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Influence of HLA DRB1 alleles in the susceptibility of rheumatoid arthritis and the regulation of antibodies against citrullinated proteins and rheumatoid factor

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ABSTRACT

Introduction: To investigate the association between HLA-DRB1 alleles with susceptibility to rheumatoid arthritis (RA) and production of antibodies against citrullinated proteins (ACPA) and rheumatoid factor (RF).

Methods: We studied 408 patients (235 with RA, 173 non-RA) and 269 controls. ACPA, RF and HLA-DR typing were determined.

Results: We found an increased frequency of HLA DRB1 alleles with the shared epitope (SE) in ACPA-positive RA. Inversely, HLA DRB1 alleles encoding DERAA sequences were more frequent in controls than in ACPA-positive RA, and a similar trend was found for HLA DR3. However these results could not be confirmed after stratification for the presence of the SE, probably due to the relatively low number of patients. These data may suggest that the presence of these alleles may confer a protective role for ACPA-positive RA. In RA patients we observed association between SE alleles and ACPA titers in a dose-dependent effect. The presence of HLA DR3 or DERAA-encoding alleles was associated with markedly reduced ACPA levels. No association between RF titers and HLA DR3 or DERAA-encoding alleles was found.

Conclusions: HLA DRB1 alleles with the SE are associated with production of ACPA. DERAA-encoding HLA-DR alleles and HLA DR3 may be protective for ACPA-positive RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a complex autoimmune disease that develops from the combined effects of genetic and environmental factors. It is estimated that the heritability of RA accounts for about 50–60%, and the most important genetic risk factors are the HLA class II molecules, which contribute to one-third of the total genetic susceptibility [1;2]. There is extensive evidence for the association between certain HLA-DRB1 alleles with a conserved amino acid sequence (Q/RK/RRAA) at residues 70 to 74 in the third hypervariable region of the DRβ1 chain, the so-called shared epitope (SE), and susceptibility to and severity of RA [3;4].

Autoimmunity in RA is characterized by the presence of autoantibodies. Rheumatoid factor (RF) is not specific to RA, as it may be present in other diseases and in healthy elderly individuals [5]. In contrast, anti–citrullinated protein antibodies (ACPA) seem to play a pivotal role in the pathogenesis of RA, as they are highly specific [6], can be detected years before the onset of symptoms [7;8], may predict progression to RA in patients with undifferentiated arthritis [9;10], are associated with the extent of joint destruction [11] and enhance disease severity in animal models of arthritis [12].

Recent studies, including our data, have demonstrated that SE alleles are associated only with ACPA-positive RA [13;14] and more strongly with ACPA than with RA itself [15], suggesting that SE alleles may influence antigen presentation pathways leading to ACPA production and have been used to subdivide patients into distinct immunopathogenetic disease classes [4]. While the association between ACPA-positive RA and SE-containing HLA class II molecules is well established, the association between HLA-DR protective versus non-predisposing alleles and ACPA-negative RA is controversial. Certain HLA-DR alleles may reduce the risk of developing RA and have been termed "protective alleles"; however, the definition of "protective alleles" differs depending on the study [16], making all of these results difficult to interpret. Alleles with the DERAA motif at positions 70–74 in the third hypervariable region have been associated with a reduced risk of RA susceptibility [17;18] and

less severe disease [17;19;20], while other studies have found conflicting results regarding HLA-DR3 [21-24].

Reproducing genetic associations is very important, and studies performed in cohorts outside of North America or Northwest Europe are especially welcome due to differences in allele frequencies and genetic background. To better understand the effect of HLA in RA in the Spanish population, we investigated the association of SE-containing HLA-DRB1 alleles with susceptibility to RA, and then we examined the possible protective effect of HLA-DR3 and DERAA-encoding alleles. Finally, the effects of these alleles on the magnitudes of RF and ACPA production were determined.

MATERIALS AND METHODS

We studied 408 patients referred to the early arthritis clinic (EAC) of La Paz University Hospital, Madrid. Data from this cohort have been previously reported [14]. At enrollment or during follow-up, 235 patients fulfilled the 1987 ACR criteria for RA, and 173 were diagnosed with non-rheumatoid arthritis (mainly undifferentiated arthritis, psoriatic arthritis, reactive arthritis and other connective tissue diseases) (Table 1). Most of the patients (91%) in the two groups have been followed up for more than 1 year (mean follow-up, 6.5 years). As controls, we included 269 healthy volunteers. The study was approved by the La Paz University Hospital ethics committee, and all subjects were of Spanish origin and provided written informed consent.

