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**Association of HLA class I markers with Multiple Sclerosis in the Italian and UK population:  
evidence of two independent protective effects**

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## ABSTRACT

**Background:** We recently confirmed the association with Multiple Sclerosis (MS) of *HLA-A\*02* and observed that the combined presence of *HLA-Cw\*05* significantly enhanced (3-fold) the protective effect of *HLA A\*02*.

**Objectives and Methods:** Since *A\*02-Cw\*05* is carried by two HLA extended haplotypes characterized by the *B\*4402* and *B\*1801* alleles, respectively, we extended the association analysis to *HLA-B\*44* and *-B\*18* in our Italian sample (1445 MS cases and 973 controls) and verified these associations in a UK cohort (721 MS cases, 408 controls and 480 family trios).

**Results:** A strong protective effect, independent of *DR15*, of the *A\*02-Cw\*05* combination carrying *B\*44* (OR=0.27  $p=3.3 \times 10^{-5}$ ), was seen in the Italian samples and confirmed in UK family trios (OR=0.33  $p=5.5 \times 10^{-4}$ ) and in a combined cohort of UK families and case/controls (OR=0.53  $p=0.044$ ). This protective effect was significantly stronger than that mediated by *A\*02* alone. Logistic regression showed that *A\*02-Cw\*05* maintained a significant protection when adjusted for B alleles (Italy: OR=0.38  $p=6.5 \times 10^{-7}$ ; UK: OR=0.60  $p=0.0029$ ) indicating that it was not secondary to linkage disequilibrium with *B\*44*. At difference from *A\*02*, the other HLA class I tested markers individually showed no significant (*Cw\*05*, *B\*18*) or a modest (*B\*44*) protection when adjusted for the remaining markers.

**Conclusions:** We identified at least two independent protective effects which are tagged by *A\*02-Cw\*05* and *A\*02*, respectively. Further studies are needed to elucidate whether this protective effect is due to the presence of an unanalyzed factor characterizing the HLA extended haplotype/s carrying *A\*02* and *Cw\*05* or to a direct interaction between these alleles.

## INTRODUCTION

Multiple Sclerosis (MS) is a complex disease characterized by inflammatory *foci* in the central nervous system (CNS) causing a progressive demyelination of axons and neuronal cell degeneration, resulting in a severe disabling condition.

The pathogenesis of MS is not well understood, but it is well established that both genetic and environmental factors contribute together to the development of the disease.

Association with the human leukocyte antigen (HLA) genes from the major histocompatibility complex on chromosome 6p21.3 was identified almost 40 years ago. It is well established that the *HLA-DRB1\*1501* allele, tagging the *DRB1\*1501-DQB1\*0602* (also known as DR15) haplotype in the HLA class II region, is the strongest genetic risk factor conferring an OR of approximately 3 in most studied populations. [1-4]

Through careful analysis of this complex region, which is characterized by an extreme polymorphism and extensive linkage disequilibrium, evidence for an independent effect on risk from the class I region has emerged. In 2007, IMSGC presented data from a large study based on UK and North American Caucasians suggesting that this signal might be determined by the *HLA Cw\*05* allele. The *HLA Cw\*05* allele was less frequent in patients than controls conferring a protective effect.[5] At the same time, two other groups identified an association between the *HLA A\*02* allele and MS with a similar OR in two populations (Swedish and Tasmanians) with a different genetic background. This allele, like *HLA Cw\*05*, conferred a protective effect.[6, 7]

We recently tested the association of both *HLA A\*02* and *HLA Cw\*05* in an Italian cohort of 1273 MS patients and 1075 controls.[8] Our analyses confirmed the protective effect of *HLA A\*02* whereas *HLA Cw\*05* did not present any independent association. However, the presence of *HLA Cw\*05* appeared to significantly enhance the protective effect due to *HLA A\*02* resulting in a 3 fold decreased OR for the *HLA A\*02-Cw\*05* combination (OR = 0.22, 95% CI: 0.13-0.38) relative to the presence of *HLA A\*02* alone (OR = 0.69, 95% CI: 0.58-0.83). In the Caucasian population, the *HLA A\*02-Cw\*05* combination is carried by two HLA extended haplotypes, namely *HLA A\*0201-Cw\*0501-B\*4402-DRB1\*0401* and *HLA A\*0201-Cw\*0501-B\*1801-DRB1\*1102* (also known as 44.1 and 18.3 haplotypes, respectively, [9-11]). These two haplotypes are present in the Italian population with approximately the same frequency (Rendine, unpublished). Thus it is possible that the strong protective effect we detected for the *A\*02-Cw\*05* combination is due to a haplotypic effect, i.e. to the presence of a primarily associated factor carried by one or both the above haplotypes. Interestingly *HLA B\*4402* was recently identified as an MS protective factor.[12, 13, 14]

In order to better understand whether the enhanced protective effect of the *HLA A\*02-Cw\*05* combination is due to a haplotypic effect or a direct interactive role of the two markers as well as test the possible role of *B\*44*, in the present study we extended the analysis in our Italian cohort to the involved *HLA-B* alleles, namely *HLA B\*44* (and its subtypes *B\*4402* and *B\*4403*) and *B\*18*. The association with MS of the various haplotypic combinations of the *A\*02-Cw\*05* set with or without *B\*44* or *B\*18* was also analysed in a UK cohort fully typed for HLA-DR, -A, -B and -C, in order to verify our results in another population with a possibly different genetic background.

## **MATERIALS AND METHODS**

### **Subjects**

A total of 1445 Italian MS patients and 973 controls were analyzed for *HLA-A\*02*, *-Cw\*05*, *-B\*18* and *-B\*44* class I markers and for *DRB1\*15*. Of these, 1433 patients and 950 controls were also typed for *B\*44* subtypes (4402 and 4403). The majority of the samples were recruited from North-West Italy and the remaining from Central Italy. Patients with Sardinian ancestors were excluded to avoid the introduction of a confounding source of heterogeneity.

Italian controls included medical students, university and hospital staff, blood donors (female:male ratio 1:1.1) matched for age and regional origin with the MS patients.

Patients (83%) and controls (91%) mostly overlap with those included in our previous paper [8] and were already typed for *HLA-A\*02*, *Cw\*05* and *DRB1\*15*. Selection for inclusion was based on DNA availability.

We also analysed a UK data set composed of 721 MS patients, 408 healthy matched controls and 480 family trios. These samples were described in detail elsewhere.[5]

Patients were diagnosed according to McDonald et al. criteria.[15] All the samples, both Italian and British, were of European ancestry.

