals for their association with risk of developing multiple sclerosis

HLA-	Cases (%)	Controls (%)	Odds ratio[a]	Odds ratio[b]	*P*-value
DRB1*01	18	20	0.9 (0.7–1.1)	0.9 (0.7–1.3)	0.6
DRB1*03	19	23	0.8 (0.6–1.0)	1.0 (0.7–1.4)	1.0
DRB1*04	29	34	0.8 (0.7–1.0)	1.0 (0.7–1.3)	0.9
DRB1*07	11	16	**0.6 (0.5–0.8)**	**0.7 (0.5–1.0)**	0.04
DRB1*08	11	8	1.3 (1.0–1.8)	1.4 (1.0–2.1)	0.06
DRB1*13	21	26	**0.8 (0.6–0.9)**	1.0 (0.7–1.3)	0.8
DRB1*15	58	29	**3.5 (2.9–4.2)**	**3.1 (2.3–4.1)**	1×10^{-14}
DRB1X[c]	15	25	**0.5 (0.4–0.6)**	**0.6 (0.4–0.8)**	0.002
A*01	28	29	1.0 (0.8–1.2)	1.0 (0.8–1.4)	0.8
A*02	43	55	**0.6 (0.5–0.7)**	**0.6 (0.6–1.0)**	0.04
A*03	40	28	**1.7 (1.4–2.1)**	**1.5 (1.2–2.0)**	0.002
A*11	11	10	1.1 (0.8–1.5)	1.4 (0.9–1.9)	0.1
A*24	19	17	1.2 (0.9–1.5)	1.2 (0.9–1.7)	0.2
AX[d]	37	36	1.1 (0.9–1.3)	1.2 (0.9–1.6)	0.2

[a]Odds ratio adjusted for age, gender, residential area and ancestry. The significantly associated alleles are shown in bold.
[b]Odds ratio adjusted for age, gender, residential area, ancestry and all alleles in the Table. The significantly associated alleles are shown in bold.
[c]DRB1X comprises all observed alleles at the HLA-DRB1 locus with frequencies of $<10\%$ among controls: DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*14, DRB1*16 and DRB1*103.
[d]AX comprises all observed alleles at the HLA-A locus with frequencies of $<10\%$ in controls: A*23, A*25, A*26, A*29, A*30, A31, A*32, A*33, A*34, A36, A*66, A*68, A*69 and A*74.

The linkage disequilibrium between HLA-A*02 and HLA-DRB1*15 was estimated in Unphased 3.0 (Dudbridge, 2003) by considering presence and absence of HLA-A*02 and HLA-DRB1*15 alleles. Linkage disequilibrium was also estimated using the same allele definitions as in Table 1, with similar results.

We then investigated the possible gene–gene interactions between alleles that had a significant influence on multiple sclerosis susceptibility and also the possible interaction between these alleles and smoking. The potential interaction between different genotypes, as well as between genotype and smoking, was analysed using departure from additivity of effects as criterion of interaction and was evaluated by calculating attributable proportion due to interaction together with a 95% confidence interval (CI) (Hosmer and Lemeshow, 1992; Rothman, 2002). Attributable proportion is the proportion of the incidence among individuals exposed to two interacting factors that is attributable to the interaction *per se*, thus an attributable proportion less than 0 indicates presence of interaction. For the most critical comparisons in our study we used permutation analysis to estimate empirical *P*-values. The empirical *P*-values for attributable proportion (Tables 3–5 and 8) were calculated by performing three separate permutations. For the *P*-value in Table 3, 10 000 new datasets of the same size as the original one were generated by permuting the HLA-A*02 status among cases and controls separately. This ensured that the frequency of HLA-A*02 was kept the same as in the original data set among cases and controls. The permutation was followed by testing how often the *P*-value for attributable proportion was as extreme as that observed in the real data. This permutation was carried out using an R-script modified from that presented by Källberg *et al.* (2006). The same permutation procedure was used for the empirical *P*-value in Table 4 but here the permutation was for HLA-DRB1*15. Similarly, the empirical *P*-values in Tables 5 and 8 were obtained by permuting smoking status.

As mentioned, smoking was considered prior to and during the index year in the cases and during the same period in the controls. In principle, this calls for a matched analysis. We performed matched analyses based on all available case–control triplets, as well as unmatched analyses of the data based on all available cases and controls. Only the results from the unmatched analyses are presented in this report since these were in close agreement with those from the matched analyses but in general had a higher degree of precision (due to a higher number of controls).

All analyses were adjusted for age, gender, residential area and ancestry. Age was categorized into the following eight intervals: 16–19, 20–24, 25–29, 30–34, 35–39, 40–45, 45–49 and 50–70 years of age. Educational level only had minor influence on the results and was not retained in the final analyses. All analyses were conducted using Statistical Analysis System (SAS) version 9.

Results

Our analyses included 843 cases and 1209 controls, all of whom had provided blood samples. Of these, 729 cases and 1018 controls were of Scandinavian origin (subjects born in Scandinavia whose parents had not immigrated). Among the cases, 72% were female and 28% were male. Ninety-nine percent of the diagnoses were supported by a positive result from MRI. The mean age at onset was 34 years and the mean duration from the initial appearance of symptoms indicative of multiple sclerosis to the diagnosis was 3.7 years.

