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Chapter 2

Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301

A meta-analysis of HLA-DRB1 associations with ACPA-positive and ACPA-negative rheumatoid arthritis in four European populations


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ABSTRACT

Objective The protective effect of HLA-DRB1 alleles on the development of rheumatoid arthritis (RA) is poorly understood. We performed a meta-analysis of four European populations to investigate which HLA-DRB1 alleles are associated with protection in anti-citrullinated protein antibody (ACPA)-positive and ACPA-negative RA.

Methods More than 2800 patients and 3000 controls with information on HLA-DRB1 typing and ACPA-status were collected from four European countries: Norway, Sweden, the Netherlands and Spain. The odds ratio’s (ORs) and 95% confidence intervals (CI) associated with the different HLA-DRB1 alleles were analyzed in a combined meta-analysis, focused on protective alleles and classifications. The analysis of ACPA-positive RA was stratified for the shared epitope (SE) alleles to correct for skewing due to this association.

Results In ACPA-positive RA, the only alleles which conveyed protection after stratification for SE, were HLA-DRB1*13 alleles (OR (95%CI): 0.54 (0.38-0.77)). The protective effect of the allele classifications based on the DERAA and D70 sequence was no longer present after exclusion of DRB1*13 (OR (95%CI) D70: 0.97 (0.75-1.25)), indicating that DRB1*13, rather than the DERAA or D70 sequence as such is associated with protection. Within the DRB1*13 alleles only DRB1*1301 was associated with protection (OR (95%CI): 0.24 (0.09-0.59)). Protection appeared to follow a North-South gradient with the strongest association in Northern countries. In ACPA-negative RA, there were no robust associations with HLA-DRB1 alleles.

Conclusion Our data do not support any of the classifications of protective alleles and indicate that protection against ACPA-positive RA is predominantly associated with HLA-DRB1*1301.
INTRODUCTION

The Human Leucocyte Antigens (HLA) are the most prominent genetic risk factor for Rheumatoid Arthritis (RA). The association between RA and the HLA region was originally discovered based on the observation that lymphocytes from RA patients were not reactive against cells from other RA patients in mixed lymphocyte cultures. This meant that RA patients had certain HLA alleles in common, which were less prevalent in control populations. Serological testing subsequently revealed that the HLA-D-Related (DR)w4 alloantigen, but not HLA-A, B or C antigens were associated with RA. Later studies demonstrated that several of the HLA-DR alleles were associated with RA, which led to the formulation of the Shared Epitope (SE)-hypothesis in 1987. This hypothesis provided a theoretical background for the observed associations between the HLA region and RA based on the fact that all HLA-DR alleles which predispose to RA have the same or a similar amino acid sequence (shared epitope) at positions 70-74 of the HLA-DRB1 molecule. This sequence is located in the peptide-binding groove of the HLA alleles and may therefore be directly involved in the presentation of peptides to arthritogenic T cells. However, due to the complexity of the HLA region, the association between the HLA region and RA is multifaceted and it is now known that not all HLA SE alleles contribute to RA to the same extent. Nonetheless, the formulation of the HLA SE hypothesis has provided a rationale for combining several HLA molecules in analyses and has thereby enabled further well-powered investigations of the contribution of the HLA region to the risk of RA.

The discovery of anti-citrullinated protein antibodies (ACPA) has led to a paradigm shift in the investigation of genetic risk factors for RA. The HLA SE alleles were found to not predispose to RA as such, but rather to ACPA-positive disease, which is present in approximately two-thirds of patients with RA according to the ACR criteria. These observations are very important for the pathophysiological concept of disease development, since they indicate that the HLA SE alleles may be involved in the induction of the ACPA-response. Several SNPs known to be associated with RA, such as PTPN22, were also discovered to be specifically associated with ACPA-positive RA. In contrast, DRB1*03 has been reported to be associated with ACPA-negative disease, although not all studies have confirmed this association. These differences in underlying risk factors indicate that ACPA-positive and ACPA-negative RA may constitute distinct disease entities with a different underlying pathogenetic background.