Features of the analyzed samples are shown in Table 1. All the samples were collected after informed consent and appropriate ethical approvals.

### **HLA typing of Italian samples**

HLA typing was performed using specific reactions in order to obtain information about the four HLA class I markers (*HLA-A\*02*, *-Cw\*05*, *-B\*44* and *-B\*18*) and *DRB1\*15*.

The methods used to analyze *HLA-A\*02*, *HLA-Cw\*05* and *DRB\*15* were described in detail elsewhere.[8]

*HLA B\*44* and *HLA B\*18* were analyzed by two distinct allele specific PCR reactions following the conditions of the 12<sup>th</sup> International Histocompatibility Workshop.[16] Specific primer pairs were used to amplify *HLA B\*44* (Forward: 5'- TACCGAGAGAACCTGCGC -3', Reverse: 5'- CCAGGTATCTGCGGAGCG -3', producing a fragment of 541 bp) and a 250 bp internal control fragment (Forward: 5'- ATGATGTTGACCTTTCCAGGG -3', Reverse: 5'- ATTCTGTAACCTTTTCATCAGTTGC -3'). The reaction mix contained 0.027 U/μl GoTaq (Promega), 0.83 pmol/μl of *HLA-B\*44* specific primers, 0.33 pmol/μl of internal control primers, 2 mM of MgCl<sub>2</sub> and 0.2 mM dNTPs. The *B\*44* subtypes *B\*4402* and *B\*4403* were typed by two distinct nested allele specific PCR reactions, utilizing an *HLA B\*4402* specific (5'- CTCCAGGTAGGCTCTGTC -3') and an *HLA B\*4403* specific (5'- CTCCAGGTAGGCTCTCAC -3') reverse primer respectively. The same forward primer and the same internal control as *HLA B\*44* was used in both reactions. The specific product size was 507 bp. Both reactions were performed with the same reaction mix, containing 0.025 U/μl GoTaq (Promega), 0.83 pmol/μl of *HLA-B\*4402/HLA-B\*4403* specific primers, 0.33 pmol/μl of internal control primers, 2 mM of MgCl<sub>2</sub> and 0.2 mM dNTPs. The *HLA B\*44* PCR product, at a 1:100 dilution factor, was used as template. *HLA B\*18* typing was performed by specific primers which allowed to amplify only the *HLA B\*18* allele (Forward: 5'- TACCGAGAGAACCTGCGC -3', Reverse: 5'- CCAGGTATCTGCGGAGCG -3', producing a fragment of 490 bp) with the same internal control as *HLA B\*44*. The reaction mix contained 0.027 U/μl GoTaq (Promega), 1 pmol/μl of *HLA B\*18* specific primers, 1 pmol/μl of internal control primers, 2 mM of MgCl<sub>2</sub> and 0.2 mM dNTPs. All these protocols, with the exception of *A\*02*, did not allow to distinguish between homozygotes and heterozygotes but only whether the samples were positive or negative for the tested markers. These methods were validated testing 45 samples previously typed with standard protocols. The results were consistent for all tested samples.

### **HLA class I and class II typing of UK samples**

All the UK samples had been previously completely typed for the *HLA-A*, *-C*, *-B* and *-DRB1* loci as described in detail elsewhere. [5]

The trio families were genotyped with a medium resolution method for all HLA class I (two-digit) and class II (four-digit) alleles. In particular for the *HLA-B* locus, all the family members were typed for both *HLA-B\*12* subtypes, namely *B\*44* and *B\*45*. The case/control samples were analyzed with a low resolution method for all class I and class II alleles. This method did not allow discrimination between the two *HLA-B\*12* subtypes. However, since *B\*44* is by far the most frequent *B\*12* subtype in the British population (0.185 versus 0.006 in the non transmitted

haplotypes of the 480 family trios) and is in strong linkage disequilibrium with *Cw\*05* ( $D'=0.85$ ,  $r^2=0.66$ , Fig.1), it is likely that most *B\*12* positive and practically all *B\*12-Cw\*05* positive individuals were *B\*44* positive.

### Statistical analysis

The association of each marker, haplotype or phenotype combination with the disease was measured by  $\chi^2$  test, the Odds Ratio (OR) and its 95% Confidence Intervals (95% CI). Allele and haplotype analyses of UK samples were performed with the UNPHASED v 3.0.13 program using the “certain haplotype only” option for family trios and the “uncertain haplotypes” option for the case/control cohort.[17]

Linkage disequilibria (LD) were calculated from phenotypes according to Mattiuz et al. [18].

Logistic 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 Odds Ratio (OR) and its 95% Confidence Intervals (95% CI). We used likelihood ratio tests (LRT) to assess the statistical significance of the models and to compare the models. The contribution of each variable was evaluated by comparing the LRTs derived from a model with and without the variable. Reported p values were not corrected for the number of comparisons.

## RESULTS

### Case/Control association analysis in the Italian population.

A total of 1445 MS patients and 973 controls, previously typed for *HLA-DRB1\*15*, *A\*02* and *Cw\*05*, were further typed for *B\*18* and *B\*44*. A schematic map of the HLA genes analysed in this study is shown in Figure 1 together with the pairwise linkage disequilibria (LD) between the tested alleles. The association with MS of the different phenotypic combinations carrying both *HLA-A\*02* and *HLA-Cw\*05* (*A\*02-Cw\*05*) with or without *B\*18* and *B\*44* was then analysed (Table 2). The complete results including all the other possible combinations of *HLA-A*, *-B*, *-C* tested alleles are shown in the Supplementary Table 1.

*A\*02-Cw\*05* conferred a strong protection particularly in the presence of *B\*44* (OR = 0.28;  $p = 4.1 \times 10^{-6}$ ) but also of *B\*18* (OR = 0.41;  $p = 0.004$ ). Conversely, in their absence, the protection conferred by *A\*02-Cw\*05* was weaker and not statistically significant, although, owing to the low frequency of this combination, this analysis had only limited power ( $\alpha = 0.05$ ;  $\beta = 0.21$ ) and cannot be considered informative. The OR values for the different phenotypic combinations in the whole population and in DR15 negative individuals were substantially the same (Table 2).



### **Association analysis in UK family trios**

To confirm and extend the results obtained in the Italian case/control study, we analysed the association with MS of *A\*02-Cw\*05* with or without *B\*44* or *B\*18* in a panel of 480 family trios from the UK population fully typed for *HLA-DR*, *-A*, *-B* and *-C* at a medium resolution. This analysis would allow us to a) test the association of “certain” haplotypes, deduced by family segregation, and b) verify the results in a population with a different genetic background, i.e. different haplotype frequencies.

