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HLA-class I markers and multiple sclerosis susceptibility in the Italian population

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Previous studies reported an association with multiple sclerosis (MS) of distinct HLA-class I markers, namely HLA-A*02, HLA-Cw*05 and MOG-142L. In this work, we tested the association with MS of A*02 and Cw*05 in 1273 Italian MS patients and 1075 matched controls, which were previously analyzed for MOG-142, and explored the relationship among these three markers in modulating MS risk. HLA-A*02 conferred a statistically robust MS protection (odds ratio, OR = 0.61; 95% confidence intervals, CI = 0.51–0.72, P < 10⁻⁵), which was independent of DRB1*15 and of any other DRB1 allele and remained similar after accounting for the other two analyzed class I markers. Conversely, the protective effect we previously observed for MOG-142L was secondary to its linkage disequilibrium with A*02. Cw*05 was not associated considering the whole sample, but its presence significantly enhanced the protection in the HLA-A*02-positive group, independently of DRB1: the OR conferred by A*02 in Cw*05-positive individuals (0.22, 95% CI = 0.13–0.38) was significantly lower than in Cw*05-negative individuals (0.69, 95% CI = 0.58–0.83) with a significant (P = 4.94 × 10⁻⁶) multiplicative interaction between the two markers. In the absence of A*02, Cw*05 behaved as a risk factor, particularly in combination with DRB1*03 (OR = 3.89, P = 0.0006), indicating that Cw*05 might be a marker of protective or risk haplotypes, respectively.

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Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system characterized by disseminated and focal damage of myelin and axons, resulting in a disabling condition. The pathogenesis of MS is not understood yet, but several studies suggest an interaction of environmental and genetic factors. The genetic factor showing the strongest association with MS is localized in the human Major Histocompatibility complex (HLA) region on chromosome 6p21.3. The risk haplotype that has been identified for the Caucasian populations is HLA DRB1*1501-DQB1*0602 (also known as DR15 haplotype) in the HLA-class II region. In the Italian as well as in other European populations, DR15 confers an odds ratio (OR) of about 3.

In recent years, several studies have searched for the presence of MS susceptibility factors in the HLA region with an effect independent of HLA-DRB1. No evidence of an HLA-A or -B association was found by Chao et al. in 294 Canadian families. Moreover, none of the 1068 single-nucleotide-polymorphisms (SNPs) from a high-density panel spanning the entire HLA genomic region showed additional association in 1185 Canadian and Finnish families after conditioning for HLA-DRB1. Conversely, other studies have detected the effect at
least of one class I genetic factor independently of HLA-DRB1. Brynedal et al. confirmed in a Nordic cohort of 1084 MS patients and 1347 controls the results of a previous study performed in a smaller Swedish panel, showing that HLA-A*02 is negatively associated with MS (OR = 0.63, P = 7 × 10^{-15}). Yeo et al. analyzed over 1600 UK MS patients and 3600 controls and found that HLA-Cw*05 exerts an MS-protective effect (OR = 0.49, 95% confidence intervals, CI = 0.34–0.69, P = 3.3 × 10^{-7}) after excluding all individuals carrying DRB1 alleles associated with MS. In a panel of 1124 Italian MS patients and 1136 controls, we found a significant association with the missense variant V142L (rs2857766) in the gene encoding the Myelin Oligodendrocyte Glycoprotein (MOG) mapping in the HLA-class I region, telomeric to HLA-A (0.3 Mb) and HLA-C (1.6 Mb). The 142L allele (MOG-142L) conferred an OR = 0.70 (95% CI = 0.60–0.82) that remained similar after accounting for HLA-DRB1*15 carrier status. Burfoot et al. showed similar data in a small Tasmanian population. Moreover, they reported a negative association with HLA-A*02, but they did not analyze if this variation was primarily associated or was dependent on MOG-142L.

The association among the three reported HLA-class I markers, A*02, Cw*05, MOG-142L and their association with MS has not been examined so far.

