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6 # *HLA-DRB1*1501 AND MULTIPLE SCLEROSIS: A FEMALE ASSOCIATION?*
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17 **Keywords:** multiple sclerosis; HLA-DRB1*1501; VDR; sex; HLA II gene expression
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19
20 ## Abstract
21 **Background**: The association between multiple sclerosis (MS) and the HLA-DRB1*15:01
22 haplotype has been proven to be strong, but its molecular basis remains unclear. Vitamin D
23 receptor (VDR) gene variants and sex have been proposed to modulate this association.
24 **Objectives**: 1) Test the association of MS with *15:01 and VDR variants; 2) check whether VDR
25 variants and/or sex modulate the risk conferred by *15:01; 3) study whether *15:01, VDR
26 variants and/or sex affect HLA II gene expression. **Methods**: Peripheral blood from 364 MS
27 patients and 513 healthy controls was obtained and DNA and total RNA were extracted from
28 leukocytes. HLA-DRB1, DRB5 and DQA1 gene expression measurements and *15:01
29 genotyping were performed by qPCR. VDR variants were genotyped by PCR-RFLP.
30 **Results**: Our data confirms that the *15:01 haplotype confers a higher risk of suffering from MS
31 (OR = 1.364; 95% CI = 1.107–1.681). No association was found between VDR variants and
32 MS, but they were shown to moderately modulate the risk conferred by *15:01. Sex confers a
33 much stronger modulation and the *15:01-MS association seems to be female specific. A higher
34 *15:01 frequency has been observed in Basques (45.1%). *15:01 positive samples showed a
35 significant overexpression of DRB1 ($p < 0.001$), DRB5 ($p < 0.001$) and DQA1 ($p = 0.004$) in
36 patients. DRB1 ($p = 0.004$) and DRB5 ($p < 0.001$) were also overexpressed in *15:01 controls.
37 **Conclusions**: We confirm the *15:01-MS association and support that it is female specific. The

relevance of ethnic origin on association studies has also been highlighted. HLA-DRB1*15:01 seems to be a haplotype consistently linked to high HLA II gene expression.

Keywords
HLA-DRB1*15: 01, HLA II gene expression, multiple sclerosis, sex,VDR

1. Introduction

Multiple sclerosis (MS) is a common inflammatory disorder of the central nervous system (CNS) characterized by demyelization, gliosis, axonal damage and progressive neurological dysfunction. It is one of the most incapacitating diseases in young people.

The pathogenic mechanisms of the disease are not yet clearly identified; however, one proposal is an activation of the lymphocytes that cross the blood–brain barrier (BBB) directly into the interstitial matrix. T cells are then reacti- vated by fragments of the myelin antigens exposed in the context of human leukocyte antigen (HLA) molecules of the surface of the antigen presenting cells.

Aetiologically, MS is a complex disease in which both genetic and environmental factors are involved. As of today, seven genome wide association studies (GWASs) have been performed in MS in order to discover the genetic factors involved in the susceptibility to suffer the disease. The strongest genetic association with MS in Northern Europeans is found to be with extended MHC haplotypes, especially those containing HLA-DRB1*15:01[1]. Out of this previous known association, GWASs have found sev- eral genetic associations. Recently a common network of different variants has been proposed for the autoimmune diseases and the model of a cooperative network of SNPs seems to be the way to understand the complex MS genetics[2]. However, each one of these susceptibility genes appears to contribute little to overall risk1 and MHC is accepted to be the key susceptibility locus in MS.

Despite the great consistency that the *15:01MS association has demonstrated since its discovery 30 years ago[3,4] the molecular mechanisms that lie behind it remain unclear. Several works have studied the role of DRB1* and DRB5* in the immune response of MS disease. Studies in EAE in mice show both the implication of both DRB1*15:01 and DRB5*0101 restricted encephalitogenic T cells which would modulate the primary T response[5]. Other studies in humans suggest that the differential expression of DRB1*15:01 and DRB5*0101 or DQA1*0102 genes of the extended haplotype associated with MS could modulate the clinical ‘phenotype’ of the disease [6]. In this sense, several genetic variants have been postulated as modulators of the risk conferred by the *15:01 allele, such as vitamin D receptor gene (VDR) variants and sex [7–9].