HLA-DRB1 and HLA-A related to multiple sclerosis risk

The strongest association with multiple sclerosis was apparent for the DRB1*15 allele (adjusted odds ratio 3.1, 95% CI 2.3–4.1). The HLA, DRB1*07, HLA-DRB1X and HLA-A*02 alleles were associated with a decreased risk (Table 1). These results are in line with previous reports indicating the risk associated with the DRB1*15 allele and the protective effect associated with the HLA-A*02 allele (Brynedal *et al.*, 2007; Burfoot *et al.*, 2008; Lorentzen *et al.*, 2009;

Table 2 Genotype counts (HLA-DRB1*15 and HLA-A*02) among all subjects and among cases and controls, respectively, presented in 3 × 3 grids

Total			
Cases	A*02/A*02	A*02/X	X/X
X/X (%)	24 (3)	128 (15)	200 (24)
15/X (%)	30 (4)	151 (18)	229 (27)
15/15 (%)	5 (1)	26 (3)	50 (6)
Controls	A*02/A2	A*02/X	X/X
X/X (%)	98 (8)	382 (32)	382 (32)
15/X (%)	33 (3)	142 (12)	142 (12)
15/15 (%)	3 (<1)	11 (1)	16 (1)
Non-smokers			
Cases	A*02/A*02	A*02/X	X/X
X/X (%)	18 (3)	93 (16)	137 (23)
15/X (%)	21 (4)	109 (18)	153 (26)
15/15 (%)	3 (<1)	19 (3)	39 (7)
Controls	A*02/A*02	A*02/X	X/X
X/X (%)	75 (8)	301 (32)	301 (32)
15/X (%)	22 (2)	107 (11)	123 (13)
15/15 (%)	2 (<1)	9 (<1)	13 (1)
Smokers			
Cases	A*02/A*02	A*02/X	X/X
X/X (%)	6 (2)	35 (14)	63 (25)
15/X (%)	9 (4)	42 (17)	76 (30)
15/15 (%)	2 (<1)	7 (3)	11 (4)
Controls	A*02/A*02	A*02/X	X/X
X/X (%)	23 (9)	81 (32)	81 (32)
15/X (%)	11 (4)	35 (14)	19 (7)
15/15 (%)	1 (<1)	2 (<1)	3 (1)

Bergamaschi *et al.*, 2010). Hence the DRB1*15 and HLA-A*02 alleles were both included in the interaction analysis. The observed linkage disequilibrium between DRB1*15 and HLA-A*02 in our subjects was low, the correlation coefficient between the alleles, r^2, was <0.0005, while the normalized deviation of observed haplotype frequency compared with expected given random assortment, D', was 0.07. The counts for the genotypes among cases and controls, respectively, are presented in 3 × 3 grids (Table 2). A supplementary table shows adjusted odds ratios with 95% CI of developing MS for subjects exposed to different combinations of HLA-DRB1*15 and HLA-A*02, compared with HLA-A*02 homozygotes without HLA-DRB1*15 alleles, for the total population, and and stratified by smoking status.

Tobacco smoking and risk of developing multiple sclerosis

The average number of pack years smoked among those classified as smokers was 11.6. Analysis of smokers versus non-smokers revealed an odds ratio of 1.6 (95% CI 1.3–2.0).

Interaction between carriage of HLA-DRB1*15 and smoking

Overall, an interaction was observed between HLA-DRB1*15 and smoking with regard to risk of developing multiple sclerosis (Table 3). However, the interaction was confined to those lacking the protective effects conferred by HLA-A*02. Among A*02 negative subjects the attributable proportion due to interaction between HLA-DRB1*15 and smoking was 0.6 (95% CI 0.3–0.8), whereas it was 0.04 (95% CI −0.4 to 0.5) among A*02 positive subjects.

Interaction between absence of HLA-A*02 and smoking

The interaction between the genetic risk factor absence of HLA-A*02 and smoking was studied in the same manner as above, i.e. by taking HLA-DRB1*15 into consideration. Among subjects carrying the HLA-DRB*15 allele, there was a significant interaction between absence of HLA-A*02 and smoking with regard to risk of developing multiple sclerosis (attributable proportion 0.6, 95% CI 0.4–0.9) (Table 4).

Interaction between carriage of HLA-DRB1*15 and absence of HLA-A*02

Having observed that the interaction between smoking and HLA-DRB1*15 depends on HLA-A*02 status and likewise that the interaction between smoking and HLA-A*02 depends on HLA-DRB1*15, a natural step was to analyse the interaction between the two genotypes taking smoking into consideration.

A significant interaction was determined between the two genotypes regarding risk of developing multiple sclerosis (attributable proportion 0.2, 95% CI 0.05–0.5) (Table 5). When taking smoking into consideration, this interaction was confined to smokers (attributable proportion 0.6, 95% CI 0.3–0.8) and was absent among non-smokers (attributable proportion 0.1, 95% CI −0.1–0.4).