In this work, we tested the association of A*02 and Cw*05 in a large cohort of Italian MS patients and controls, most of whom had been previously typed for MOG-142 polymorphism, and we explored the relationship among MOG-142L, HLA-A*02 and HLA-Cw*05 in modulating MS risk.

Results

Association with MS of MOG-142L, HLA-A*02 and HLA-Cw*05 in the Italian population

The MS association of MOG-142L, HLA-A*02 and HLA-Cw*05 was tested in a case–control study (Table 1). The frequency of MOG-142L and HLA-A*02-positive individuals was significantly lower in cases than in controls, confirming the previously reported protective effect, whereas the association with Cw*05 was not significant.

The comparison of genotype frequencies suggested a dominant-protective effect of MOG-142L and of HLA-A*02, as their frequency was significantly decreased both among homozygous (OR = 0.55, 95% CI = 0.34–0.89 for 142L and OR = 0.55, 95% CI = 0.37–0.82 for A*02) and heterozygous (OR = 0.75, 95% CI = 0.62–0.90 for 142L and OR = 0.68, 95% CI = 0.57–0.81 for A*02) MS patients. The results did not change after adjustment for sex and were not different among male and female cases in a stratified analysis (data not shown). The results were not significantly different in relapsing remitting and primary progressive patients (data not shown).

To eliminate the possible confounding effect of linkage disequilibrium (LD) with HLA-DRB1*15, we performed the same analysis in DRB1*15-negative individuals. The OR values for the three tested markers remained substantially the same (Table 1).

The relative effect of the three tested HLA-class I markers on MS risk was evaluated by logistic regression analysis (Table 2). DRB1*15 was also included in the model. All individuals were categorized according to the presence or absence of each of the four considered alleles. The OR conferred by A*02 remained similar after accounting for each of the other markers included in the model and for all the markers together, showing that the protective effect was independent of any other analyzed HLA marker. Conversely, the protective effect of MOG-142L was abolished by adjustment for A*02. The lack of a significant association with Cw*05 and the significant risk effect of DRB1*15 were similar with and without the adjustment for the remaining markers. This analysis clearly showed that the association with MOG-142L was not independent of HLA-A*02. This was also indicated by an analysis stratified for HLA-A*02: MOG-142L was not significantly associated either in the A*02 positive (OR = 0.96 95% CI = 0.74–1.23, P = 0.76) or in the A*02 negative (OR = 0.80, 95% CI = 0.60–1.07, P = 0.13). These results were explained by the strong LD (D = 0.59; r² = 0.5) between the two alleles both in the control and in the MS patient population (Table 3).

Considerable LD was also detected between A*02 and Cw*05 in the control population (Table 3). Notably, it was completely absent in the MS patients. This difference was explained by the significantly decreased frequency of the

Table 1 Frequency of MOG-142L, HLA-A*02 and HLA-Cw*05-positive individuals among MS patients and controls

| Marker | Sample | Frequencies | P-value | OR (95% CI) |
|--------|--------|-------------|---------|-------------|
|        | Cases  | Controls    |         |             |
| Total  | N = 1273 | N = 1075 |         |             |
| MOG-142L | 0.285 | 0.364 | 4.93 × 10^{-5} | 0.70 (0.59–0.83) |
| HLA-A*02 | 0.331 | 0.448 | 5.28 × 10^{-9} | 0.61 (0.51–0.72) |
| HLA-Cw*05 | 0.101 | 0.112 | NS | 0.85 (0.65–1.10) |
| DRB1 negative | N = 883 | N = 935 |         |             |
| MOG-142L | 0.293 | 0.377 | 1.42 × 10^{-4} | 0.68 (0.56–0.84) |
| HLA-A*02 | 0.334 | 0.464 | 1.44 × 10^{-8} | 0.58 (0.48–0.70) |
| HLA-Cw*05 | 0.108 | 0.119 | NS | 0.89 (0.66–1.21) |

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio; NS, not significant.