To eliminate the possible confounding effect of linkage disequilibrium (LD) with *HLA-DRB1\*15*, we considered the transmission of *DRB1\*15*-negative haplotypes only (Table 3). Pairwise LD values between *DRB1\*15* and the tested HLA Class I markers in the UK population are shown in Figure 1.

The large majority of haplotypes carrying both *A\*02* and *Cw\*05* were *B\*44* positive and conferred a strong protection (OR = 0.36;  $p = 5.5 \times 10^{-4}$ ), comparable to that seen in the Italian population. The association with the *A\*02-Cw\*05-B\*44* haplotype remained significant ( $p=0.0057$ ) after conditioning on the *DRB1* locus by the UNPHASED program, main effect option.

The *A\*02-Cw\*05-B\*18* and *A\*02-Cw\*05-B\*44/B\*18neg* haplotypes were very rare in the UK population and would require testing of a much larger panel of families to yield informative results. The complete results including all the other possible combinations of *HLA-A\*02*, *-B\*44*, *-B\*18* and *-Cw\*05* alleles are shown in the Supplementary Table 2.

### **Dissection of the protective effect of the *A\*02-Cw\*05* haplotypes**

To dissect the effect of the different components of the *A\*02-Cw\*05* protective combinations both in the Italian and UK samples we a) performed a logistic regression analysis and b) tested the association of all the possible combinations of the *A\*02*, *Cw\*05* and *B\** alleles. The same procedure was applied to the analysis both of Italian phenotypes and UK haplotypes.

a) For the logistic regression analysis (Table 4) all Italian individuals and UK haplotypes were categorized according to the presence or absence of each of the considered alleles (*A\*02*, *Cw\*05*, *B\*18*, *B\*44*) and also of the *A\*02-Cw\*05* combination. *DRB1\*15* was always included in the model. To increase the power of the UK study, haplotype frequencies deduced from an additional case-control dataset of the UK population were combined with those of family trios, with a total of 2106 case and 1676 control haplotypes (see note to Table 4) fully typed for *HLA-DR*, *-A*, *-B* and *-C*. This second UK database was typed for *B\*12* instead of *B\*12* subtypes, *B\*44* and *B\*45* (see Materials and Methods).

In the Italian sample, the significant protection conferred by *A\*02* (OR=0.63; CI95% 0.53-0.74) and by the *A\*02-Cw\*05* combination (OR=0.39; CI95% 0.27-0.57) remained similar after accounting for *B\*44* and *B\*18*. In addition we observed a significant protective effect for *B\*44* (OR=0.70; CI95% 0.56-0.88) that was not substantially modified by adjustment for the other markers included in the model. No significant effect was detected for *B\*18* and for *Cw\*05*. The contribution to the model of *A\*02* and of *A\*02-Cw\*05* relative to *B\*44*, as calculated by the Likelihood Ratio Test (LRT), was highly significant ( $p=1.3 \times 10^{-7}$  and  $p=7.3 \times 10^{-6}$  respectively). As for *B\*44*, its contribution was still significant but weaker relative to the effect of *A\*02* ( $p=0.005$ ) and borderline relative to *A\*02-Cw\*05* ( $p=0.050$ ).

In the UK sample we replicated the protective effect of *A\*02* (OR=0.72; CI95% 0.62-0.83) and of *A\*02-Cw\*05* (OR=0.56; CI95% 0.42-0.76) which remained similar after accounting for the other markers. *A\*02* showed a significant contribution to the model relative to *Cw\*05* ( $p=0.0003$ ) and *B\*12* ( $p=0.00013$ ), while, at difference from Italians, the *A\*02-Cw\*05* combination yielded only a modest ( $p=0.0272$ ) contribution relative to *B\*12*. *B\*12*, in its turn, showed a significant protection (OR=0.70; CI95% 0.58-0.85) which was not modified by adjustment for *A\*02* but whose contribution was no longer significant after accounting for *Cw\*05* ( $p=0.06$ ). The contribution of *B\*12* to MS protection maintained a borderline effect relative to *A\*02-Cw\*05* ( $p=0.047$ ), similarly to what seen in the Italian sample. The protection of *Cw\*05* was statistically significant, at difference from the Italians, but it was no longer significant after adjustment for *B\*12* ( $p=0.06$ ) or both *B\*12* and *A\*02* ( $p=0.21$ ). No protective effect was observed for *B\*18*.

To elucidate whether the effect of *A\*02-Cw\*05* was distinct from that of *A\*02* alone, we analysed the odds ratio of the *A\*02+Cw\*05+* vs the *A\*02+Cw\*05-* combination. The combined presence of *A\*02* and *Cw\*05* was significantly more protective than *A\*02* without *Cw\*05* in both populations (Italy: OR = 0.49, 95% CI = 0.34-0.73,  $p=0.0004$ ; UK: OR = 0.69, 95% CI = 0.50-0.95,  $p = 0.023$ ), after adjustment for *DRB\*1501*. When adjusting also for *B\*44/12*, the protection contributed by the *A\*02+Cw\*05+* combination remained significantly higher than that of *A\*02* without *Cw\*05* in the Italian population (OR = 0.55, 95% CI = 0.37-0.82,  $p = 0.003$ ) while it was no longer significant in UK ( $p = 0.3$ ).

The above results indicate the presence of three distinct protective effects, namely *A\*02-Cw\*05*, *A\*02* and *B\*44* with a decreasing protective strength. While this is evident in Italians, in the UK population only two effects are clearly detectable since it is not possible to distinguish the effect of *B\*12* from that of *A\*02-Cw\*05*. This is likely explained by the fact that most (>90%) *A\*02-Cw\*05* haplotypes carry *B\*44* in the British as against about 50% in the Italian population.

b) In order to better understand the effect of the different allele combinations we tested their association in DR15 negative Italian (1005 MS cases and 848 controls) and UK (261 MS patients, 293 controls and 131 DR15 negative families) samples. The complete results including all the possible combinations of *HLA-A\*02*, *-B\*44*, *-B\*18* and *-Cw\*05* alleles of the Italian and British data sets are shown in the Supplementary Tables 1, 2, 3 and 4.