For every patient, laboratory tests were performed on blood samples that were obtained during the patient's first visit to the clinic, before treatment with disease-modifying antirheumatic drugs, and were stored at $-40°C$. Rheumatoid factor (RF) was measured by nephelometry (Behring, Nephelometer Analyzer II) with a detection limit of 15 U/ml, and ACPA were determined by a second-generation anti-CCP-2 antibody ELISA (Immunoscan RA Mark 2; Eurodiagnostica, Arhem, The Netherlands) with a cut-off level of 25 arbitrary units/ml, according to the manufacturer's instructions. The range of

measurement was 0–1600 U/ml, and all values higher than this upper limit were truncated and considered as 1600 U/ml for the analysis.

In DNA samples obtained from peripheral blood of controls and cases, HLA class II alleles were genotyped using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO HLA-DRB1 typing kit, Dynal Biotech, Bromborough, UK). When necessary, high-resolution typing of HLA-DRB1*03 samples was performed using Dynal AllSetTM SSP DRB1*03. The following alleles were considered SE-positive: DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1402. DERAA-encoding alleles were HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304.

Statistical analysis was performed using the Statistical Package for the Social Sciences version 10.0 (SPSS, Chicago, IL). Odds ratios (OR) and proportions were compared by the chi-square test. Differences in values between groups were analyzed using Kruskal-Wallis and Mann-Whitney U tests for non-parametric data.

RESULTS

Of the 253 patients with RA, 143 (61.9%) had ACPA and 169 (72.5%) RF (Table 1). The presence of both autoantibodies was strongly associated with RA (OR 27.4, 95% confidence interval (CI) 14.08–53.34, p<0.001 and OR 14.99, 95% CI 9.18–24.45, p<0.001, respectively). In non-RA patients, only 9 (5.6%) and 27 (15.9%) had ACPA and RF, respectively (Table 1). Double positivity for both ACPA and RF were found in 137 (59.3%) RA patients and in 6 (3.5%) patients with other arthropathies.

The distribution of our study population with respect to HLA-DRB1 genotypes and alleles is shown in Tables 2 and 3. As described previously, an increased frequency of HLA-DRB1 alleles encoding the SE was found in patients with RA compared with non-RA and control subjects (56.2% vs. 35.5%, OR 2.33, 95% CI 1.56–3.5, p<0.001 and 56.2% vs. 41.6%, OR 1.8, 95% CI 1.26–2.56,

p=0.001, respectively). There was a dose-dependent effect of SE-encoding HLA-DRB1 alleles: 29.8% of RA cases and 24.9% of controls had one allele (OR 1.89, 95% CI 1.13–3.13), while 14% of RA cases and 5.6% of controls were homozygous for SE-encoding HLA-DRB1 alleles (OR 3.97, 95% CI 1.93–8.16) (Table 3).

The frequency of HLA-DR3 alleles was lower in the RA than in the non-RA group (18.3% vs. 28.3%, OR 0.57, 95% CI 0.36–0.90, p= 0.012) but similar to the healthy controls (18.3% and 19.7%, respectively). No differences in the DERAA-encoding allele distribution were found among the three groups (RA 23.4%, non-RA 26.6% and controls 26.8%, p=0.39). Next, we studied the effect of the presence of HLA-DR3 or DERAA-encoding alleles in combination with the SE on susceptibility to RA. In the presence of one SE allele, a HLA-DR3 or DERAA-encoding allele reduced the risk of developing RA, although the effect was not statically significant (OR 1.11, 95% CI 0.51–2.43 for HLA-DR3 and OR 1.04, 95% CI 0.46–2.36 for DERAA-encoding alleles) (Table 3). Finally, to study the effect of the presence of HLA-DR3 or DERAA-encoding alleles on susceptibility, we combined homozygous and heterozygous patients and controls for these alleles and compared them with "neutral" alleles. No differences in susceptibility were found for HLA-DR3 (OR 1.53, 95% CI 0.76–3.04) or DERAA-encoding alleles (OR 1.33, 95% CI 0.74–2.39).

To investigate whether HLA-DR alleles were associated with different RA phenotypes, we divided our RA population according to ACPA status (Table 4). We found a higher frequency of SE alleles in ACPA-positive RA compared with healthy controls (67.8% vs. 41.6%, OR 2.95, CI 1.93–4.53, p<0.001), but we did not observe this relationship with ACPA-negative RA patients and controls (35.2% vs. 41.6%, p=0.31), indicating that SE alleles are associated with ACPA production in RA. Inversely, HLA-DRB1 alleles encoding the DERAA sequence were more frequent in controls than in ACPA-positive RA patients (26.8% vs. 17.5%, OR 0.58, CI 0.34–0.96, p=0.03), and a similar trend was found for HLA-DR3 (19.7% vs. 12.6%, OR 0.32–1.04, p=0.07). However, the frequency of HLA-DR3 was similar in controls and ACPA-negative RA patients, indicating that the presence of these alleles confers a protective role only for ACPA-

positive RA. To further investigate whether, in ACPA-positive RA patients, the decreased frequencies of DERAA and HLA-DR3 alleles were secondary to the increased frequencies of SE, we stratified our study population by the presence of SE. Only a trend was observed for HLA DR3, and no effect was observed for DERAA-encoding alleles, probably because of the low numbers of patients remaining (Table 5). No effect on RF of HLA-DR3 (OR 1.10, 95% CI 0.43–2.83, p=0.8) or DERAA-encoding alleles (OR 0.78, 95% CI 0.28–2.15 p=0.5) was observed.