Finally, we calculated the odds ratio associated with the most susceptible genotype (carriage of DRB1*15 but not HLA-A*02) in smokers and non-smokers, respectively, compared with non-smokers without these genetic risk factors (Table 6). Non-smokers with the two risk genotypes displayed an odds ratio of 4.9 (95% CI 3.6–6.6) whereas the same genotype for smokers rendered an odds ratio of 13.5 (95% CI 8.1–22.6). Among those with both genetic risk factors, smoking thus increased the risk by a factor of 2.8 in comparison with a factor of 1.4 among those without the genetic risk factors. The interaction between carriage of the risk factor HLA-DRB1*15, absence of HLA-A*02 and smoking is illustrated in Fig. 1.

Genetic model of HLA-A*02 associated protection

Even though the odds ratio for HLA-A*02 homozygotes (0.50, 95% CI 0.4–0.7) and heterozygotes (0.60, 95% CI 0.5–0.8) are numerically similar and the CIs largely overlap, we cannot exclude the possibility that HLA-A*02 acts multiplicatively, rather than dominantly, with regard to protection against multiple sclerosis. With a dominant protective association, HLA-A*02 homozygotes and heterozygotes can be grouped together with ensuing odds

Table 3 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects exposed to different combinations of smoking and HLA-DRB1*15 alleles compared with non-smokers without HLA-DRB1*15 alleles, total and by HLA-A*02 status

	No HLA-DRB1*15			HLA-DRB1*15		
	Cases/controls	OR (95% CI)	*P*-value	Cases/controls	OR (95% CI)	*P*-value
Total						
Non-smokers	248/677	1.0		344/276	3.4 (2.8–4.3)	4×10^{-29}
Current smokers	104/185	1.6 (1.2–2.1)	0.002	147/71	5.8 (4.2–8.0)	1×10^{-26}
AP					0.3 (0.07–0.5)	0.01
HLA-A*02 positive						
Non-smokers	111/376	1.0		152/140	3.8 (2.7–5.1)	3×10^{-16}
Current smokers	41/104	1.4 (0.9–2.1)	0.1	60/49	4.3 (2.8–6.7)	8×10^{-11}
AP					0.04 (−0.4–0.5)	0.9
HLA-A*02 negative						
Non-smokers	137/301	1.0		192/136	3.1 (2.3–4.2)	3×10^{-13}
Current smokers	63/81	1.7 (1.2–2.6)	0.006	87/22	8.9 (5.3–14.9)	8×10^{-17}
AP					0.6 (0.3–0.8)	8×10^{-7}
P permutation**						0.016

Attributable proportion due to interaction between HLA-DRB1*15 and smoking.
***P*-value achieved after 10 000 permutations.
All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
AP = attributable proportion; OR = odds ratio.

Table 4 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects exposed to different combinations of smoking and HLA-A*02 alleles compared with non-smokers carrying HLA-A*02, total and by HLA-DRB1*15 status

	HLA-A*02			No HLA-A*02		
	Cases/controls	OR (95% CI)	*P*-value	Cases/controls	OR (95% CI)	*P*-value
Total						
Non-smokers	263/516	1.0		329/437	1.5 (1.2–1.8)	0.0001
Current smokers	101/153	1.3 (1.0–1.8)	0.06	150/103	2.9 (2.2–3.9)	1×10^{-12}
AP					0.4 (0.2–0.6)	0.0007
HLA-DRB1*15 negative						
Non-smokers	111/376	1.0		137/301	1.5 (1.2–2.1)	0.004
Current smokers	41/104	1.4 (0.9–2.1)	0.1	63/81	2.8 (1.8–4.1)	8×10^{-7}
AP					0.3 (−0.04–0.6)	0.08
HLA-DRB1*15 positive						
Non-smokers	152/140	1.0		192/136	1.3 (0.9–1.8)	0.1
Current smokers	60/49	1.1 (0.7–1.8)	0.6	87/22	3.7 (2.2–6.3)	1×10^{-6}
AP					0.6 (0.4–0.9)	6×10^{-7}
P permutation**						0.003

Attributable proportion due to interaction between absence of HLA-A*02 and smoking.
***P*-value achieved after 10 000 permutations.
All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
AP = attributable proportion; OR = odds ratio.

ratio as shown in Fig. 1. However, with other modes of action, other combinations may be of relevance. Irrespective of the mode of HLA-A*02 association to protection and in view of both of them being protective, we also found it of interest to compare X/X with A*02/A*02, and X/X with A*02/X, in their interaction with DRB1*15 among smokers, where X denotes any A allele with the exception of A*02. Hereby, X/X versus A*02/A*02 provided a statistically significant interaction with DRB1*15 (attributable proportion 0.6, 95% CI 0.3–0.9, nominal $P < 2 \times 10^{-5}$, permuted $P < 0.03$). The same procedure repeated for X/X versus A*02/X provided a similar result (attributable proportion 0.6, 95% CI 0.3–0.8, nominal $P < 2 \times 10^{-5}$, permuted $P < 0.02$). No sign of interaction was evident between HLA-DRB1*15 and HLA-A*02 among non-smokers (Table 7 and Fig. 2).