Genotype frequencies (+/+, +/-, /-, /-) in the total MS patient sample vs total controls were 0.02, 0.26, 0.72 vs 0.04, 0.32, 0.64 for MOG-142L and 0.04, 0.29, 0.67 vs 0.07, 0.38, 0.55 for HLA-A*02. These frequencies did not deviate from Hardy Weinberg equilibrium either in MS patients or controls.
Table 2  Logistic regression analysis of MOG-142L, HLA-A*02, HLA-Cw*05 and DRB1*15 alleles

| Markers | MOG-142L | HLA-A*02 | HLA-Cw*05 | DRB1*15 | All markers in the model |
|---------|-----------|-----------|-----------|---------|-------------------------|
| MOG-142L | 0.70 (0.59–0.83) | 0.87 (0.71–1.06) | 0.70 (0.59–0.83) | 0.73 (0.61–0.87) | 0.90 (0.73–1.10) |
| HLA-A*02 | 0.65 (0.53–0.79) | 0.61 (0.51–0.72) | 0.61 (0.52–0.72) | 0.63 (0.53–0.75) | 0.66 (0.54–0.81) |
| HLA-Cw*05 | 0.87 (0.67–1.13) | 0.90 (0.69–1.17) | 0.85 (0.65–1.10) | 0.88 (0.67–1.14) | 0.92 (0.71–1.21) |
| DRB1*15 | 2.90 (2.34–3.59) | 2.89 (2.33–3.58) | 2.94 (2.37–3.64) | 2.95 (2.38–3.65) | 2.87 (2.32–3.56) |

Abbreviations: CI, confidence intervals; OR, odds ratio.

Each marker in row is adjusted for markers in columns. In the last column the model includes all the markers.
The bolded diagonal values contain the crude values for each marker.

$P$-values are obtained from likelihood ratio test comparing the likelihood of the two-gene additive vs the single marker model considering as single marker the marker used for adjustment.

Table 3  Linkage disequilibria among the three considered HLA-class I markers and DRB1*15 in the control and in the MS patient population

|          | MOG-142L | A*02 | Cw*05 | DRB1*15 | MOG-142L | A*02 | Cw*05 | DRB1*15 |
|----------|----------|------|-------|---------|----------|------|-------|---------|
| MOG-142L | –        | 0.587*** (0.469) | –   | –       | –        | 0.588*** (0.510) | –   | –       |
| A*02     | 0.194*** (0.091) | 0.403*** (0.160) | –   | 0.002 (0.001) | –        | 0.006 (0.028) | –   | –       |
| Cw*05    | –        | –    | 0.001 (0.012) | –       | –        | –    | –0.003 (0.031) | –   |
| DRB1*15  | –0.011*** (0.074) | –0.014** (0.082) | –0.001 (0.012) | –       | –        | –0.006 (0.028) | –0.003 (0.011) | –0.003 (0.031) |

Abbreviation: MS, multiple sclerosis.

Numbers represent $D'$ (Lewontin’s delta) and $r^2$ (in brackets) values.

$^*P<0.05$, $^{**}P<0.005$ and $^{***}P<0.00001$.

Table 4  Phenotypic combinations of HLA-A*02 and HLA-Cw*05 in MS patients and controls

| A*02 | Cw*05 | Sample | Frequency | P-value | OR (95% CI) |
|------|-------|--------|-----------|---------|-------------|
|      |       |        | Cases     | Controls |             |
| Total | N = 1273 | 0.599 | 0.513 | 2.4 $\times$ 10$^{-5}$ | 1.42 (1.21–1.68) |
| +    | 0.031 | 0.078 | 4.1 $\times$ 10$^{-7}$ | 0.38 (0.26–0.56) |
| +    | 0.299 | 0.370 | 2.8 $\times$ 10$^{-4}$ | 0.73 (0.61–0.86) |
| -    | 0.070 | 0.039 | 1.85 (1.27–2.69) |
| DR15 negative | N = 883 | 0.589 | 0.498 | 1.1 $\times$ 10$^{-4}$ | 1.44 (1.20–1.73) |
| +    | 0.031 | 0.081 | 1.8 $\times$ 10$^{-4}$ | 0.36 (0.23–0.56) |
| +    | 0.305 | 0.383 | 3.6 $\times$ 10$^{-4}$ | 0.70 (0.58–0.85) |
| -    | 0.077 | 0.038 | 2.4 $\times$ 10$^{-4}$ | 2.15 (1.41–3.26) |

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio.