A comparison of the results of the various tested populations and data sets is summarized in Table 5. *HLA-B\*18* was not included because in the British population some of its combinations with *A\*02* and *Cw\*05* were very rare and none was significantly associated to MS (Table 3 and Supplementary Tables 2, 3, 4) and in both populations the logistic regression analysis ruled out a protective role of this marker (Table 4).

The main conclusions that can be drawn from Table 5 are the following:

- The *A\*02-Cw\*05-B\*44/12* combination conferred a significant protection in both populations. Actually, this was the only allele combination consistently associated in both populations and in all the tested panels.

- A significant protection was also observed for the *Cw\*05-B\*12* combination in the absence of *A\*02*, but only in the UK case/control (OR = 0.31, 95% CI: 0.10-0.81, p = 0.0324, Supplementary Table 3) and combined haplotype (OR = 0.37, p = 0.0087) panels.

- The *A\*02-Cw\*05* combination in the absence of *B\*44* was significantly protective only in the Italian population (OR = 0.36, p = 0.007). This haplotypic combination was practically absent (<1%) in the British population (supplementary Tables 2, 3, 4).

- In both populations *A\*02* also conferred a significant protection in the absence of *Cw\*05* and *B\*44*. The OR conferred by *A\*02* was significantly different from that of the *A\*02-Cw\*05-B\*44* combination in the DR15 negative Italian dataset (p= 0.002) and in the UK family trios (p= 0.007) suggesting the presence of two distinct protective effects, while **this difference was not statistically different in the UK case- control cohort.**

- ***B\*44* in the absence of *A\*02-Cw\*05* consistently showed OR values <1 although, at difference from the logistic regression results, the lower number of included samples (only those DR15 negative) did not allow to reach statistical significance.** In the Italian samples it was possible to test the association with MS also of the two *B\*44* subtypes (*B\*4402* and *B\*4403*), (Supplementary Table 1). The two subtypes were differently distributed according to the presence or absence of *Cw\*05*: about 86% of *Cw\*05* positive as against about 35% of *Cw\*05* negative individuals were *B\*4402*, similarly in patients and in controls. In the absence of *A\*02-Cw\*05* neither of the two *B\*44* subtypes was significantly associated with MS (Supplementary Table 1).

- *Cw\*05*, in the absence of *A\*02* and *B\*44*, was not significantly protective in any of the tested data sets. On the contrary, in the Italian population it behaved as a strong risk factor, confirming our previous results.[8]

## DISCUSSION

This work stems from our previous study which found that the combination of two HLA class I alleles, *HLA-A\*02* and *-Cw\*05*, conferred a significantly stronger protective effect towards MS than *A\*02* alone.[8] The *A\*02-Cw\*05* combination is carried by two HLA extended haplotypes, namely 44.1 [*HLA A\*0201-Cw\*0501-B\*4402-DRB1\*0401*] and 18.3 [*HLA A\*0201-Cw\*0501-B\*1801-DRB1\*1102*], respectively. Our result was particularly intriguing since a strong protective association with the *B\*4402* allele characterizing the 44.1 haplotype was recently reported in three studies.[12, 13, 14] Several interpretations of these results were possible: i) the stronger protection we detected for *A\*02 -Cw\*05* was secondary to linkage disequilibrium with *B\*4402*; ii) a primarily involved protective factor is carried by the 44.1 haplotype; iii) the presence of an epistatic interaction (i.e. an interaction between 2 alleles (*in cis* or *trans*) in which the risk associated with a particular allele depends on the presence or absence of a second allele,[19]) between *A\*02* and *Cw\*05* and/or *B\*4402*.

To answer to these questions we evaluated the association of the above involved HLA markers in two genetically different populations. Specifically, in the UK population the *A\*02-Cw\*05* positive combination is carried mostly by the *A\*02-Cw\*05-B\*44* haplotype (91% of *A\*02-Cw\*05* control haplotypes) while, the Italian *A\*02-Cw\*05* positive individuals frequently carry this combination also with *B\*18* (41%) or in the absence of both B markers (8%).

The typing strategy utilized for the Italian sample did not distinguish homozygotes from heterozygotes, making it impossible to deduce the haplotypic combinations of the tested alleles. Therefore the detected associations were with phenotypic rather than haplotypic allele combinations and it was not possible to determine whether the involved alleles were carried in *cis* or in *trans*. The second population compensated for this limitation, since in the UK samples it was possible to determine the allele phase, either by family segregation (trios) or by computer algorithms (case-control).

Association analysis in the Italian population showed a very strong protective effect of the *A\*02-Cw\*05-B\*44* phenotypic combination, independently of *DRB1\*15*. The protective effect of the *A\*02-Cw\*05-B\*44* haplotype was replicated in British family trios and shown to be independent of *DRB1* locus. In this dataset the *A\*02-Cw\*05-B\*44* haplotype conferred a protection similar to the effect observed for the phenotypic allele combination in Italy. In both populations the protection

was significantly higher than that of *A\*02* alone, *i.e.* in the absence of *Cw\*05* and *B\*44* ( $p = 0.002$  and  $0.007$  respectively in Italy and UK trios), indicating two distinct protective effects.

The logistic regression analysis showed that the *A\*02-Cw\*05* combination maintained a significant protective effect in both populations when adjusting for the B locus indicating that this effect was not secondary to linkage disequilibrium with *B\*44*. Moreover, in the Italian population *A\*02-Cw\*05* maintained a significant protective effect also in the absence of *B\*44*, suggesting that this effect was not restricted to the *A\*02-Cw\*05-B\*44* combination. This result cannot be replicated in the UK sample though, since the *A\*02-Cw\*05* combination without *B\*44* is virtually absent in this population. Finally, *A\*02-Cw\*05* was significantly more protective than *A\*02* without *Cw\*05* in both populations.

In both populations *B\*44* showed significant protection independent of the *A\*02* effect, paralleling the result recently published in a US sample. However, *B\*44* maintained only a borderline effect relative to *A\*02-Cw\*05*, indicating that most of its protection is mediated by the haplotype. The recent studies [12,13,14] reporting the protection of *B\*44* did not evaluate the effect of the *A\*02-Cw\*05* haplotype. Since two of the above studies [12, 13] included a large overlap of individuals from UK where the majority of *A\*02-Cw\*05* haplotypes also carry the *B\*44* allele, it is possible that their data indicate the same effect observed in the present paper. These results highlight the importance of testing the association of allele combinations in addition to single alleles within the HLA region as well as highlight the usefulness of testing populations with different haplotype frequencies to dissect the associated alleles.