Interaction between HLA-DRB1*15 and HLA-A*02 among smokers, with HLA-A*02 homozygotes as reference, is also observed when HLA-A*02 heterozygotes are classified together with subjects who do not carry any copy of HLA-A*02

Table 5 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects exposed to different combinations of the genetic risk factors carriage of the HLA-DRB1*15 allele and absence of the HLA-A*02 allele, compared with subjects carrying none of these genetic risk factors, for the total population and stratified by smoking status

	No HLA-DRB1*15			HLA-DRB1*15		
	Cases/controls	OR (95% CI)	*P*-value	Cases/controls	OR (95% CI)	*P*-value
Total						
HLA-A*02	152/480	1.0		212/189	3.6 (2.7–4.7)	2×10^{-20}
No HLA-A*02	200/382	1.7 (1.3–2.1)	7×10^{-5}	279/158	5.6 (4.3–7.4)	1×10^{-36}
AP					0.2 (0.05–0.5)	0.01
Non-smokers						
HLA-A*02	111/376	1.0		152/140	3.7 (2.7–5.0)	4×10^{-16}
No HLA-A*02	137/301	1.6 (1.2–2.1)	0.003	192/136	4.8 (3.5–6.5)	1×10^{-23}
AP					0.1 (−0.1–0.4)	0.4
Smokers						
HLA-A*02	41/104	1.0		60/49	3.3 (1.9–5.7)	1×10^{-5}
No HLA-A*02	63/81	2.1 (1.3–3.4)	0.004	87/22	10.3 (5.6–18.9)	5×10^{-14}
AP					0.6 (0.3–0.8)	7×10^{-6}
P permutation**						0.017

Attributable proportion due to interaction between HLA-DRB1*15 and absence of HLA-A*02.
***P*-value achieved after 10 000 permutations.
All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
AP = attributable proportion; OR = odds ratio.

Table 6 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects with different combinations of smoking habits and the genetic risk factors carriage of the HLA-DRB1*15 allele and absence of the HLA-A*02 allele, compared with non-smokers carrying none of the genetic risk factors

HLA-DR15 +	HLA-A*02-	Smoking	Cases/controls	OR (95% CI)	*P*-value
−	−	−	111/376	1.0	
−	+	−	137/301	1.6 (1.2–2.1)	0.003
+	−	−	152/140	3.7 (2.7–5.1)	3×10^{-16}
+	+	−	192/136	4.9 (3.6–6.6)	5×10^{-24}
−	−	+	41/104	1.4 (0.9–2.1)	0.1
−	+	+	63/81	2.7 (1.8–4.0)	7×10^{-7}
+	−	+	60/49	4.3 (2.8–6.6)	6×10^{-11}
+	+	+	87/22	13.5 (8.1–22.6)	5×10^{-23}

All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
OR = odds ratio.

(attributable proportion 0.5, 95% CI 0.1–0.9, nominal $P < 0.01$, permuted $P < 0.1$) (Table 8). The data in Table 8 are further illustrated in Fig. 3 and Table 9, where each exposure combination has been compared with the same reference category (non-smoking HLA-A*02-homozygotes carrying no HLA-DRB1*15 alleles). The results are similar to those observed in Fig. 1.

Discussion

We herein describe a gene-lifestyle/environment interaction involved in the risk for multiple sclerosis. Thus, an interaction between presence of HLA-DRB1*15 and absence of HLA-A*02 was observed among smokers, whereas such an interaction was absent among non-smokers. Hereby, HLA-A*02 heterozygotes and homozygotes were considered as one group and our conclusion is based on the assumption that the HLA-A*02 associated protection acts dominantly, although this awaits definitive confirmation. However, irrespective of this uncertainty, each group consisting of A*02 homozygotes or A*02 heterozygotes displayed protection compared with other HLA-A alleles, and each group displayed a significant interaction with HLA-DR*15 among smokers. The difference in multiple sclerosis risk between the extremes was considerable; smokers carrying HLA-DRB1*15 and lacking HLA-A*02 had a 14-fold increased risk compared with non-smokers without these genetic risk factors (odds ratio 13.5, 95% CI 8.1–22.6). When ex-smokers were excluded, the corresponding odds ratio was 16.6 (9.7–28.6). Since allele frequencies of HLA genes differ between different ethnic groups we also conducted analyses restricted to subjects of Scandinavian origin. The results with regard