A*02 +, Cw*05 + phenotypic combination in the MS patient as opposed to the control population (Table 4).
When analyzing in more detail the effect of the different phenotypic combinations of the A*02 and Cw*05 alleles on MS risk (Table 4), it appeared that the A*02 +, Cw*05 + combination conferred an OR (0.38) significantly lower than that (OR = 0.73) consequent to the presence of A*02 in the absence of Cw*05. Conversely, in the absence of HLA-A*02, Cw*05 showed a significant positive association with MS (OR = 1.85). The same results were seen when considering only DRB1*15-negative individuals (Table 4).

Stratification for the HLA-Cw*05 revealed a substantial difference in risk conferred by the A*02 among
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Cw*05-positive individuals (OR = 0.22, 95% CI = 0.13–0.38) and Cw*05-negative individuals (OR = 0.69, 95% CI = 0.58–0.83). When stratifying for A*02, Cw*05 behaved as a protective factor among A*02-positive individuals (OR = 0.50, 95% CI = 0.33–0.74) and as a risk factor among A*02-negative individuals (OR = 1.53, 95% CI = 1.04–2.24). The likelihood ratio test used to compare the full model (including the two markers and the interaction term) to the additive model (including only the two markers) was statistically significant (P = 4.9 × 10⁻⁷). Thus, although HLA-Cw*05 and A*02 are not reciprocally confounders, as there was no difference between crude and adjusted ORs (Table 2), the stratification analysis showed a significant multiplicative interaction between the two markers.

From the analysis in the case-control population, it was not possible to distinguish whether the A*02+, Cw*05+ individuals carried the two alleles in cis or in trans. We, therefore, analyzed the transmission of these two alleles in 201 family trios. No Mendelian errors were observed. Notably, out of 10 haplotypes that carried both A*02 and Cw*05, none were transmitted to the MS patients (TNT = 0.10; P = 0.002). The only A*02+, Cw*05+ MS patient present in this panel inherited the two alleles in trans. A*02-positive individuals of the family trios were sequenced to define the A*02 alleles at a higher resolution. In agreement with the literature (http://www.allelefrequencies.net/), in these samples, A*0201 was the most frequent A*02 allele (90%) followed by A*0205 (8%) and A*0217 (2%). A*0201 was the most represented A*02 allele both in the A*02+ and, Cw*05+ and in the A*02+, Cw*05– haplotypes. The same proportion of A*02 subtypes was seen among transmitted and non-transmitted haplotypes (data not shown).

The joint effect of class I (A*02, Cw*05) and class II (DRB1*15) markers on MS risk is reported in Figure 1. All the combinations of these three alleles showed a significantly increased risk relative to the A*02+, Cw*05+, DRB1*15– phenotype, which is characterized both by the presence of the class I markers conferring the highest protection and by the absence of the class II allele (Global Χ² = 4.9 × 10⁻⁷). The three combinations carrying DRB1*15 in the absence of the highly protective A*02+, Cw*05+ phenotype (that is carrying only A*02 or only Cw*05 or none of them) showed a similar eightfold increased risk. In the presence of both A*02 and Cw*05, the DRB1*15 risk approximately halved to 4.57, although this difference was not statistically significant owing to the low frequency of this phenotype (N = 13 MS patients and 8 controls). In the absence of DRB1*15, a differently increased risk was observed according to the presence of only A*02 (OR = 2.11) or only Cw*05 (OR = 5.47) or none of them (OR = 3.14).