The protective effect of *Cw\*05* evident in the UK population was not replicated in the Italian sample and was not distinguishable from that of *B\*44* because the LD between these two markers is particularly high in the UK population (Figure 1). While our results clearly show that the *A\*02-Cw\*05* combination maintains a protective effect independently of the tested B alleles, we were not able to elucidate whether this protective effect was due to the presence of a non analyzed factor characterizing the HLA extended haplotype/s carrying *A\*02* and *Cw\*05* (haplotypic effect) or to a direct interaction between the two alleles. There is a preliminary indication in favour of the “haplotypic effect” hypothesis derived from the observation that the *Cw\*05-B\*44* haplotype not carrying *A\*02* still maintains a significant protective effect. This result would be against the hypothesis of an *A\*02/Cw\*05* interaction and indicates the presence of a protective factor in the region of the haplotype closer to *HLA-B* and *HLA-C* loci. However, this information mainly comes from the UK case/control data set where the haplotypic combinations are not certain (ascertained through family segregation) but are deduced by an algorithm.

Further studies are needed to definitively demonstrate which of the two hypotheses is correct. A direct cooperation between A\*02 and Cw\*05 molecules in MS protection remains an attractive hypothesis since they present antigenic peptides and interact with NK receptors.[20, 21]. Evidence in other autoimmune or infective diseases indicate that molecules encoded by different HLA loci might cooperate in the disease mechanism.[19, 22, 23] A direct functional effect of A\*02 is suggested by recent results showing that A\*02 prevents an MS-like disease in transgenic humanized mice.[24]

In conclusion, this study contributes to the demonstration that the HLA class I region contains additional factors modulating MS susceptibility, independently of the well established *DRB1* locus. At least two independent protective effects are proposed which are tagged by the A\*02-Cw\*05 combination and the A\*02 allele, respectively. The protective effect of the A\*02-Cw\*05-B\*44 haplotype has a strength comparable to that of *DRB1\*15*, which is the strongest MS risk factor, thus behaving as an important modulator of MS susceptibility. The A\*02-Cw\*05-B\*44 combination is relatively rare in both tested populations (4.6% and 15.0% of *DRB1* negative Italian and British controls respectively). However, it is likely that its highly protective effect is genuine, and not a spurious signal due to its low frequency or to stratification, since it was replicated in two independent samples belonging to two populations with a different genetic background.

The identification of the primarily associated factor/s requires an extended analysis of the involved haplotype/s in populations with different genetic background. This will help to elucidate the mechanism mediating the protective effect and hence to shed new light on MS pathogenesis.

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## FIGURE LEGEND

Figure 1

Map of the HLA alleles analysed in this study and their linkage disequilibrium relationship.

Pairwise  $D'$ ,  $r^2$  and p values were calculated according to Mattiuz et al. [18] in the Italian (IT) and UK controls. Significant values are in boldface.

Regarding  $B^*44/B^*12$ , results are referred to  $B^*44$  for the Italians and  $B^*12$  for the UK .

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**Competing Interest :**

"None to declare"



Table 1: Characteristics of the patients

Origin	N	Age at Diagnosis	Female:Male Ratio	Disease Course (%)			
				RR	SP	PP	PR
Italy	1445	31±10.5	2:1	89	11	7	1
UK	1201 <sup>a</sup>	35.6±9.8	2.8:1	57	34	9	0

<sup>a</sup> of whom 480 with both parents

RR: Relapsing Remitting; SP: Secondary Progressive; PP: Primary Progressive; PR: Progressive Relapsing.

Table 2: Combinations of the “*HLA-A\*02- HLA-Cw\*05*” phenotype with *HLA-B* in Italian MS patients and controls

HLA phenotype <sup>a</sup>		Sample	Frequency <sup>b</sup>		OR (95%CI)	P value <sup>c</sup>
<i>A*02 - Cw*05</i>	<i>B*<sup>d</sup></i>		MS patients	Controls		
		total	N = 1445	N = 973		
+	-		0.008	0.013	0.59 (0.24-1.41)	0.317
+	44		0.013	0.044	0.28 (0.15-0.50)	4.1x10 <sup>-6</sup>
+	18		0.014	0.032	0.41 (0.22-0.75)	0.0040
all other combinations			0.966	0.917	1 <sup>e</sup>	2x10 <sup>-7</sup>
		DR15 negative	N = 1005	N = 848		
+	-		0.007	0.012	0.56 (0.19-1.59)	0.400
+	44		0.013	0.046	0.27 (0.13-0.52)	3.3x10 <sup>-5</sup>
+	18		0.013	0.037	0.33 (0.16-0.67)	0.0015
all other combinations			0.968	0.913	1 <sup>e</sup>	5x10 <sup>-7</sup>

<sup>a</sup> Presence of the indicated alleles

<sup>b</sup> Frequency of individuals carrying the indicated alleles

<sup>c</sup> Calculated from 2x2 tables versus all the other possible allele combinations

<sup>d</sup> *HLA-B* was considered as a triallelic locus including *HLA-B\*44*, *HLA-B\*18* or none of them. The *A\*02 - Cw\*05* individuals positive for both *B\*44* and *B\*18* were counted twice (cases N=2, controls N=6 in the total samples; cases N=1, controls N=6 in DR15 negative samples).

<sup>e</sup> Reference for OR calculation.

Table 3: Combinations of the “*HLA-A\*02- HLA-Cw\*05*” haplotype with *HLA-B* in UK family trios

HLA Haplotype <sup>a</sup>		Frequency		OR (95% CI)	p <sup>b</sup>
<i>A*02 - Cw*05</i>	<i>B*<sup>c</sup></i>	Transmitted N = 531 <sup>d</sup>	Non transmitted N = 720 <sup>d</sup>		
+	-	0.0018	0.0041	0.43 (0.01-5.40)	0.641
+	44	0.028	0.075	0.36 (0.19-0.67)	5.5x10 <sup>-4</sup>
+	18	0.0094	0.0028	3.24 (0.53-34.12)	0.142
all other combinations		0.960	0.918	1 <sup>e</sup>	0.0036

<sup>a</sup> Haplotypes were calculated with the UNPHASED v 3.0.13 program using the “certain haplotype only” option.[17] All typing information, including DR, were utilised to deduce haplotypes.

<sup>b</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

<sup>c</sup> *HLA-B* was considered as a triallelic locus including *HLA-B\*44*, *HLA-B\*18* or none of them.

<sup>d</sup> *HLA-DRB1\*15* negative haplotypes from 480 families.