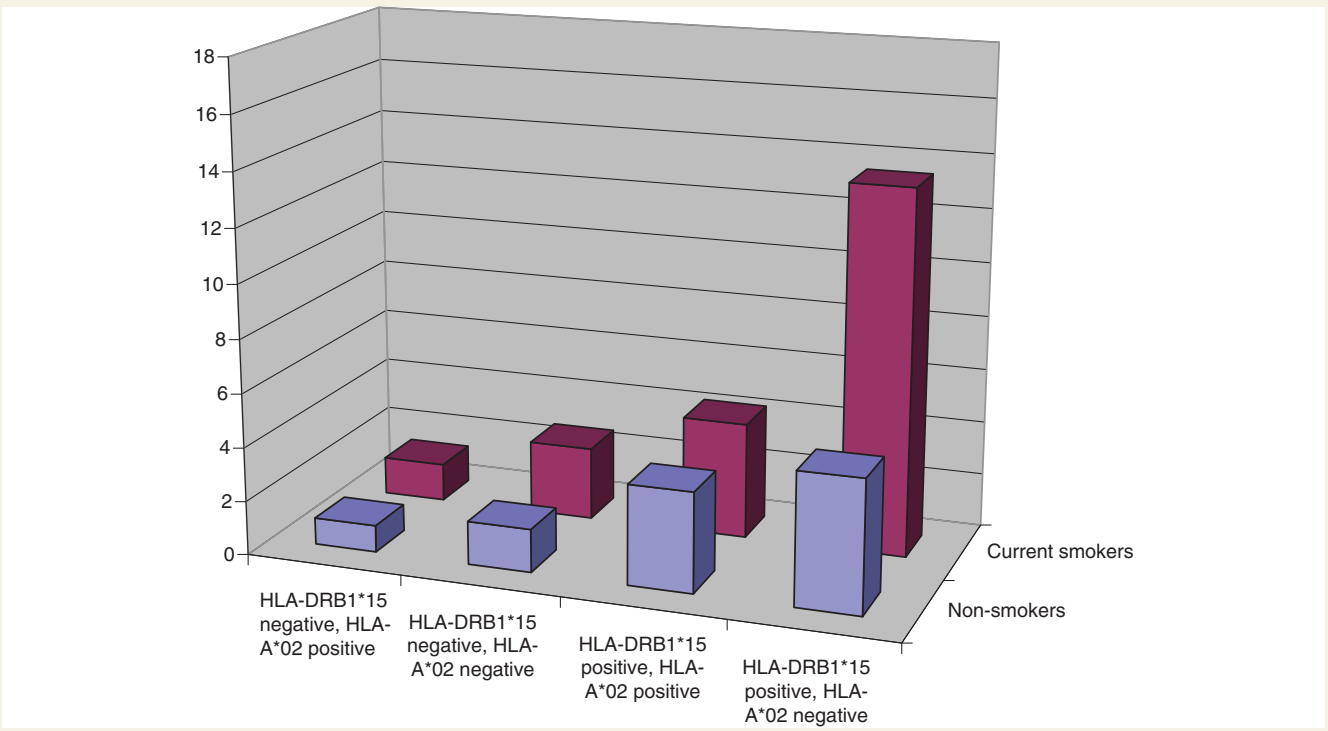

Figure 1 Odds ratios for different combinations of two genetic risk factors (absence of HLA-A*02 and carriage of HLA-DRB1*15) compared with non-smokers carrying none of the genetic risk factors, among smokers and non-smokers. Statistics are shown in Table 6.

Table 7 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects exposed to different combinations of smoking habits, HLA-DRB1*15 and HLA-A*02, compared with non-smoking A2-homozygotes carrying no HLA-DRB1*15 alleles

Smoking	HLA-DRB1*15	A*02 status[a]	HLA-Cases/controls	OR (95% CI)	*P*	AP
–	–	A*02/A*02	18/75	1.0		
–	–	A*02/X	93/301	1.3 (0.7–2.3)	0.4	
–	–	A*02/X or X/X	230/602	1.6 (0.9–2.8)	0.08	
–	–	X/X	137/301	1.9 (1.1–3.3)	0.02	
–	+	A*02/A*02	24/24	4.1 (1.9–8.9)	3×10^{-4}	
–	+	A*02/X	128/116	4.7 (2.6–8.3)	1×10^{-7}	0.04 (−0.5–0.6)[b], $P = 0.9$
–	+	A*02/X or X/X	320/252	5.4 (3.1–9.2)	1×10^{-9}	0.1 (−0.4–0.6), $P = 0.6$
–	+	X/X	192/136	6.0 (3.4–10.5)	4×10^{-10}	0.1 (−0.3–0.6)[c], $P = 0.5$
+	–	A*02/A*02	6/23	1.1 (0.4–3.2)	0.8	
+	–	A*02/X	35/81	1.8 (1.0–3.5)	0.07	
+	–	A*02/X or X/X	98/162	2.6 (1.5–4.6)	0.001	
+	–	X/X	63/81	3.3 (1.8–6.2)	1×10^{-4}	
+	+	A*02/A*02	11/12	4.0 (1.5–10.6)	0.005	
+	+	A*02/X	49/37	5.7 (2.9–11.1)	4×10^{-7}	0.4 (−0.2–1.0)[b], $P = 0.2$
+	+	A*02/X or X/X	136/59	9.7 (5.3–17.8)	1×10^{-13}	0.5 (0.1–0.9), $P = 0.01$
+	+	X/X	87/22	16.5 (8.2–33.2)	4×10^{-15}	0.6 (0.3–0.9)[c], $P = 5 \times 10^{-5}$

Attributable proportion due to interaction between HLA-DRB1*15 and HLA-A*02 status among non-smokers and smokers, respectively. All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
[a]A2/A2 = homozygotes for HLA-A*02, A2/X = heterozygotes for HLA-A*02, X/X = absence of HLA-A*02.
[b]Attributable proportion calculated among non-smokers and smokers, respectively, based on a model excluding X/X.
[c]Attributable proportion calculated among non-smokers and smokers, respectively, based on a model excluding A*02/X.
AP = attributable proportion; OR = odds ratio.

to interaction between HLA-DRB1*15 and HLA-A*02 were similar (attributable proportion 0.6, 95% CI 0.4–0.9), whereas the difference between extremes was higher among Scandinavians; smokers carrying both the genetic risk factors had a 17-fold increased risk compared with non-smokers without the genetic risk factors. The risk increased further when the analysis was restricted to comparing current smokers with never-smokers (odds ratio 21.7, 95% CI 11.6–40.4).