**Stratification for DRB1 alleles**

To test whether the detected associations were consequent on LD with DRB1 alleles different from DRB1*15, we evaluated the relationship of HLA-A*02, Cw*05 and their combinations with the DRB1 locus in a random subset of 562 MS cases and 888 controls fully typed for DRB1 at low resolution. In this subgroup, DRB1*15 was positively MS associated with OR = 2.86 (95% CI = 2.14–3.83; P = 8.4 × 10⁻⁴). When considering DRB1*15-negative individuals, there was an additional significantly positive association with DRB1*04 (OR = 1.88, 95% CI = 1.34–2.63, P = 1.6 × 10⁻⁴) and a negative association with DRB1*07 of borderline significance (OR = 0.69, 95% CI = 0.49–0.98, P = 0.036). At variance with other studies, DRB1*03 was not associated (OR = 1.0, 95% CI = 0.72–1.39). A modest global LD was detected between HLA-A*02 and DRB1 (Global D’ = 0.149, Cramer’s V = 0.176) and between HLA-Cw*05 and DRB1 (Global D’ = 0.280, Cramer’s V = 0.194). The association with A*02 remained significant after conditioning on the DRB1 locus. The joint effect of HLA class I markers involved in MS susceptibility

**Discussion**

This work stems from previous studies undertaken by our group and others, each reporting an MS association with distinct HLA-class I markers, namely HLA-A*02,⁹,¹³ HLA-Cw*05¹¹ and MOG-142L.¹² We here analyze the relationship among these three markers for MS susceptibility.
We confirmed a strong protective effect of HLA-A*02, which was independent of DRB1*015 as well as of any other DRB1 allele. This association has now been detected, with similar ORs, in three populations with a different genetic background, respectively, from Italy (this paper), Sweden and Tasmania based on a total of over 2600 MS patients and 2700 controls. These data concur to demonstrate a role in MS of this allele (or of a variation in LD with it). Conversely, Chao and colleagues failed to observe a transmission distortion of the HLA-A*02 allele in a large family sample from Canada and no SNPs in the HLA-A region showed association with MS independently of HLA-class II loci in Canadian and Finnish families. As both these studies were based on an intrafamilial association method, an obvious explanation of the discrepancy could be a stratification problem related to the case–control approach used in the present as well as the other two studies. However, as a significant HLA-A*02 association was seen in independent studies and in three different populations, this simple explanation seems unlikely and further studies are needed to address this point.

The HLA-A*02 protection remained similar after accounting for the other two analyzed class I markers. Conversely, the protective effect of MOG-142L was secondary to its high linkage disequilibrium with HLA-A*02. This excluded a direct role of the missense V142L variation in the MOG gene, a previously suggested strong MS susceptibility candidate (reviewed in D’Alfonso et al.).

In this study, Cw*05 was not associated with MS either in the whole sample or in DRB1*15-negative individuals. However, when stratifying the results separately for all DRB1 alleles, Cw*05 conferred a significant protection for MS in the subgroup of individuals negative for DRB1*03. This is partially in line with the data reported by Yeo et al., who observed a protective effect of Cw*05 in the whole sample as well as in individuals negative for DRB1*03 and other MS-associated DRB1 alleles (DRB1*15, DRB1*0103). Conversely, in DRB1*03-positive individuals, and in the absence of A*02, Cw*05 was a risk marker and conferred a risk similar to DRB1*15 (OR = 3.89, P = 0.0006). Moreover, Cw*05 significantly enhanced the protection effect of HLA-A*02: the OR conferred by A*02 among Cw*05-positive individuals (OR = 0.22, 95% CI = 0.13–0.38) was about 1/3 smaller than among Cw*05-negative individuals (OR = 0.69, 95% CI = 0.58–0.83). Thus, although Cw*05 itself was not significantly associated to MS, it behaved as a modifier of the HLA-A*02-mediated protection. The protective effect of the Cw*05-A*02 combination was independent of the presence of DRB1*03 (Table 5).