In RA patients, we observed a dose-dependent association between the presence of SE alleles and titers of RF and ACPA (Figure 1). With two SE alleles, ACPA had a median value of 915 Au/ml in our ELISA, with an interquartilic range (IR) of 124–1500. With one SE allele, there was a median ACPA of 400 (0–1050), and with no alleles it was 0 (0–650; p<0.001). The presence of HLA-DR3 or DERAA-encoding alleles was associated with markedly reduced ACPA levels. In the presence of HLA-DR3, ACPA had a median value of 0 (0–594), while in the absence of HLA-DR3, ACPA had a median value of 360 (0–1000) (p=0.002). ACPA was 0 (0–653) in the presence of DERAA-encoding alleles vs. 310 (0–1000) in the absence of any DERAA-encoding alleles (p=0.013). No association between RF titers and HLA-DR3 or DERAA-encoding alleles was found.

Next, we investigated the influence of DERAA-encoding alleles or HLA-DR3 together with one SE allele on RF and ACPA production. Although ACPA levels were lower in the presence of HLA-DR3 or DERAA alleles in combination with one SE allele (SE/DR3 or SE/DERAA) than in HLA-DR SE/– patients, these differences were not statistically significant (median 500 IQR (34–1250) in SE/– vs. 468 (0–760) in SE/DERAA and 32 (0–900), p=0.11. No similar trend for RF was found (Figure 2).

DISCUSSION

Since Gregersen et al. formulated the SE hypothesis in 1987 [25], several authors have investigated the relationship between RA and SE-encoding HLA DRB1 alleles. An important discovery is that these alleles are associated with ACPA in RA, irrespective of RF status [26]. Moreover, it has recently been demonstrated that SE alleles are not associated with RA but are specific for a disease characterized by ACPA [27]. Our results confirm these observations, as ACPA-positive RA was strongly associated with the SE, with a gene-dose effect.

It was proposed that SE alleles function as immune response genes in the development of ACPA, as no differences in ACPA levels were found between RA patients with one or two SE copies [15]. In our cohort, as in other studies, a risk hierarchy in ACPA production with a significant dose effect in patients with two predisposing alleles was found [23]. Some frequent SE alleles in Northern Europe, such as DRB1*0401 and *0404, appear to confer a much greater degree of risk for RA [28-30] and are associated with higher levels of ACPA than the DR1 or DR10 alleles are [29;30]. We have reported that in our Spanish inception cohort, DR1 and DR4 alleles with the SE are equally represented [31], and DR10 is a frequent SE allele in the Spanish population [32]. Therefore, it is possible that DR1- and/or DR10-heterozygous patients have lower levels of ACPA than DR4 patients and that DR1- and/or DR10-homozygous patients have higher levels of ACPA.

A lack of association between ACPA and SE alleles, when they were analyzed together, has been described, with 68% of SE-negative patients having ACPA, compared to 32% in our cohort [30]. One reason for the lack of association may be the influence of external risk factors. Tobacco exposure is a well-known environmental risk factor for the development of ACPA in the presence of SE alleles [33] but also in their absence [34]. Increased ACPA levels in tobacco users have been reported, but these effects were only relevant for DR1 and DR10 alleles, with no significant effect on DR4 patients who had higher levels regardless of tobacco exposure [29]. Other reasons may be that not only inherited SE alleles but also non-inherited SE alleles from the mother (NIMA)

can be associated with RA susceptibility [35], and SE-positive cells persist in SE-negative women as a consequence of pregnancy (microchimerism) [36].

There are no well-established genetic risk factors for ACPA-negative RA [4]. Two studies have reported an increased risk of ACPA-negative RA associated with the presence of HLA-DR3 [21;22]; however, our results, as those of two independent groups [23;24], do not support that finding. Instead, in HLA-DR3 carriers, we have only found a reduced risk of ACPA-positive RA. Another interesting finding of our study is that, as reported previously [21], HLA-DR3 carriers had lower levels of ACPA compared with DR3 non-carriers. As this result may have been due to the reduced risk of ACPA production, we further analyzed the effect of HLA-DR3 in the presence of one SE allele and found a reduction in ACPA levels. The fact that we did not observe levels above the upper limit of detection of our ELISA may limit our capacity to demonstrate statistically significant differences.