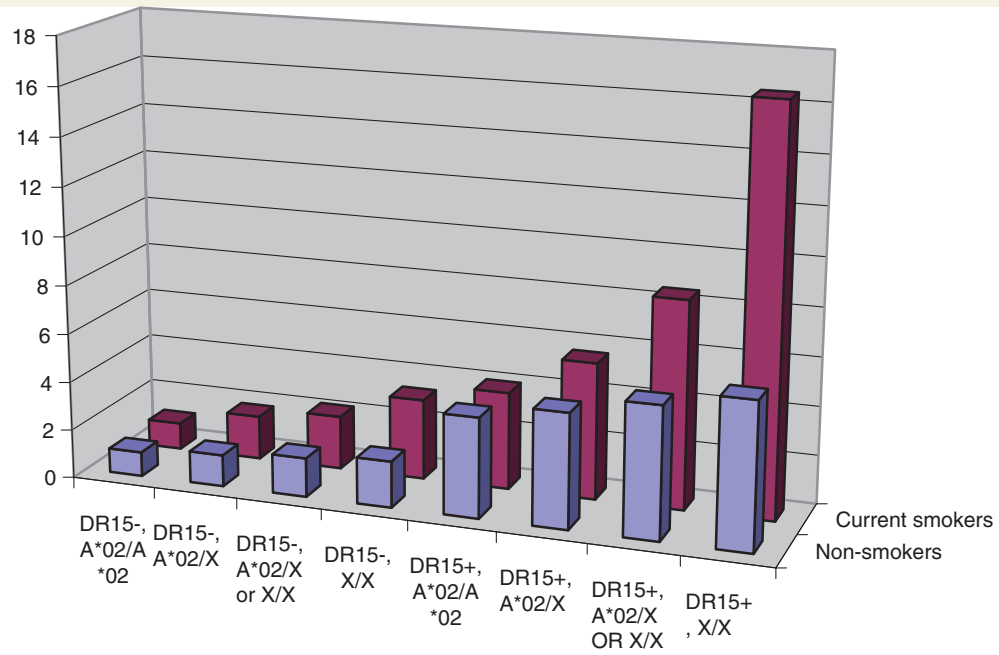

Figure 2 Odds ratios for different combinations of HLA-DRB1*15 and HLA-A*02 compared with non-smoking A2-homozygotes carrying no HLA-DRB1*15 alleles, among smokers and non-smokers. Statistics are shown in Table 7.

Table 8 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects with different combinations of HLA-DRB1*15 and HLA-A*02 compared with A2-homozygotes carrying no HLA-DRB1*15 alleles, for the total population and stratified by smoking status

	No HLA-DRB1*15			HLA-DRB1*15		
	Cases/controls	OR (95% CI)	*P*-value	Cases/controls	OR (95% CI)	*P*-value
Total						
A*02/A*02	24/98	1.0		35/36	4.0 (2.1–7.6)	3×10^{-5}
A*02/X or X/X	328/764	1.8 (1.1–2.8)	0.02	456/311	6.0 (3.8–9.7)	7×10^{-14}
AP					0.2 (−0.1–0.6)	0.2
Non-smokers						
A*02/A*02	18/75	1.0		24/24	4.1 (1.9–8.9)	3×10^{-4}
A*02/X or X/X	230/602	1.6 (0.9–2.8)	0.08	320/252	5.4 (3.1–9.2)	1×10^{-9}
AP					0.1 (−0.4–0.6)	0.6
Smokers						
A*02/A*02	6/23	1.0		11/12	3.2 (0.9–11.3)	0.06
A*02/X or X/X	98/162	2.2 (0.8–5.7)	0.1	136/59	8.5 (3.2–22.5)	2×10^{-5}
AP					0.5 (0.1–0.9)	0.01
P permutation**						0.1

Attributable proportion due to interaction between HLA-DRB1*15 and HLA-A*02 negativity/heterozygosity.
***P*-value achieved after 10 000 permutations.
All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.
AP = attributable proportion; OR = odds ratio.

The study was designed as a case–control study with incident cases, in which information regarding smoking habits was collected retrospectively. In order to minimize recall bias, we primarily included incident cases of multiple sclerosis who had received the diagnosis within the past year. Moreover, the questionnaire contained a wide range of questions regarding many potential environmental risk factors and no section in the questionnaire was given prime focus.

Another potential methodical problem is that the recruitment of cases and controls may introduce selection bias. Considering the structure of the public Swedish health care system, which provides equal free of charge access to medical services for all Swedish citizens, it is most likely that almost all cases of multiple sclerosis are referred to neurological units.