These data suggest that the association of Cw*05 with MS varies according to its haplotypic context. Cw*05 is a marker of a three ancestral or conserved extended HLA haplotypes, one of which carries DRB1*03 and is negative for A*02, namely 18.2 [A*30, Cw*05, B*18, DRB1*03], and the other two carry A*02, but not DRB1*03, namely 44.1 [A*0201, Cw*0501, B*4402, DRB1*0401] and 18.3 [A*0201, Cw*0501, B*1801, DRB1*1102].

The 18.2 HLA-extended haplotype is typical of the Sardinian population and, to a lesser extent, of other Mediterranean populations. Conversely, in the populations of northern European origin, this DR3 haplotype is very rare and the majority of DRB1*03-positive individuals carry the 8.1 [A*1, Cw*07, B*08, DRB1*03] extended haplotype. The positive association of the A*02, Cw*05 +, DRB1*03 + combination observed in this study may reflect the effect of the 18.2 haplotype. It is tempting to speculate that the strong positive association of MS with DRB1*03 observed in Sardinia is related to the 18.2 haplotype and not only to DRB1*03 itself.

As stated above, the A*02-Cw*05 combination is carried by two HLA ancestral haplotypes (44.1 and 18.3). However, as the individuals included in this study were not typed for HLA-B and other HLA markers, from our data it is not possible to conclude whether the enhanced MS-protective effect of the A*02-Cw*05 combination is due to an haplotype effect (that is the presence of a primarily associated protective factor carried by the extended haplotype marked by A*02 and Cw*05) or to a direct interactive role of the two markers.

The evidence in favor of an haplotype effect is as follows: (i) Cw*05 was not associated with MS in the absence of A*02. Actually, Cw*05 behaved as a protective factor among A*02-positive individuals (OR = 0.50, 95% CI 0.33–0.74) and as a risk factor among A*02-negative individuals (OR = 1.53, 95% CI 1.04–2.24). (ii) In family trios, out of 10 haplotypes that carried both A*02 and Cw*05, none were transmitted to the MS patients (T:NT = 0:10; P = 0.002). The only A*02 +, Cw*05 + MS patient present in this panel inherited the two alleles in trans. (iii) Each of the A*02, B*44 and Cw*05 alleles

| DRB1*03+ | A*02 | Cw*05 | Cases N (%) | Controls N (%) | OR | 95% CI | P-value |
|----------|------|-------|-------------|----------------|----|--------|---------|
| – –      | 20 (0.19) | 10 (0.06) | 3.89 | 1.74–8.66 | 0.0006 |
| – –      | 57 (0.54) | 82 (0.46) | 1.34 | 0.83–2.18 | NS    |
| + –      | 5 (0.05) | 26 (0.15) | 0.28 | 0.11–0.77 | 0.006 |
| – +      | 24 (0.23) | 59 (0.33) | 0.59 | 0.33–1.01 | NS    |
| DRB1*03– | – –      | 16 (0.04) | 26 (0.04) | 0.96 | 0.51–1.81 | NS    |
| – –      | 274 (0.60) | 360 (0.51) | 1.46 | 1.15–1.86 | 0.002 |
| + +      | 13 (0.03) | 49 (0.07) | 0.39 | 0.21–0.74 | 0.002 |
| – +      | 153 (0.34) | 276 (0.39) | 0.79 | 0.62–1.01 | NS    |

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio; NS, not significant.
(characterizing the 44.1 ancestral haplotype) was significantly decreased in the study of Yeo et al. However, the association with the A*02-B*44-Cw*05 haplotype combination was not investigated by the authors.

On the other hand, evidence reported in this study and from work undertaken using an experimental animal model points to a direct role of MS pathogenesis, as their encoded genes in this region might be primarily associated with MS. The 1.3 Mb interval within HLA-A and -C genes contains 46 genes (24% of which encoding molecules involved in immune functions) and about 4000 SNPs, according to the genome-wide association study in about 1000 individuals (http://www.hapmap.org/). A genome-wide association study recently reported that the MS-like disease developed by double transgenic mice expressing both a human HLA-class I allele (A*03) and a human myelin-specific autoreactive T-cell receptor is completely prevented by further adding an HLA-A*0201 transgene. Thus, A*02, which protects against MS in human populations, also prevents an MS-like disease in transgenic humanized mice. This protection resulted from thymic deletion of autoreactive T cells, which greatly reduced their number in the periphery.