Several studies have found a possible association between VDR variants and MS. However, the results of these studies have been contradictory. While some works have not revealed any association between VDR variants and MS[10] some others have found a clear link. According to

these, the ff allele of VDR seems to have a protective effect;[11,12] in contrast, Taq I and Apa I restriction marker variants of the gene have been found to be linked to a higher risk of suffering the disease.13 Moreover, a recent study has shown that the activation of several members of the nuclear receptor family, VDR among them, is suppressed in the pre-disease state of MS, which impairs apoptosis mediated depletion of activated T cells[14].

In the context of genetic association studies, we have to remember that allele frequencies are known to vary widely within and between populations, irrespective of disease status. Consequently, population stratification [15,16] must be taken into account in this kind of study and the characteri- zation of their distribution in the specific populations may provide valuable information.

The aim of this work is to test the association of MS with *15:01 in a sample set that contains a subgroup of Basque population, to check the influence of VDR and sex in this association and to study whether all of these variables affect HLA II gene expression.

2. Methodology

2.1 Blood sample collection

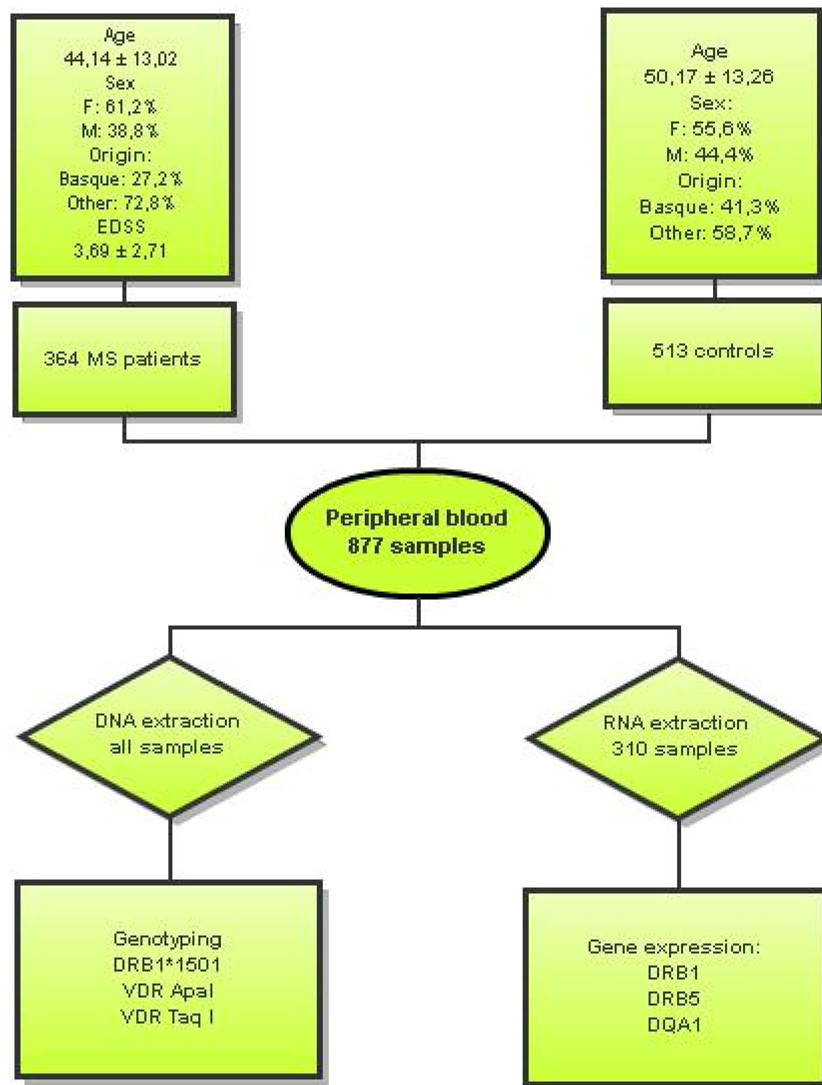
Three hundred and sixty-four MS patients and 513 controls were included in this study (Figure 1). All MS patients were diagnosed with multiple sclerosis according to Polman.17 Peripheral blood of patients and healthy controls was obtained in the Neurology Department of Hospital Donostia after informed consent was given. Blood extraction was always performed in the early morning and RNA extraction was carried out no more than 2 h after the blood was col- lected and during this time was kept at 4oC. In all of the cases, 10 ml of blood was collected in EDTA tubes by veni- puncture. All procedures have been approved by the hospi- tal’s ethic committee.