The higher frequency of HLA-DR3 found in non-rheumatoid arthritis patients in our cohort was not unexpected. HLA-DR3 has been associated with several autoimmune diseases and with autoantibody production, mainly as a part of an extended haplotype designated A1;B8;DR3 [37;38]. Whether the association with autoimmune diseases is caused by HLA-DR3 itself or by other nearby genes in linkage disequilibrium remains to be determined [21].

It has been proposed that some HLA class II alleles with a neutral or negative electric charge in their P4 pocket, such as DERAA-encoding alleles, reduce the risk of developing RA [17;23;39]; however, while the RA shared epitope is consistently and reproducibly associated with RA in Caucasians, the "protective" effect of some HLA alleles is far from fully accepted [16]. Our results are in accord with recent reports [28] and do not confirm that hypothesis. As with HLA-DR3, HLA DERAA-encoding alleles were only associated with a reduced risk of ACPA-positive RA, but this effect disappeared after stratification for the presence of the SE. As these same alleles have been associated with a reduced risk of RA [17;18], it is possible that these results are due to the low

number of patients remaining after stratification, and new studies including greater numbers of patients are needed to clarify this effect.

HLA-DR3 and HLA DERAA-encoding alleles were associated with lower levels of ACPA, and this finding may explain the association with less severe disease [19;20]. The progression of joint damage has been related to levels of ACPA, both in RA patients [40;41] and in animal models of arthritis [12], so the previously described less severe disease in DERAA allele carriers may be due to lower levels of ACPA. The reason for the reduced levels of ACPA in the presence of HLA-DR3 or DERAA-encoding alleles is not clear, but it has been suggested that some non-associated MHC class II molecules may contain P4 pockets that lack the proper size or charge to effectively accommodate the large polar side chains of citrulline, and they would be unable to bind to and present modified citrullinated peptides [42;43].

Overall, our data confirm the considerable complexity of HLA class II associations with RA. Many of the previously reported inconsistencies may be due not only to genetic differences or the influence of other genes in linkage disequilibrium but to the interaction with environmental risk factors. In summary, in this study confirmed the previously reported observation that ACPA-positive RA is strongly associated with the SE, with a gene-dose effect in a Spanish population. However, our results do not support the hypothesis that HLA-DR3 or DERAA-encoding alleles protect against RA or are associated with ACPA-negative RA, but rather they only reduce the risk of ACPA-positive RA.

CONCLUSIONS
In this study, we have examined the relationship between HLA-DRB1 alleles and RA in an inception cohort of early arthritis patients, and we confirm previous evidence of an association between SE-containing HLA-DRB1 alleles and ACPA-positive RA. Our results do not support a protective effect of HLA-DR3 or DERAA-encoding alleles against RA, nor are these alleles associated with ACPA-negative RA in the Spanish population. Instead, we suggest that these alleles reduce the risk of ACPA-positive RA, but these results must be confirmed with higher number of patients or by analyzing pooled data from

reported cohorts. The presence of theses alleles was associated with lower levels of ACPA antibodies, but in the presence of one SE allele, this interaction could not be demonstrated.

Abbreviations

RA, rheumatoid arthritis; SE, shared epitope; RF, rheumatoid factor; ACPA, anti–citrullinated protein antibodies; EAC, early arthritis clinic.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB, AC, JM and DP-S participated in the design of the study, helped in the statistical analysis and helped draft the manuscript. TC and EM-C participated in data collection and helped draft the manuscript. GO helped in the statistical analysis and participated in the design of the study. MAL-N and JLV carried out the HLA determination, analyzed and interpreted the data and helped in the manuscript preparation. All authors read and approved the final manuscript.

<div align="center">Reference List</div>

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Table 1. Baseline characteristics of the patients in the study

	RA (n=253)	Non-RA (n=173)
Age (mean ± SD)	62 ± 12	50 ± 14
Women, %	64	55
ACPA positive	143 (61.9%)	9 (5.6%)
RF positive	169 (72.5%)	27 (15.9%)
Duration of symptoms, median (range) weeks	20 (3–52)	15 (2–52)

RA = rheumatoid arthritis; Non-RA = other arthropathies; RF = IgM rheumatoid factor;

ACPA = anti–citrullinated protein antibodies.