Both HLA-A and -C genes are interesting candidates for a direct role in MS pathogenesis, as their encoded molecules present antigenic peptides and interact with NK receptors. However, several other genes in this region might be associated with MS. The 1.3 Mb interval within HLA-A and -C genes contains 46 genes (24% of which encoding molecules involved in immune functions) and about 4000 SNPs, according to the recently reported sequence of eight HLA ancestral haplotypes and HapMap data (http://www.hapmap.org/). A genome-wide association study in about 1000 US MS patients and 1000 matched controls identified several MS-associated SNPs in the HLA-class I region after conditioning for HLA-DRB1*15. Among these, the two signals were localized around members of the tripartite motif (TRIM) gene family, mapping about 200 kb centromeric to HLA-A. Although their function is unknown, the presence of a RING domain suggests DNA-binding activity.

In conclusion, this study provides additional supportive evidence indicating that the HLA-class I region does indeed exert an additional influence on the risk of MS, analogous to that reported for other autoimmune diseases. Moreover, it identifies haplospecific markers conferring a high MS protection. Notably, although in general DRB1*15-negative individuals have an about threefold lower MS risk relative to DRB1*15-positive individuals, their risk was significantly decreased (about eightfold) if they also carried A*02 and Cw*05 (Figure 1). The highly protective A*02-Cw*05 combination is rare (0.078 phenotypic frequency in our controls, 0.025 haplotype frequency in our family trios) and, therefore, unless it tags a primary factor with higher frequency, it confers a modest modification of the HLA attributable MS risk in the population. In any case, the identification of the mechanism mediating the protective effect might throw new light on MS pathogenesis.

Materials and methods

Subjects
A total of 1273 Italian MS patients (female:male ratio 2:1) diagnosed according to McDonald et al., were genotyped for the three HLA-class I markers considered in this study and for DRB1*15. For 201 of these, a DNA sample of the parents was also available (family trios). The mean age of the MS patients at disease onset was 31 ± 10.16 years, the mean age at time of analysis was 40 ± 15.52 years and the mean disease duration was 12 ± 9.31 years. Eighty-four percent of the patients were affected by the relapsing remitting, 7% by the secondary progressive and 9% by the primary progressive form of the disease, defined according to Lublin and Reingold. MS patients with Sardinian ancestors were excluded to avoid the introduction of confounding sources of heterogeneity. Enrolment of the MS patients followed their informed consent. The study was approved by the Ethical Committees of the collaborating clinical centers.

Controls included 1075 Italian individuals (medical students, university and hospital staff, blood donors; female:male ratio 1:1) matched for age and regional origin with the MS patients and also typed for all considered markers.

MS patients (82%) and controls (30%) in part overlap with those included in a previous paper and were selected for inclusion on the basis of the availability of DNA and of HLA genotypes.

MOG-V142L typing

The newly included samples were typed using a pre-designed TaqMan SNP Genotyping Assay (probe code: C_25474376_10). Reactions were performed according to the manufacturer’s protocol using 25 ng of DNA. The PCR reaction was set up on a 7000 Applied Biosystems instrument. Genotypes were detected using the 7000 System Software (Applied Biosystems, Foster City, CA, USA).

A sub-sample of 60 individuals were typed both with this method and with the method used in the previous paper. The results were consistent for all tested samples.

HLA-A*02 typing

HLA-A exon 2 was amplified using a specific couple of primers (Forward: 5'CGACGCCGCCGACGCCAGARTGAT3', Reverse: 5'GGGCGCTCCGTGGGGGATGA3'). The PCR product (213 bp) was digested using the restriction enzyme KpnI. This enzyme recognizes and cuts the rs3173427-T sequence that is specific of HLA-A*02. This approach, it was possible to distinguish HLA-A*02 homozygotes (displaying two fragments of 159 and 54 bp) from heterozygotes (displaying three fragments of 159, 54 and 213 bp). The digestion was performed at 55 °C for 4 h, and then the enzyme was inactivated at 80 °C for 20 min.