2.2 Ethnic origin determination

Ethnic origin determination of patients and controls was car- ried out based on the linguistic root of the first two surnames. People whose first two surnames had a Basque linguistic root were considered to have a Basque origin. The rest of the peo- ple were classified as Spaniards (Otaegui et al, 2004).

2.3 DNA and RNA extraction

DNA extraction from white blood cells was carried out fol- lowing a manual protocol (all samples). 5Prime’s Perfect Pure RNA Blood Kit was used for total white blood cell RNA extraction according to the manufacturer’s instruc- tions (170 patients and 140 controls).



115
116 ***Fig 1. Workflow and sample description.***
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118 *2.4 Genotyping*
119 HLA-DRB1*15:01 genotyping of the samples was carried out as previously described19
120 using a 7300 Real Time PCR System (Applied Biosystems). Amplification detection of DRB1
121 was considered as a synonym of the existence of at least one *15:01 copy. Even though unable
122 to distinguish between heterozygous and homozygous carriers of the hap- lotype, this method
123 was chosen because we considered that the information obtained by this technique was
124 sufficient for the achievement of our goals; also the method was cheaper and faster than other
125 alternatives.
126 The deeper DRB1 and DRB5 genotyping was performed by both exon 2 PCR and high
127 definition (HD) reverse SSO microbead arrays making use of Lab type kits (One Lambda, Inc.)
128 and a Luminex system, respectively. The presence of DRB5 was tested by PCR-SSP, using the
129 MicroSSP generic class II DNA typing tray (One Lambda, Inc.).
130 The VDR ApaI and TaqI polymorphisms (Table 1) were identified using PCR-RFLP. A 740-
131 bp fragment generated by PCR was digested with the restriction endo- nuclease TaqI (Takara

132 Bio) to yield 490 bp and 250 bp long fragments for the 'T' allele and 290, 250 and 200 bp long
133 fragments for the 'C' allele. After digestion with ApaI, the same PCR product was cut into 515
134 and 225 bp fragments for the 'a' allele, whereas the 'A' allele was undigested. The PCR
135 products and the restriction fragments were separated in a 3% agarose gel stained with
136 ethidium bromide, and visualized by a Gene Flash Syngene Bio Imaging system (Syngene).
137 The amplifications were performed using the following primer pair: F-
138 CAGAGCATGGACAGGGAGCAAG; R-GCAACTCCTCATGGCTGAGGTCTCA.
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141 **Table 1**: a summary of the genotyped VDR variants and the nomenclature used to designate them
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Analyzed VDR polymorphisms			
Marker	**Allele**		
	Nomenclature used in paper	Methodical nomenclature	db SNP
ApaI	A	1025 - 49 G	rs7975232
	a	1025 - 49 T	
TaqI	T	c.1056 T	rs731236
	C	c.1056 C	