The proportion of responders with regard to participation in the study was 93% for cases and 73% for controls. A potential

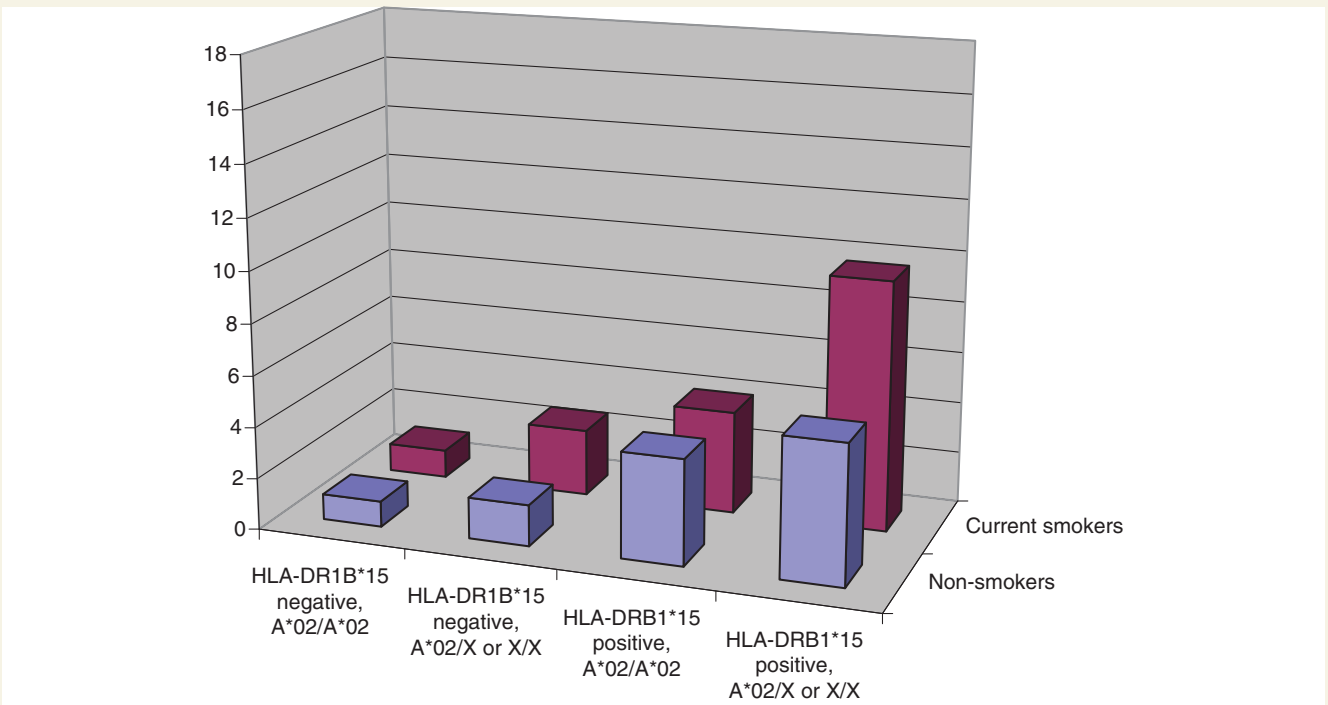

Figure 3 Odds ratios for different combinations of HLA-DRB1*15 and HLA-A*02 compared with non-smoking A2-homozygotes carrying no HLA-DRB*15 alleles, among smokers and non-smokers. Statistics are shown in Table 9.

Table 9 Adjusted odds ratio with 95% confidence interval of developing multiple sclerosis for subjects with different combinations of smoking habits, HLA-DRB1*15 status and HLA-A*02 status, compared with A2-homozygotes carrying no HLA-DRB*15 alleles

HLA-DR15+	A2/X or X/X	Smoking	Cases/controls	OR (95% CI)	*P*-value
–	–	–	18/75	1.0	
–	+	–	230/602	1.6 (0.9–2.7)	0.09
+	–	–	24/24	4.1 (1.9–8.9)	3×10^{-4}
+	+	–	320/252	5.4 (3.1–9.2)	1×10^{-9}
–	–	+	6/23	1.1 (0.4–3.2)	0.8
–	+	+	98/162	2.6 (1.5–4.6)	0.001
+	–	+	11/12	4.0 (1.5–10.6)	0.005
+	+	+	136/59	9.7 (5.3–17.7)	1×10^{-13}

All analyses were adjusted for age, gender, residential area and ancestry. Further adjustment for HLA-DRB1*07, HLA-DRB1X and HLA-A*03 only marginally influenced the results.

selection bias may result from the relatively high proportion of non-responders among the controls. However, this bias is probably modest because the prevalence of smoking among the controls was consistent with that of the general population in similar ages (www.scb.se). Only controls that had responded to the questionnaire were asked to provide a blood sample, and 58% of these controls donated blood. There were no differences with respect to age, gender, residential area or smoking habits between those who provided blood and those who did not.

In summary, we consider it highly unlikely that our main finding on the interaction between particular HLA types and smoking would be affected by bias to a large extent, especially since such a bias would then depend on HLA types.