Table 2. Distribution of HLA-DRB1 alleles in the study population. Number and percent of patients in each group

		RA (235 patients)	Non-RA (173 patients)	Controls (n=269)
SE	*0101	49 (10.4%)	15 (4.3%)	32 (5.9%)
	*0102	17 (3.6%)	13 (3.7)	16 (3.0%)
	*0401	27 (5.7%)	10 (2.9%)	23 (4.3%)
	*0404	19 (4%)	7 (2.0%)	10 (1.8%)
	*0405	27 (5.7%)	9 (2.6%)	17 (3.1%)
	*0408	4 (0.8%)	5 (1.4%)	8 (1.5%)
	*1001	18 (3.8%)	7 (2.0%)	18 (3.3%)
	*1402	4 (0.8%)	2 (0.6%)	3 (0.5%)
DR3	*03	43 (9.1%)	54 (15.6%)	55 (10.2%)
DERAA	*0103	4 (0.8%)	9 (2.6%)	14 (2.6%)
	*0402	7 (1.5%)	6 (1.7%)	10 (1.8%)
	*1102	6 (1.3%)	5 (1.4%)	11 (2.0%)
	*1103	7 (1.5%)	7 (2.0%)	12 (2.3%)
	*1301	25 (5.3%)	20 (5.8%)	27 (5.0%)
	*1302	11 (2.3%)	1 (0.3%)	5 (0.9%)
	*1304	-	-	-

RA = rheumatoid arthritis; Non-RA = other arthropathies; SE = shared epitope alleles; DERAA = HLA with DERAA-encoding alleles.

Table 3. Distribution of HLA-DRB1 genotypes

	RA (235 patients)		Non-RA (n=173)		Controls (n=269)	
	Number	%	Number	%	Number	%
SE/SE	33	14.0	7	4.0	15	5.6
SE/–	70	29.8	31	17.9	67	24.9
SE/DR3	15	6.4	12	6.9	16	5.9
DR3/–	21	8.9	25	14.4	23	8.6
DR3/DR3	-	-	5	2.9	2	0.7
SE/DERAA	14	6.0	11	6.3	14	5.2
DERAA/–	29	12.3	26	15.0	39	14.5
DERAA/DERAA	5	2.1	2	1.1	7	2.6
DR3/DERAA	7	3.0	7	4.0	12	4.5
No alleles (–/–)	41	17.4	47	27.1	74	27.5

RA = rheumatoid arthritis; Non-RA = other arthropathies; SE = shared epitope alleles; DR3 = HLA-DR3; DERAA = HLA with DERAA-encoding alleles.

Table 4. Association of HLA-DRB1 alleles with the SE, HLA-DR3 and DERAA motif with ACPA-positive or ACPA-negative RA

		Controls (n=269)	ACPA$^+$ (n=143)	p	OR (95% CI)	ACPA$^-$ (n=88)	p	OR (95% CI)
SE	absence n (%)	157 (58.4)	46 (32.2)	0.0001	2.95 (1.93–4.53)	57 (64.8)	0,31	0.76 (0.46–1.27)
	presence n (%)	112 (41.6)	97 (67.8)			31 (35.2)		
DR3	absence n (%)	216 (80.3)	125 (87.4)	0.07	0.58 (0.32–1.04)	63 (71.6)	0,10	1.61 (0.93–2.80)
	presence n (%)	53 (19.7)	18 (12.6)			25 (28.4)		
DERAA	absence n (%)	197 (73.2)	118 (82.5)	0.03	0.58 (0.34–0.64)	58 (65.9)	0,22	1.41 (0.84–2.37)
	presence n (%)	72 (26.8)	25 (17.5)			30 (34.1)		

RA = rheumatoid arthritis; SE = shared epitope allele; DR3 = HLA-DR3; DERAA = DERAA-encoding allele; ACPA = anti–citrullinated protein antibodies.