150 *2.5 Gene expression*
151 cDNA synthesis from total white blood cell RNA was per- formed using the High Capacity
152 cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions.
153 For gene expression measurement, DRB1 (Hs99999917_m1), DRB5 (Hs03046116_m1) and
154 DQA1 (Hs03007426) specific Applied Biosystems taqman probes were used. qPCRs were
155 performed in a volume of 10 µl, using the following mix: 5 µl 2x Universal Master Mix (Applied
156 Biosystems), 0.5 µl Specific TaqMan Assay, 2.5 µl H2O and 2 µl of 25 ng/µl cDNA (50 ng). The
157 amplifications were done employing a 7900 Real Time PCR System under the following thermal
158 programme: stage 1, 50oC/2 min; stage 2, 95oC/10 min; stage 3, (95oC/15 s, 60oC/1 min) ! 40
159 repeats. 18S was used as the endogenous control gene, as was previously proven to show great
160 stability among our samples. Ct values were obtained using SDS 2.2.2 software. To ensure that
161 the high sequence variability of the HLA II genes had not impaired our gene expression
162 measurements, we performed a qPCR efficiency assay and found that efficiencies were similar
163 among the groups under comparison for each of the genes making the expression value of the
164 different groups comparable.
165
166 *2.6 Data analysis*
167 Frequencies of each genotype (DRB1*15:01, VDR ApaI and VDR TaqI) were calculated for
168 MS patients and controls and a chi-square analysis was performed in order to find out whether
169 differences in genotype frequencies were marked enough to reach statistical significance and
170 calculate the OR for MS.
171 dCt values for gene expression were calculated using Microsoft Excel. Statistical analyses
172 were performed by the PASW Statistics 18 (SPSS Inc.). For the statistical analysis, a data

distribution normality Kolmogorov–Smirnov test was performed first and, as none of the variables showed a normal distribution, they were subjected to a Mann– Whitney *U* test (a non-parametric variable distribution comparing analysis) in order to find whether DRB1, DRB5 and DQA1 expression could be affected by *15:01.

3. Results

3.1. Genotyping

Our genotyping data (Table 2) confirms the *15:01MS association and a higher risk of developing the illness for *15:01 carriers is seen (odds ratio, OR = 1.364; 95% CI = 1.107–1.681). However, no association has been found between VDR variants and the disease when considering all samples together. After data subdivision based on ethnic origin, instead, a statistical tendency of association has been observed between ApaI genotypes and MS ("2= 5.535; *p* = 0.063) in Basques.

Table 2: frequencies of each genotype in patients and controls, chi-square analysis results and MS odds ratio between

		MS	**Controls**	**All**	**χ2 (p)**	**OR (95% CI)**
HLA*1501	**+**	101 (45,9%)	133 (34,1%)	234 (38,4%)	8,292 (0,004)	1.364 (1,107-1,681
	-	119 (54,1%)	257 (65,9%)	376 (61,6%)		
	total	**220**	**390**	**610**		
VDR Apa I	**AA**	39 (29,1%)	76 (22,4%)	115 (24,3%)	2,93 (0,231)	
	Aa	60 (44,8%)	178 (52,4%)	238 (50,2%)		
	aa	35 (26,1%)	86 (25,3%)	121 (25,5%)		
	total	**134**	**340**	**474**		
VDR Taq I	**TT**	55 (40,4%)	145 (43,0%)	200 (42,3%)	1,161 (0,56)	
	TC	70 (51,5%)	157 (46,6%)	227 (48,0%)		
	CC	11 (8,1%)	35 (10,4%)	46 (9,7 (%)		
	total	**136**	**337**	**473**		

Modulating factors of the *15:01-MS association.

On the other hand, the *15:01-MS association was separately tested on each VDR genotype with the aim of detecting possible modulation phenomena. Optimal modulation detection was reached when putting AA and Aa genotypes (A+) together in Apa I and TT and TC (T+) in Taq I (Table 3). A certain degree of modulation of ORs has been detected in both markers (A+ = 1.361 vs. A– = 0.974; T+ = 1.265 vs. T– = 0.874). However, the statistical significance of the *15:01-MS association was lost in all cases.

The same analysis was performed after sex-based subdivision and a much clearer modulation was found. In fact, in our data, the *15:01-MS association seems to be female specific, with an OR of 1.656, and drops from statistical significance in males (*p* = 0.784).

213 Finally, the effect of ethnic origin on *15:01 distribution and the *15:01-MS association has
214 been tested (Figure 2). A significantly tendency of presenting a higher *15:01 frequency was found
215 in Basques when compared with non- Basques (45.1% *vs*. 34.4%; p = 0.02), a tendency
216 maintained both in patients and controls. However, that tendency did not reach statistical
217 significance (p = 0.087 and p = 0.059). In addition, no remarkable modulation of ORs has been
218 detected (1.392 *vs*. 1.246) and the *15:01-MS association lost statistical significance in both
219 subgroups (p = 0.159 and p = 0.102), probably due to sample size decrease.
220
221 **Table 3. *15:01-MS association modulation by VDR variants and sex.**