HLA-A*02-positive individuals of family trios were also analyzed by sequencing exon 2 and exon 3 to define HLA-A*02 alleles at a higher resolution. Exon 2 and 3 were amplified together in the same fragment (Forward: 5'CGACGCCGCCGACGCCAGARTGAT3', Reverse: 5'AA CGGGAAGGAAGCGCTGC-3'). The reaction mix was performed using 0.02 U µl-1 TaqAB, 0.2 pmol µl-1 of every primers, 1.75 mM MgCl2 and glycerol to 7.4%. PCR was made at 60 °C annealing temperature for 35 cycles. PCR products were sequenced using nested primers: 5'-GGGCGCTCCGTGGGGGATGA3' for exon 2 and 5'-TCAGTTTAGGCCAAAATCC-3' for exon 3. Sequences were analyzed with the automatic sequencer Applied Biosystems (ABI) 3100.
All the samples were typed for HLA-Cw*05 by an allele-specific PCR after the conditions of the 12th International Histocompatibility Workshop. In detail, specific primer pairs were used to amplify in the same tube HLA-homozygous typing cell lines from the reference panel. The genotype methods used to type A*02, Cw*05 and DRB1*15 were already available.

For 562 MS cases and 888 controls, a complete low-resolution DRB1 typing was already available. DRB1 alleles were typed by the DR low-resolution PCR-SSP (Sequence Specific Primer amplification) kit (Dynal or B&G, Formedic, Milan, Italy).

The remaining MS patients and controls were typed only for DRB1*15 by an allele-specific PCR (Forward primer: 5’-TCCTGAGCCAGCTTACAGG-3’, Reverse primer: 5’-CGGCCCCTGCTTACAGGA-3’) with an internal control fragment (Forward: 5’-TGTTCTGTATTTGTGTTG-3’, Reverse: 5’-GCATCTTGCTCTGTGCA-3’). The reaction mix contained 0.02 U μl⁻¹ TaqAB (AB Analitica), 1 pmol μl⁻¹ of HLA-Cw*05-specific primers, 0.33 pmol μl⁻¹ of internal control primers and 2 mM of MgCl₂. By this approach, it was possible to specifically identify all the samples positive for HLA-Cw*05, but not to distinguish between Cw*05 homozygotes and heterozygotes.

The genotype methods used to type A*02, Cw*05 and DRB1*15 alleles were validated by typing 51 HLA homozygous typing cell lines from the reference panel of the 12th International Histocompatibility Workshop and 55 individuals previously typed with a commercial kit.

Unconditional logistical regression was carried out to determine the effect of the considered markers on MS susceptibility. The association of each polymorphism with the disease was measured by the OR and its 95% CI. Reported P-values were not corrected for the number of comparisons.

The potential confounding variables were assessed individually by comparing the log-likelihood ratios derived from a model with and without the variable. This analysis was set up using multivariate models using the four considered markers. All analyses were adjusted for sex. The different models were compared by the likelihood ratio test.

The interaction (modification) effect was assessed by comparing ORs across levels of potential modifying variables. Inclusion of appropriate interaction terms in the logistic regression model was used to assess the statistical significance of the interactions. For each marker, the potential effect modification by sex variable was also tested.

The main-effects test of the COCAPHASE program, part of the UNPHASED suite, was used for conditional analysis on DRB1. This program provides association tests conditioning on additional loci, which may already be associated and in linkage disequilibrium with the test loci. The EM algorithm is used to obtain maximum-likelihood estimates of haplotypes.

LD were calculated from phenotypes according to Mattiuz et al. Estimates for Global D’ and Cramer’s V (measures of LD between multiallelic loci) were calculated using the COCAPHASE program.

The authors declare no conflict of interest.

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Conflict of interest

Acknowledgements

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