Table 5. Association of HLA-DR3 and DERAA-encoding alleles stratified by the presence of shared epitope alleles with ACPA-positive RA
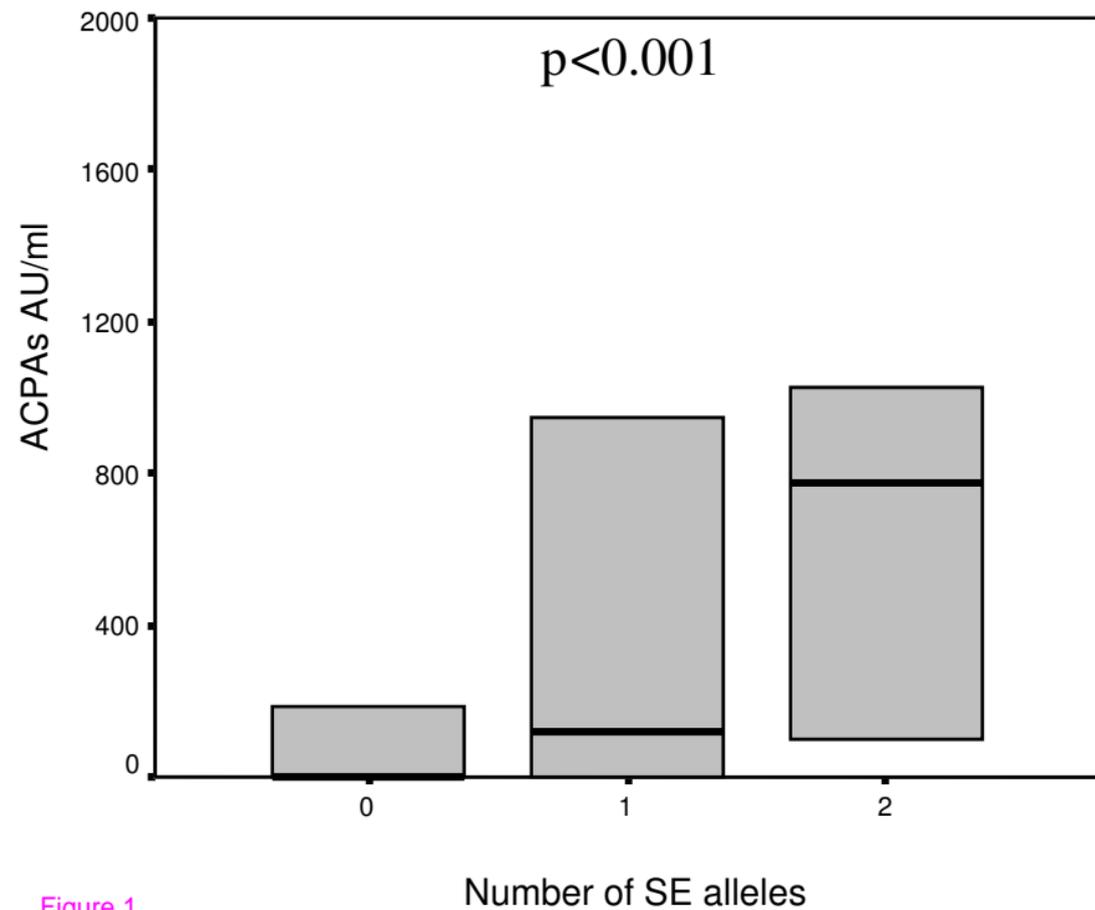
		Controls (n=269)	ACPA-positive RA patients (n=143)		
				p	OR (95% CI)
SE-positive	DR3-positive	16 (14.3)	8 (8.2)	0.17	0.53 (0.22–1.22)
	DR3-negative	96 (85.7)	89 (91.8)		
SE-negative	DR3-positive	37 (23.6)	10 (21.7)	0.84	0.90 (0.40–1.98)
	DR3-negative	120 (76.4)	36 (78.3)		
SE-positive	DERAA-positive	14 (12.5)	10 (10.3)	0.85	0.58 (0.34–1.90)
	DERAA-negative	98 (87.5)	87 (89.7)		
SE-negative	DERAA-positive	58 (36.9)	15 (32.6)	0.72	0.82 (0.41–1.65)
	DERAA-negative	99 (63.1)	31 (67.4)		

RA = rheumatoid arthritis; SE = shared epitope allele; DR3 = HLA-DR3; DERAA = DERAA-encoding allele; ACPA = anti–citrullinated protein antibody.

Figure 1. Box plots of ACPA levels **(a)** in arbitrary units and rheumatoid factor **(b)** in UI/ml in rheumatoid arthritis patients depending on the number of shared epitope alleles. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles. Lines inside the boxes represent medians. Differences in values between groups were analyzed using the Kruskal-Wallis test for non-parametric data.