Subgroup		DRB1 haplotype	MS patients	Controls	All	X^2(p)	OR (95%CI)
VDR ApaI	A+	1501+	41 (42,7%)	74 (32,5%)	115 (35,4%)	3,196 (0,077)	1,361 (0,974-1,902)
		1501-	55 (57,3%)	155 (67,7%)	210 (64,6%)		
		Total	96	229	325		
	A-	1501+	14 (41,2%)	32 (42,1%)	46 (41,8%)	0,008 (1,000)	0,974 (0,552-1,719)
		1501-	20 (58,8%)	44 (57,9%)	64 (58,2%)		
		Total	34	76	110		
VDR TaqI	T+	1501+	51 (41,8%)	91 (33,7%)	142 (36,2%)	2,386 (0,140)	1,265 (0,942-1,699)
		1501-	71 (58,2%)	179 (66,3%)	250 (63,8%)		
		Total	122	270	392		
	T-	1501+	4 (36,4%)	13 (40,6%)	17 (39,5%)	0,062 (1,000)	0,874 (0,301-2,537)
		1501-	7 (63,6%)	19 (59,4%)	26 (60,5%)		
		Total	11	32	43		
Sex	F	1501+	67 (51,9%)	57 (30,8%)	124 (39,5%)	14,197 (**<0,001**)	**1,656** (1,276-2,150)
		1501-	62 (48,1%)	128 (69,2%)	190 (60,5%)		
		Total	129	185	314		
	M	1501+	34 (37,8%)	58 (40,0%)	92 (39,1%)	0,115 (0,784)	0,944 (0,674-1,321)
		1501-	56 (62,2%)	87 (60,0%)	143 (60,9%)		
		Total	90	145	235		

Table 3: *1501-MS association modulation by VDR variants and sex. 1501+ and 1501- sample frequencies in both patients and controls, chi-square analysis results and odds ratio values are shown for each subgroup. **A+** = AA/Aa; **A-** = aa; **T+** = TT/TC; **T-** = CC; **F** = females; **M** = males.

222 15:01+ and 15:01- sample frequencies in both patients and controls, chi-square analysis results and
223 odds ratio values are shown for each subgroup. A+:AA/Aa,A-: aa,T+:TT/TC,T-: CC, F: females, M: males
224
225 ***3.2. Gene expression***
226 Gene expression data of DRB1, DRB5 and DQA1 obtained from qPCR experiments was
227 converted to dCt values using Microsoft Excel. In order to visualize the data and extract more
228 biologically relevant conclusions, all expression values (-dCt-s) of the three genes have been
229 represented in a plot after *15:01 and condition-based data organization (Figure 3). All expression
230 values can visually be grouped into three main clusters: cluster A, high expression values for the
231 three genes that match with *15:01 positive sam- ples; cluster B, high expression values of DRB1
232 and DQA1 in some *15:01 negative samples; and cluster C, low expression values of DRB1 and
233 DQA1 in some *15:01 negative samples and of DRB5 in all *15:01 negative samples. However,

234 some exceptions to that rule can be seen in Figure 3 (encircled in red: two *15:01- samples with
235 high DRB5 expression values).
236 After the statistical analysis, *15:01 positive samples showed a significant overexpression of
237 DRB1 (p < 0.001), DRB5 (p < 0.001) and DQA1 (p = 0.004) in *15:01+ patients (Table 4). No
238 other variable (age, sex, VDR variants and disease state (relapse/remission) has been found to
239 be linked to HLA II gene expression.
240 We then wondered whether the disease could have an effect in HLA II gene expression
241 independent from and masked by the *15:01 effect. In order to isolate the *15:01 variable, we
242 segmented the data based on it and performed again a Mann–Whitney U test (Table 5). Patients
243 showed a significant underexpression of DRB5 (p = 0.02) in the *15:01 positive group. In contrast,
244 when focusing on *15:01 negative samples, DQA1 was the only gene show- ing a significant
245 underexpression (p = 0.006) in patients and DRB5 was found to be overexpressed (p = 0.043).