<sup>e</sup> Reference for OR calculation.



**Table 4 Logistic regression analysis in the Italian and UK population**

		OR (95% CI) adjusted values for:							
		<i>DRBI*15</i>	<i>DRBI*15</i> and:						
Population	Markers:		<i>A*02</i>	<i>Cw*05</i>	<i>B*44/B*12</i> a	<i>B*18</i>	Two markers b		<i>A*02-Cw*05</i> c
<b>ITALY</b> <sup>d</sup>	<i>A*02</i>	0.63 (0.53-0.74) p=6.8x10 <sup>-8</sup>		0.63 (0.53-0.75) p=9.9x10 <sup>-8</sup>	0.63 (0.53-0.75) p =1.3x10 <sup>-7</sup>	0.62 (0.52-0.74) p=4.4 x10 <sup>-8</sup>	<i>Cw*05 B*44</i> 0.63 (0.53-0.75) p=1.4x10 <sup>-7</sup>	<i>Cw*05 B*18</i> 0.63 (0.53-0.74) 6.9 x10 <sup>-8</sup>	NA
	<i>Cw*05</i>	0.84 (0.65-1.09) p=0.2024	0.88 (0.68-1.14) p=0.3445		0.94 (0.72-1.23) p=0.6503	0.79 (0.60-1.03) p=0.0885	<i>A*02 B*44</i> 0.97 (0.74-1.28) p=0.8593	<i>A*02 B*18</i> 0.82 (0.63-1.08) p=0.1555	NA
	<i>B*44</i>	0.70 (0.56-0.88) p=0.0026	0.72 (0.57-0.90) p=0.0051	0.71 (0.56-0.91) p=0.0056		NA	<i>A*02 Cw*05</i> 0.72 (0.57-0.92) p=0.0082		0.79 (0.62-1.00) p=0.0503
	<i>B*18</i>	1.17 (0.95-1.44) p=0.1389	1.20 (0.97-1.49) p=0.0817	1.23 (0.99-1.52) p=0.0626	NA		<i>A*02 Cw*05</i> 1.25 (1.01-1.56) p=0.0415		1.25 (1.01-1.55) p=0.036
	<i>A*02-Cw*05</i> c	0.39 (0.27-0.57) p=4.7 x10 <sup>-7</sup>			0.42 (0.29-0.62) p=7.3x10 <sup>-6</sup>	0.37 (0.26-0.54) p=1.5x10 <sup>-7</sup>	<i>B*44 B*18</i> e 0.38 (0.26- 0.56) p=6.5x10 <sup>-7</sup>		
<b>UK</b> f	<i>A*02</i>	0.72 (0.62-0.83) p=1.2x10 <sup>-5</sup>	-	0.75 (0.65-0.88) p=0.0003	0.74 (0.64-0.87) p=0.00013	0.72 (0.62-0.83) p=1.3x10 <sup>-5</sup>	<i>Cw*05 B*12</i> 0.76 (0.65-0.88) p=0.0004	<i>Cw*05 B*18</i> 0.76 (0.65-0.88) p=0.0004	NA

	Cw*05	0.65 (0.52-0.82) p=0.0002	0.72 (0.57-0.91) p=0.0058	-	0.76 (0.57-1.01) p=0.0636	0.62 (0.49-0.78) p=6.3x10 <sup>-5</sup>	A*02 B*12 0.83 (0.62-1.11) p=0.2104	A*02 B*18 0.69 (0.54-0.87) P=0.0021	NA
	B*12	0.70 (0.58-0.85) p=0.0002	0.75 (0.62-0.90) p=0.0026	0.80 (0.63-1.01) p=0.0586	-	NA	A*02 Cw*05 0.81 (0.64-1.02) p=0.0808		0.80 (0.64-1.00) p=0.047
	B*18	1.29 (0.93-1.79) p=0.1221	1.28 (0.92-1.77) p=0.1416	1.45 (1.04-2.03) p=0.0275	NA	-	A*02 Cw*05 1.40 (1.00-1.96) p=0.0459		1.30 (0.93-1.80) p=0.118
	A*02- Cw*05 <sup>c</sup>	0.56 (0.42-0.76) p=0.0001			0.68 (0.48-0.96) p=0.0272	0.56 (0.42-0.76) p=0.0001	B*12 B*18 <sup>c</sup> 0.60 (0.43-0.84) p=0.0029		

Italian individuals and UK haplotypes were categorized according to the presence or absence of the indicated alleles

Each marker in row is adjusted for markers in columns

p-values for adjusted models are obtained from Likelihood Ratio Test (LRT) comparing the likelihood of the two-gene additive vs the single marker model considering as single marker the marker used for adjustment.

NA = not applicable

<sup>a</sup> Results are referred to *B\*44* for the Italian individuals and *B\*12* for the UK haplotypes

<sup>b</sup> values adjusted for *DRB1\*15* plus the indicated additional two class I markers

<sup>c</sup> subjects positive for both *A\*02* and *Cw\*05* (*A2+* and *Cw5+*).

<sup>d</sup> 1445 MS patients and 973 controls carrying the indicated alleles.

<sup>e</sup> since the two markers are allelic, they were considered as a single variable. Italian individuals and UK haplotypes were categorized according to the presence or absence of any of them.

<sup>f</sup> 2106 cases (860 transmitted haplotypes in family trios +1246 haplotypes from MS patients) and 1676 controls (860 non-transmitted haplotypes in family trios + 816 haplotypes from controls). Haplotypes were calculated from individuals, fully typed for *HLA-A*, *-Cw*, *-B* and *-DRB1* loci,

with the UNPHASED v 3.0.13 program using the “certain haplotype only” option in family trios and the “uncertain haplotypes” option in the case/control cohort.[17] All typing informations, including *DR*, were utilised to deduce haplotypes.