Figure 2. Box plots of ACPA levels **(a)** in arbitrary units and rheumatoid factor **(b)** in UI/ml in rheumatoid arthritis patients depending on the presence of one shared epitope allele combined with a HLA-DR3 or DERAA-encoding allele. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent medians. Differences in values between groups were analyzed using Kruskal-Wallis test for non-parametric data. N.S., not significant.
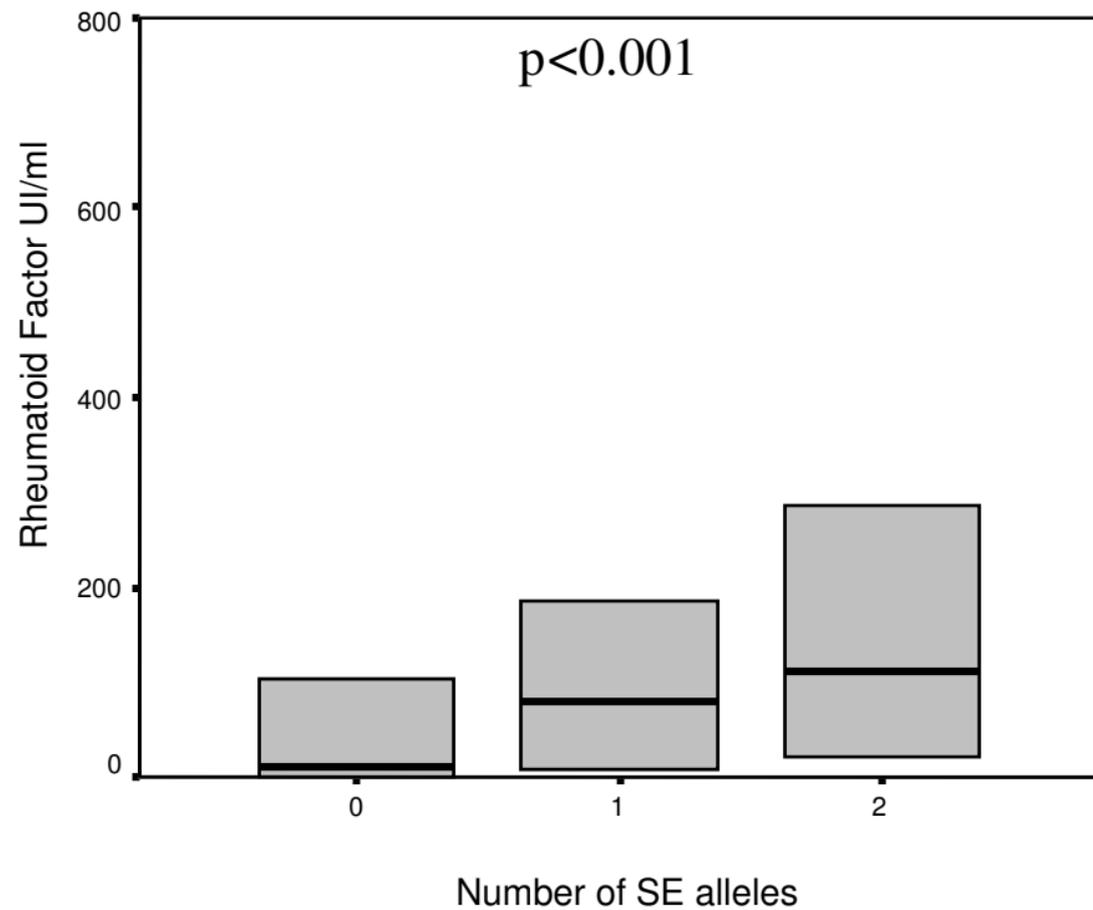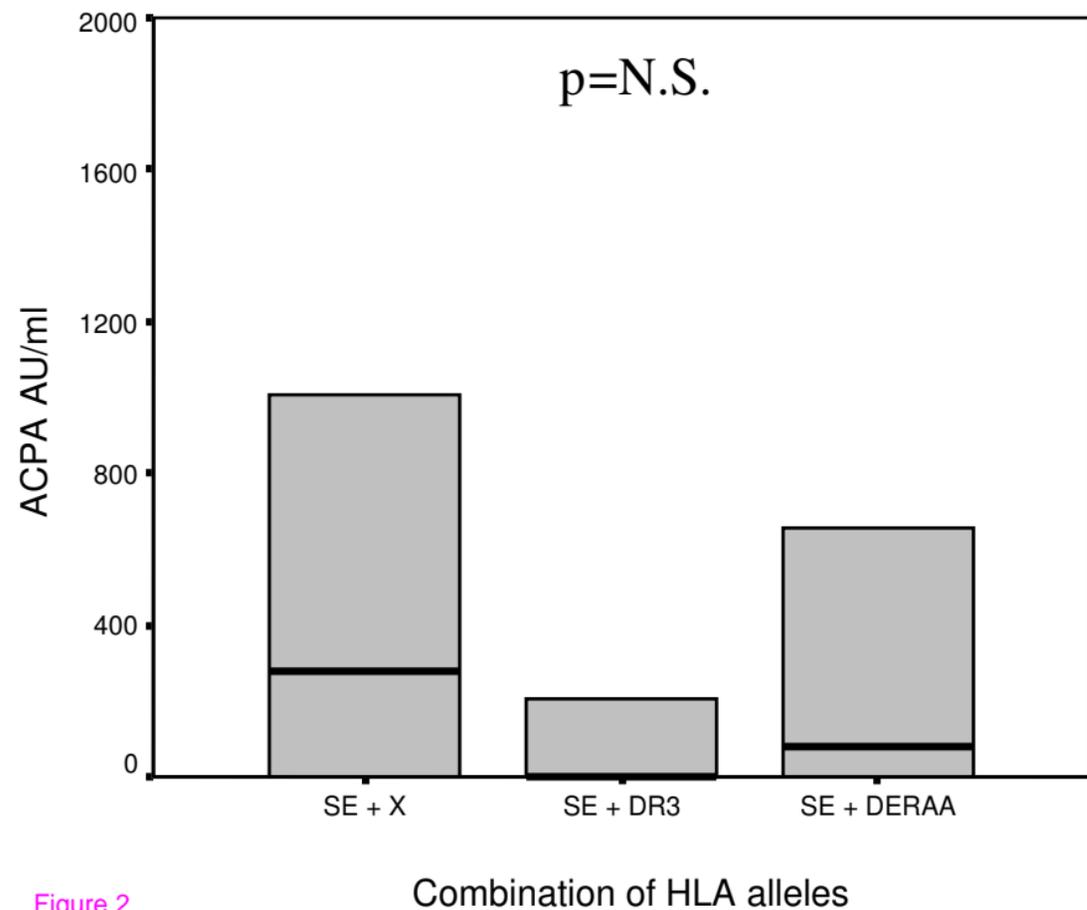
(a)