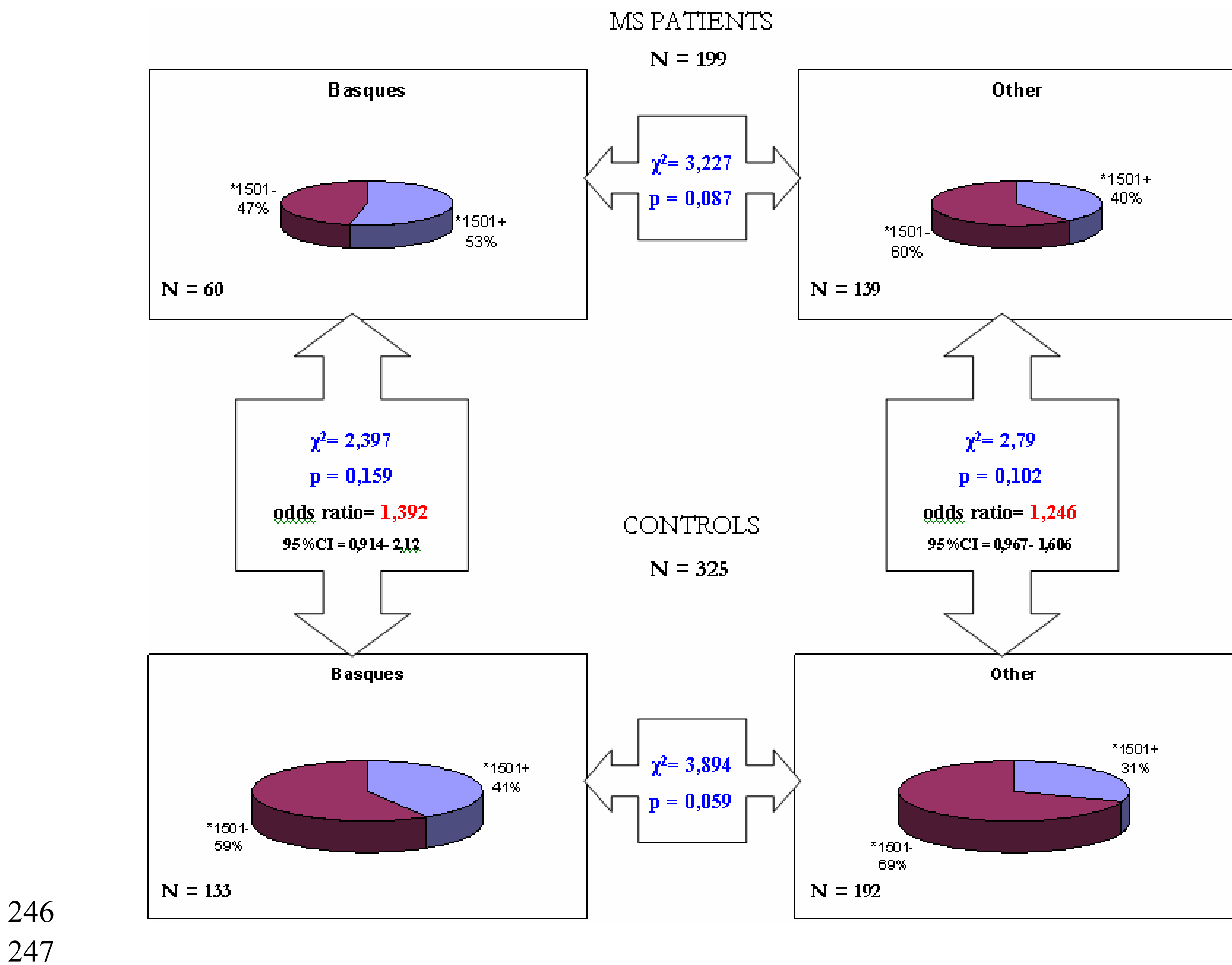

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Figure 3: *15:01 distribution differences between Basques and non-Basques both in patients and controls. Chi-square analysis results and odds ratio values are also shown.

Figure 3: gene expression values after data organization based on *1501 and condition. The dashed line divides *1501 positive and *1501 negative samples.