NA = not applicable

Table 5:

## Comparative analysis of all the possible combinations of the A\*02, Cw\*05 and B\*44 alleles

HLA alleles			Population					
A*	Cw*	B*	Italian		UK		UK	
02	05	44/12 <sup>a</sup>	Case/control <sup>b</sup>		Family trios <sup>c</sup>		Combined case/control and family trios <sup>d</sup>	
			OR <sup>e</sup>	P value <sup>f</sup>	OR <sup>e</sup>	P value <sup>f</sup>	OR <sup>e</sup>	P value <sup>f</sup>
+	-	-	<b>0.63</b> (0.50-0.78)	<b>0.0007</b>	0.80 (0.59-1.08)	0.509	<b>0.65</b> (0.49-0.84)	<b>0.0099</b>
-	+	-	<b>3.41</b> (1.74-7.32)	<b>1.7x10<sup>-6</sup></b>	1.18 (0.42-3.27)	0.680	1.30 (0.58-2.93)	0.335
-	-	+	0.62 (0.41-0.92)	0.182	0.79 (0.49-1.29)	0.763	0.75 (0.49-1.13)	0.561
-	+	+	0.77 (0.40-1.47)	0.977	0.49 (0.24-1.00)	0.108	<b>0.37</b> (0.19-0.72)	<b>0.0087</b>
+	-	+	0.74 (0.45-1.23)	0.866	0.62 (0.25-1.50)	0.538	0.83 (0.33-2.13)	0.856
+	+	-	<b>0.36</b> (0.19-0.67)	<b>0.007</b>	1.42 (0.36-5.94)	0.543	0.39 (0.07-1.74)	0.346
+	+	+	<b>0.22</b> (0.11-0.44)	<b>3.3x10<sup>-5</sup></b>	<b>0.33</b> (0.17-0.61)	<b>5.5x10<sup>-4</sup></b>	<b>0.53</b> (0.32-0.86)	<b>0.044</b>

<sup>a</sup> Results are referred to B\*44 for the Italians and the UK family trios and B\*12 for the combined UK case/control and family trios. HLA-B\*18 was not considered in this table; therefore HLA-B\*18 positives are included among B\*44/12 negatives.

<sup>b</sup> 1005 DR15 negative MS patients and 848 DR15 negative controls carrying the indicated alleles.

<sup>c</sup> 531 DR15 negative transmitted and 720 DR15 negative non-transmitted haplotypes from 480 family trios.

<sup>d</sup> 784 cases (derived from 262 transmitted haplotypes in DR15 negative families + 522 haplotypes from DR15 negative MS patients) and 848 controls (derived from 262 non-transmitted haplotypes in DR15 negative families. + 586 haplotypes from DR15 negative controls). Haplotypes

were calculated with the UNPHASED v 3.0.13 program using the “certain haplotype only” option in family trios and the “uncertain haplotypes” option in the case/control cohort.[17] All typing information, including *DR*, were utilised to deduce haplotypes.

<sup>e</sup> The *A\*02-*, *Cw\*05-*, *B\*44/12-* combination was the reference for OR calculation.

<sup>f</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

Significantly protective allele combinations are bolded.

Supplementary Table 1

A\*02, Cw\*05 and B\*(44/18) phenotypic combinations in Italian *DR15* negative samples

HLA phenotype <sup>a</sup>			Frequency <sup>b</sup>		OR	95%CI	p <sup>c</sup>
A*02	Cw*05	B*d	MS patients N=1005 <sup>e</sup>	Controls N=848 <sup>e</sup>			
-	-	-	0.433	0.360	1 <sup>f</sup>	1	0.0016
-	-	44	0.056	0.072	0.64	0.43-0.97	0.1823
-	-	4402	0.022	0.021	0.91	0.45-1.85	0.948
-	-	4403	0.031	0.046	0.57	0.34-0.97	0.886
-	-	18	0.092	0.061	1.24	0.84-1.83	0.0196
-	+	-	0.010	0.001	7.01	0.99-305	0.0319
-	+	44	0.024	0.025	0.80	0.42-1.53	0.9774
-	+	4402	0.021	0.021	0.87	0.43-1.78	0.934
-	+	4403	0.0030	0.0013	2.10	0.17-110.8	0.631
-	+	18	0.050	0.015	2.70	1.39-5.32	8.0x10 <sup>-05</sup>
+	-	-	0.212	0.276	0.64	0.50-0.81	0.0016
+	-	44	0.040	0.042	0.78	0.47-1.28	0.8656
+	-	4402	0.015	0.013	0.96	0.41-2.26	0.905
+	-	4403	0.022	0.025	0.73	0.38-1.43	0.761
+	-	18	0.068	0.077	0.73	0.50-1.08	0.5115
+	+	-	0.007	0.012	0.49	0.17-1.42	0.4001
+	+	44	0.013	0.046	0.23	0.12-0.46	3.3x10 <sup>-05</sup>
+	+	4402	0.013	0.031	0.35	0.16-0.72	0.011
+	+	4403	0.0010	0.0072	0.12	0-0.97	0.051
+	+	18	0.013	0.037	0.29	0.14-0.59	0.0015

<sup>a</sup> Presence of the indicated alleles.<sup>b</sup> Frequency of individuals carrying the indicated alleles.<sup>c</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

<sup>d</sup> *HLA-B* was considered as a triallelic locus including *HLA-B\*44*, *HLA-B\*18* or none of them. Individuals positive for both *B\*44* and *B\*18* were counted twice (Cases N=16; Controls N=20).

<sup>e</sup> 995 *DR15* negative patients and 827 *DR15* negative controls were typed for *B\*44* subtypes *B\*4402* and *B\*4403*.

<sup>f</sup> Reference for OR calculation.

Supplementary Table 2

*DRBI\*15* negative haplotypic combinations in UK family trios

Haplotype <sup>a</sup>			Frequency		OR	95% CI	p <sup>b</sup>
<i>A*02</i>	<i>Cw*05</i>	<i>B*</i>	Transmitted N = 531 <sup>c</sup>	Non transmitted N = 720 <sup>c</sup>			
-	-	-	0.637	0.556	1 <sup>d</sup>	1	0.0048
-	-	44	0.055	0.064	0.75	0.45 - 1.25	0.57
-	-	18	0.021	0.013	1.45	0.55 - 3.84	0.36
-	+	-	0	0.003	0	0 - 6.33	0.51
-	+	44	0.024	0.043	0.5	0.24 - 1	0.11
-	+	18	0.017	0.010	1.52	0.51 - 4.58	0.38
+	-	-	0.186	0.201	0.81	0.6 - 1.1	0.56
+	-	44	0.015	0.022	0.59	0.23 - 1.49	0.48
+	-	18	0.006	0.007	0.71	0.11 - 3.68	1
+	+	-	0.002	0.004	0.39	0.01 - 4.94	0.64
+	+	44	0.028	0.075	0.33	0.17 - 0.61	5.5x10 <sup>-4</sup>
+	+	18	0.009	0.003	2.96	0.48 - 31.21	0.14

<sup>a</sup> Haplotypes were deduced with the UNPHASED v 3.0.13 program using the “certain haplotype only” option.[17] The complete *HLA-A, B, C, DR* typing was considered for haplotype reconstruction.