Gene–environment and gene–gene interactions were examined by quantification of departure from additivity of effects, described as the method most appropriate for identifying specific biological interactions (Rothman *et al.*, 1980). Two risk factors can either be independent (i.e. no pathway to disease requires the involvement of both risk factors) or have biological interaction (i.e. at least one pathway towards disease requires the involvement of both risk factors). Independent risk factors adhere to an additive model and biological interaction results in departure from additivity of the disease rates (Rothman *et al.*, 2008; VanderWeele, 2009). The product term in a logistic regression model, a commonly used alternative when studying interaction, has in general no such specific biological interpretation. When we calculated the

product term for the interaction between carriage of HLA-DRB1*15, absence of HLA-A*02 and smoking in a logistic model, this was statistically significant (odds ratio 1.9, 95% CI 1.1–3.3, $P = 0.04$). Considering genotypes rather than allele carriage, the additional degrees of freedom introduced into the analysis limit the power to such an extent that a statistically significant interaction is no longer apparent (odds ratio interaction term 1.5, 95% CI 0.7–3.3).

The HLA complex has a strong linkage disequilibrium (Caillier *et al.,* 2008; Ramagopalan *et al.,* 2009*a, b*) between the different loci that may be sufficient to produce an association signal at class I loci secondary to DRB1 effects. Therefore, there is a possibility that the observed association with HLA-A in our study is secondary to another genetic variant in linkage disequilibrium with HLA-A*02. However, the linkage disequilibrium between HLA-A and HLA-DRB1 was small (overall D' 0.19) in our study population, which has been previously reported (Brynedal *et al.,* 2007). Our results thus confirm that HLA class I, or closely located variants, indeed have an influence on multiple sclerosis susceptibility, independently of and in interaction with the HLA-DRB1 locus. It is noteworthy that this interaction is only apparent among smokers. Despite the solid statistical support for the interaction we describe, a replication of our findings would have given further support. However, we have not been able to localize any other group in the field that have comprehensive information on smoking habits and HLA genotype, though our study may stimulate groups in the field to gather such data.

Our findings provide new testable hypotheses regarding the role of HLA gene influences in multiple sclerosis. The HLA-A allelic effects as studied in mouse transgenic models could either be due to disease-promoting roles of the non-A*02 alleles or to protective effects by the A2 allele, or both (Friese *et al.,* 2008). Other experimental findings demonstrate that certain allelic variants of class I molecules evoke CD8+ cells to produce anti-inflammatory cytokines (Mustafa *et al.,* 1993, 1994; Issazadeh *et al.,* 1997). Preferences in peptide binding by allelic variants of class II molecules, acting either in the periphery or at the level of thymic deletion, are likely to be critical for the HLA class II influences on autoimmune diseases (Olsson and Hillert, 2008). The potent interaction between smoking and DRB1*15 in the absence of the presumed protection by class I HLA-A*02 can primarily be considered in the context of preferences in peripheral antigen presentation. Since tobacco in the form of moist snuff does not increase the risk for multiple sclerosis (Hedström *et al.,* 2009), the prime interactive event may take place in the lung, possibly due to the irritative actions of smoking or to the increased incidence of respiratory infections that is secondary to smoking. Interestingly, in context with emphysema, smoking results in activation of dendritic cells in the lungs that induce T cell producing proinflammatory cytokines (Shan *et al.,* 2009). Furthermore, it has been demonstrated that smokers have increased levels of post-translationally modified proteins in the lungs (Makrygiannakis *et al.,* 2008). A variety of post-translational modifications of proteins may render them more autoantigenic, through antigen presentation or enhanced uptake by antigen presenting cells, leading to breakdown of self-tolerance (Doyle and Mamula, 2002; Cloos, 2004). Since T cell recognition of peptides is degenerate and T cell-mediated autoimmune disease has been documented due to low-affinity cross-reactivity to common microbial peptides (Harkiolaki *et al.,* 2009), reactivity to CNS specific antigens is feasible.

Interestingly, carriage of the so-called share epitope, comprising HLA-DRB1*04, interacts with smoking to increase the risk of rheumatoid arthritis (Klareskog *et al.,* 2006; Linn-Rasker *et al.,* 2006; Källberg *et al.,* 2007; Lundström *et al.*, 2009; Mahdi *et al.,* 2009; Karlsson *et al.,* 2010). Thus, in two different organ-specific inflammatory diseases, rheumatoid arthritis and, as demonstrated here, multiple sclerosis, there are now indications of strong interactions between smoking and HLA genes whose basic function is to present peptides to T cells. The fact that the interactions take place between smoking and different class II alleles supports the hypothesis that the association with disease depends on the classical function of class II molecules since these have different preferences in peptide binding. We thus hypothesize that smoking in the context of HLA-DRB1*15, with an absence of HLA-A*02 protection, may post-translationally modify peptides cross-reactive with CNS antigens, promoting a CNS directed auto-aggressive immunity that results in multiple sclerosis.

In conclusion, in this population-based case–control study we have confirmed the independent influence of HLA-DRB1*15 and HLA-A*02 on multiple sclerosis susceptibility, and demonstrated a statistically significant interaction between HLA DRB1*15, HLA A*02, and smoking in the development of multiple sclerosis.

Our study emphasizes the need to include data regarding environmental exposures in genetic analyses of complex diseases, as illustrated by our results where the effects of the main genetic risk factors for multiple sclerosis seem to be influenced by the presence of smoking in the population at risk.

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Supplementary material

Supplementary material is available at *Brain* online.

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