Table 4: RQ values and Mann–Whitney U test results for a DRB1, DRB5 and DQA1 gene expression comparison between *15:01 positive and *15:01 negative samples both in patients and controls. *P<0.05: **p<0.001. RQ; relative quantification (2-(mean dCt1 - mean dCt2)).

HLA II gene expression comparison 1501+ vs 1501 -		
	MS	*CON*
DRB1	$2.12*10^{4}$ **	$5.6*10^{3}$ *
DRB5	$1.18*10^{5}$ **	$3*10^{5}$ **
DQA1	$2.22*10^{2}$ *	25.53

Table 5: RQ values and Mann–Whitney U test results for an HLA II gene expression comparison between patients and controls in both *15:01+ and *15:01– samples. RQ; relative quantification (2-(mean dCt1 - mean dCt2)).

HLA II gene expression comparison MS vs C		
	**1501+*	**1501-*
DRB1	0,44	0,12
DRB5	0,62 *	1,59 *
DQA1	0,70	0,08 *

4. Discussion

Our results confirm the already well established HLADRB1* 15:01–MS association[1],[20] as we obtained a 1.364 OR for *15:01 haplotype carriers. However, this value lies well below those published in previous works as they range between 2 and 3, approximately[20–24]. It is known that population stratification may create a smaller bias in association studies such as the ones involving HLA genes, as they present great allele frequency variability among different ethnic groups[15] . We checked this possibility in our samples and we found no significant change in the OR when comparing Basques with non-Basques (1.39 *vs*. 1.25).

However, we have to take into account that the ethnic background between cases and controls is very different and that could explain the low OR obtained for the *15:01 haplotype. As Basques have a clearly higher *15:01 frequency and the proportion of Basques is much higher in the controls (41.3% *vs*. 27.2%), this makes the difference of *15:01 frequency between patients and controls much lower and, thus, the power of the association analysis is decreased. This, in fact, could also explain the relatively low value obtained in the neighboring Biscayan province.24,25.

As mentioned, the present study has also revealed a tendency towards a higher *15:01 carrier frequency both in Basque patients and controls when compared with non- Basques, although that tendency did not reach statistical significance. Indeed the *15:01 carrier frequencies both in patients (53%) and controls (41%) are higher than these observed for other European populations such as Swedish (39% and 16%), Serbian (34.6% and 19.7%), German (14.6% in controls) and Spaniards (18% in controls).20,26–28.

Our data also shows that *15:01 confers risk for MS only in females. We wondered whether these results could be a consequence of a sampling artefact and tested whether *15:01 distribution is biased by sex in our sample. No statistical differences have been found in *15:01 distribution between females and males ("2 = 0.007; p = 1.0) and, thus, the *15:01–MS association modulation by sex has been accepted as a reliable conclusion. These results strongly suggest that either *15:01 has no effect on disease appearance in males or its effect is completely diluted by other variables. Thus, it can be concluded that in our data all of the susceptibility linked to the *15:01-MS association comes from females.

Multiple sclerosis is a disease that affects differently males and females,29 two-thirds of the patients being female. Several causes have been suggested to explain the gender issue such as sex hormones, genetic factors, immune bias and environment,30 but the underlying cause remains elusive. With regard to that problem, several studies have appeared in recent years pointing to immunological differences as a plausible cause. While some researchers have seen a bigger inflammatory component in females,30 others have focused their efforts on trying to elucidate the relationship between the HLA genes, sex and MS and have shed some light on the issue. The *15:01 containing HLA-DR2,DQ6 haplotype, which confers risk of undergoing MS, has been found to be more frequent in female than in male patients,8 the statistically significant difference in *15:01 frequency between MS patients and controls seems to be female specific7 and the HLA-DR15 phenotype has been found to be associated to sex in MS.9 Moreover, this