<sup>b</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

<sup>c</sup> *DRBI\*15* negative haplotypes from 480 families.

<sup>d</sup> Reference for OR calculation.



Supplementary Table 3  
Haplotypic combinations in UK case/control *DR15* negative samples.

Haplotype <sup>a</sup>			Frequency		OR	95% CI	p <sup>b</sup>
<i>A*02</i>	<i>Cw*05</i>	<i>B*</i>	MS patients N = 522	Controls N = 586			
-	-	-	0.657	0.567	1 <sup>c</sup>	1	0.0025
-	-	12	0.069	0.072	0.83	0.52-1.36	0.954
-	-	18	0.021	0.017	1.06	0.41-2.75	0.789
-	+	-	0.004	0.002	1.94	0.10-114.55	0.604
-	+	12	0.011	0.032	0.31	0.10-0.81	0.0324
-	+	18	0.021	0.012	1.52	0.54-4.40	0.336
+	-	-	0.149	0.212	0.61	0.44-0.85	0.0094
+	-	12	0.013	0.009	1.36	0.37-5.47	0.623
+	-	18	0.010	0.009	0.97	0.22-4.25	1.000
+	+	-	0.002	0.002	0.97	0.01-76.21	1.000
+	+	12	0.042	0.061	0.59	0.33-1.06	0.192
+	+	18	0	0.007	0	0-1.48	0.127

<sup>a</sup> Haplotypes were deduced by the UNPHASED v 3.0.13 program using the “uncertain haplotypes” option.[17] Also the information of the complete DR typing was considered for haplotype reconstruction.

<sup>b</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

<sup>c</sup> Reference for OR calculation.

Supplementary Table 4

Combined analysis of haplotypic combinations derived from UK *DR15* negative family trios and case/controls

Haplotype <sup>a</sup>			Frequency		OR	95% CI	p <sup>b</sup>
<i>A*02</i>	<i>Cw*05</i>	<i>B*</i>	MS patients N = 784 <sup>c</sup>	Controls N = 848 <sup>d</sup>			
-	-	-	0.663	0.565	1 <sup>e</sup>	1	5.7x10 <sup>-05</sup>
-	-	12	0.0612	0.0696	0.75	0.49-1.14	0.56
-	-	18	0.0179	0.013	1.17	0.5-2.79	0.55
-	+	-	0.0025	0.0035	0.61	0.05-5.39	1
-	+	12	0.018	0.041	0.37	0.19-0.72	0.0087
-	+	18	0.019	0.011	1.54	0.63-3.83	0.22
+	-	-	0.156	0.206	0.64	0.49-0.84	0.0096
+	-	12	0.013	0.013	0.84	0.33-2.14	0.86
+	-	18	0.008	0.008	0.79	0.22-2.77	0.89
+	+	-	0.0013	0.004	0.31	0.01-3.84	0.63
+	+	12	0.038	0.061	0.53	0.32-0.87	0.044
+	+	18	0.0026	0.005	0.46	0.04-3.23	0.69

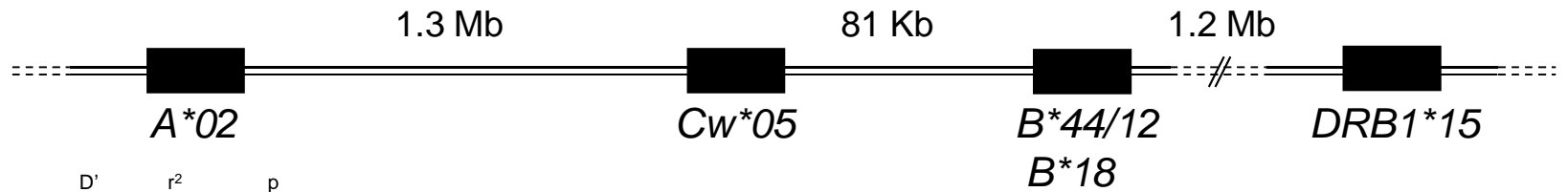
<sup>a</sup> Haplotypes were calculated with the UNPHASED v 3.0.13 program using the “uncertain haplotypes” option.[17] Also the information of the complete DR typing was considered for haplotype reconstruction.

<sup>b</sup> Calculated from 2x2 tables versus all the other possible allele combinations.

<sup>c</sup> 262 transmitted haplotypes in *DR15* negative families + 522 haplotypes from *DR15* negative MS patients.

<sup>d</sup> 262 non-transmitted haplotypes in *DR15* negative families + 586 haplotypes from *DR15* negative controls.

<sup>e</sup> Reference for OR calculation.



*Cw\*05*

	D'	r <sup>2</sup>	p
IT	<b>0.39</b>	<b>0.16</b>	<b>1.5x10<sup>-6</sup></b>
UK	<b>0.46</b>	<b>0.24</b>	<b>&lt;10<sup>-7</sup></b>

*B\*44/12*  
*B\**

	D'	r <sup>2</sup>	p
IT	0.030	0.014	ns
UK	<b>0.26</b>	<b>0.17</b>	<b>&lt;10<sup>-7</sup></b>

*18*

	D'	r <sup>2</sup>	p
IT	0.19	0.095	ns
UK	-0.008	0.012	ns

	D'	r <sup>2</sup>	p
IT	<b>0.46</b>	<b>0.36</b>	<b>&lt;10<sup>-7</sup></b>
UK	<b>0.85</b>	<b>0.66</b>	<b>&lt;10<sup>-7</sup></b>

	D'	r <sup>2</sup>	p
IT	<b>0.26</b>	<b>0.20</b>	<b>&lt;10<sup>-7</sup></b>
UK	<b>0.22</b>	<b>0.12</b>	<b>1.7x10<sup>-5</sup></b>

	D'	r <sup>2</sup>	p
IT	-0.004	0.041	ns
UK	<b>-0.012</b>	<b>0.12</b>	<b>7.0x10<sup>-7</sup></b>

	D'	r <sup>2</sup>	p
IT	-0.0005	0.005	ns
UK	0.049	0.023	ns

*DRB1\*15*

	D'	r <sup>2</sup>	p
IT	<b>-0.015</b>	<b>0.084</b>	<b>0.011</b>
UK	-0.002	0.016	ns

	D'	r <sup>2</sup>	p
IT	0.004	0.004	ns
UK	<b>-0.008</b>	<b>0.11</b>	<b>2.4x10<sup>-4</sup></b>