p<0.001

ACPAs AU/ml
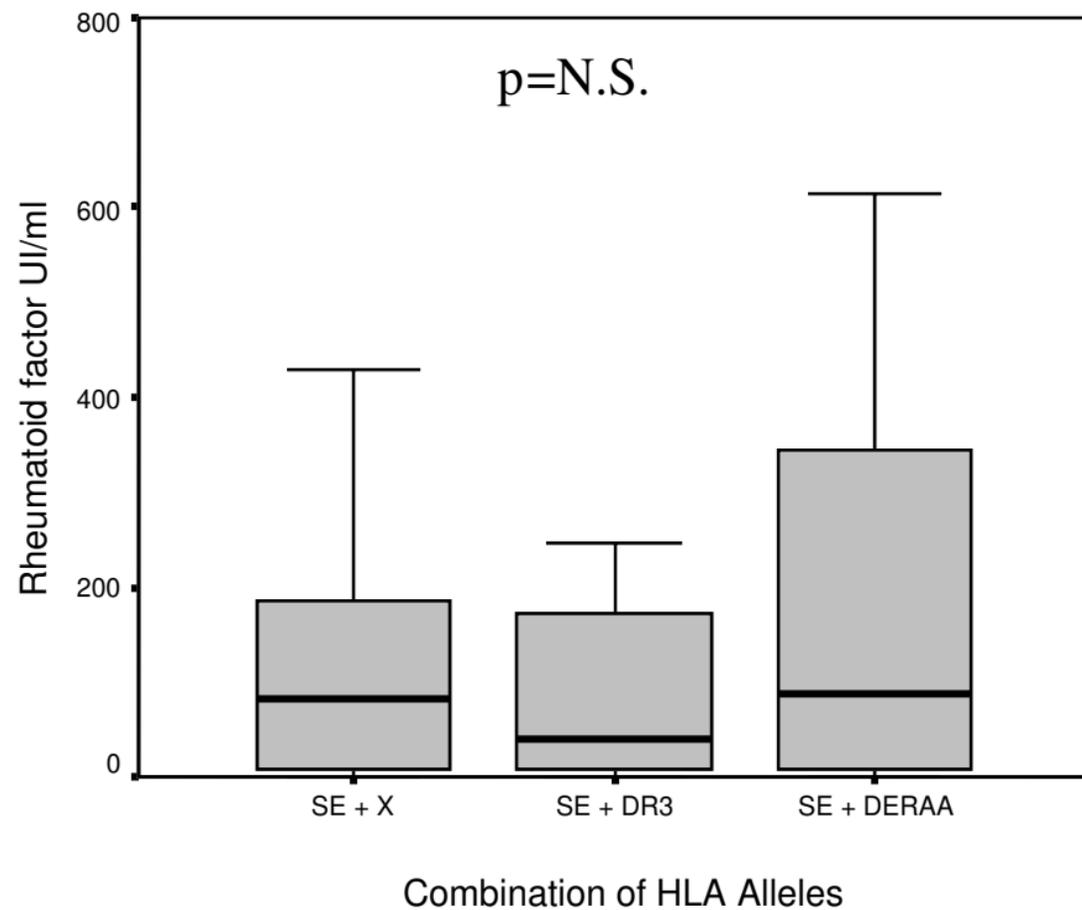
Number of SE alleles

(b)

p<0.001

Rheumatoid Factor UI/ml

Number of SE alleles

Figure 1

(a) | (b)

ACPA AU/ml — Combination of HLA alleles

Rheumatoid factor UI/ml — Combination of HLA Alleles

Figure 2