modulation by sex of the risk conferred by HLA genes has been reported in other diseases such as narcolepsy- cataplexy31 and type 1 autoimmune hepatitis, 31 and oestrogen receptor gene polymorphisms have also been found to alter the *15:01-MS association.23 Concerning the VDR analysis, we have not been able to confirm an association between variants in ApaI or TaqI markers of VDR and the disease when considering all samples together, but a statistical tendency of association between ApaI variants and MS has been observed in Basques. These results highlight, once again, the strong effect of ethnic origin on genetic associations and demonstrate that when ethnic stratification of samples is suspected it must be taken into account to achieve correct interpretation of the results, despite the consequent loss of statistical power. In fact, ethnic origin along with environmental factors such as sunlight exposure and vitamin D intake could help to explain the controversy on the association between VDR variants and MS. VDR variants have also been proposed to modulate the *15:01-MS association.33–35 Niino et al. observed a higher risk of suffering the disease in samples containing both the 'A' allele of ApaI and the *15:01 haplotype, compared with 'A' negative and *15:01 positive samples. We also made that observation and, in addition, our data suggests that TaqI seems to perform a similar modulation, where *15:01 confers risk only in 'T' positive individuals, which is in contradiction to the protective role of the 'T' allele proposed by Agliardi et al.

Expression

We found HLA II gene expression differences between patients and controls after *15:01-based data subdivision. We thought that these differences in HLA II gene expression may not be an effect of the disease itself but an effect of the treatments patients were receiving, as most of drugs used in MS treatment work via immunomodulation. To assess that question, we compared gene expression values between treated and non-treated patients, but no statistically significant differences were found in any of the genes (data not shown). On the other hand, seeing that the trend differs between the *15:01+ and the *15:01- samples, a chi-square analysis was performed with the aim of testing whether treatment frequencies (the proportions of treated patients) were different between the two groups. That analysis did not report significant differences either ("$_2$ = 0.232; p = 0.789). Thus, treatment does not seem to be the underlying cause of the HLA II gene expression differences observed between MS patients and controls in our sample. However, specific studies are needed in larger samples in which the effect of different drugs may be analysed separately.

Although the *15:01-MS association was well established more than 30 years ago,[3,4] the molecular mechanisms underlying this link are unknown. Ramagopalan et al.[34] hypothesized that, as the promoter of HLA-DRB1*1501 contains a vitamin D response element (VDRE), in a vitamin D deficient environment the gene expression of HLADRB1* 1501 would be low enough to impair auto-reactive T cell depletion in the thymus and lead to MS development. Our results do not support this theory. According to our results, the gene expression of the three HLA II genes in the

study, DRB1, DRB5 and DQA1, is consistently high in *15:01 positive samples, both in patients and controls. Moreover, VDR variants seem to have no effect on the expression of these genes. Finally, we have found that DRB5 gene expression is nearly *15:01 specific. While all *15:01 positive samples showed a high DRB5 expression, in just two of the 117 *15:01 negative samples was DRB5 found to be overexpressed, being nearly absent in most of the samples. These results are consistent with the already well known fact that the DRB5 locus is carried exclusively on DRB1*15 and *16 haplotypes.[35,36] In fact, DRB5 expression could be used as a DRB1*15:01 screening tool.

However, some exceptions to the general expression pattern described above have been observed. In two *15:01-negative samples (exception numbers 1 and 2) DRB5 has been found to be highly expressed. In order to clarify the basis of these exceptions, a deeper DRB1 and DRB5 allele determination was performed. For the first exception, the genotyping assays revealed that it is DRB1*0103/*0301, which is completely concordant with our previous *15:01-result, but no presence of the DRB5 locus could be found; even the expression value was clearly high. By contrast, the SSP analysis of the second exception revealed the presence of DRB5, which is compatible with the high expression result, and the DRB1 genotyping revealed it to be *1201/*1601, which is consistent both with the previous *15:01- result and with the literature, as the presence of the DRB5 locus has been described to be closely linked to the DRB1*16 haplotype.

In summary, this study confirms the *15:01-MS female specific association. Even though not directly connected with the disease, the vitamin D receptor seems to act synergistically with the *15:01 haplotype in the development of MS. More efforts must be directed in the future towards clarifying the role of VDR in MS pathogenesis. Finally, *15:01 has proven to be a haplotype consistently linked to a high HLA II gene expression, with a nearly exclusive expression of DRB5.

Disclosure Statement

The authors have no financial conflicts of interest.

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394 **Conflict of interest statement**

395 The authors have no financial conflicts of interest.

396

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