In addition to the HLA-DRB1 alleles which contribute to RA susceptibility, other HLA-DRB1 alleles confer protection against disease. These protective HLA-DRB1 alleles have been categorized according to several different classifications, analogous to the shared epitope classification of susceptibility alleles. The three best-known classifications postulate that HLA-DRB1 alleles with a protective effect harbor the “shared sequence.”
DERAA at position 70-74, or an aspartic acid (D) at position 70, or an isoleucine (I) at position 67 of the HLA-DRB1 molecule. The DERAA and D70 alleles have been shown to be protective both in the presence and the absence of SE alleles, which demonstrates that their protective effect is not solely due to the absence of SE alleles. A new classification of HLA-DRB1 alleles has recently been put forward which incorporates both predisposing and protective effects. Although this has provided some interesting nuances with regard to the predisposing effect of the different shared epitope alleles, it is unclear if this classification accurately describes the protective HLA effects.

The multitude of classifications of protective HLA alleles illustrates that a protective effect of HLA alleles in RA is now well-accepted. However, it is still unclear which HLA alleles exactly are protective. Geographical differences in the prevalence of HLA alleles have led to conflicting results, which have been further complicated by the use of different classifications. Furthermore, it is as yet unclear whether protective effects are present in ACPA-positive as well as in ACPA-negative disease.

For these reasons, we wished to determine the contribution of individual HLA-DRB1 alleles to RA, both with respect to susceptibility and protection in a meta-analysis across European populations. Using data from four different populations, being from Norway, Sweden, The Netherlands and Spain, we investigated the HLA-DRB1 associations with ACPA-positive and ACPA-negative RA in more than 2800 patients and 3000 controls. A significant protective effect of HLA-DRB1*13 was found which remained present after stratification for the SE effect. Moreover, the protective effect of HLA-DRB1*13 was only observed for ACPA-positive and not for ACPA-negative disease. An in-depth analysis of the protective classifications revealed that the protective effect of DERAA and D70 alleles was limited to the HLA-DRB1*13 alleles and was in fact only observed for DRB1*1301. Together, our data do not support any of the classifications described above and indicate that protection is mainly associated with DRB1*1301.

MATERIALS AND METHODS

Populations

Data on cases and controls were contributed by cohorts from four different European countries: Norway, Sweden, the Netherlands and Spain. The protocol of each cohort was approved by the relevant local ethics committee and all participants provided informed consent. More than 97% of cases and controls in all four cohorts was of Caucasian descent.

The Norwegian dataset consisted of RA patients who participated in the Oslo RA registry (ORAR) or the European Research on Incapacitating Disease and Social Support (EURIDISS) cohort. For the ORAR, which was initiated in 1992, inclusion criteria were a diagnosis of RA according to the 1987 ACR classification criteria and a residential address...
in Oslo. The EURIDISS, which commenced in 1991, enrolled consecutive RA patients with a maximum disease duration of 4 years at baseline. Both RA cohorts have been described in detail elsewhere \(^{25,26}\). Controls were randomly selected from the Norwegian Bone Marrow Registry and cases and controls were matched for gender at a group level.

The Swedish Epidemiological Investigation of RA (EIRA) cohort recruited cases and controls aged 18-70 from May 1996 to December 2003 from a geographically defined area in the south and central regions of Sweden. Patients were seen by rheumatologists at private as well as general health care units and were eligible for inclusion if they fulfilled the 1987 ACR criteria for RA. Controls were randomly selected from a national population register and were matched to the cases for age, sex and residential area. More details on the EIRA have been described in previous publications \(^{27}\).

Data on Dutch cases were provided by two inception cohorts of early arthritis patients, the Leiden Early Arthritis Clinic (EAC) and the BehandelStrategieén (BeSt) trial. The Leiden EAC was initiated in 1993 and included patients with recent-onset arthritis (less than 2 years of complaints) treated at the Leiden University Medical Center (LUMC). For the BeSt study, patients with arthritis with a maximum disease duration of 2 years and active disease were recruited at 20 centers in the western part of the Netherlands from 2000 to 2002. Only patients with a diagnosis of RA were included in the present study. These cohorts are described in further detail elsewhere \(^{28,29}\). Dutch controls were randomly selected by the section Immunogenetics and Transplantation Immunology (ITI) of the Department of Immunohematology and Blood Transfusion, LUMC.

The Spanish dataset consisted of RA patients fulfilling the ACR criteria for RA, who were recruited from 4 Spanish hospitals in Granada, Seville, Lugo and Madrid. Blood donors and bone marrow donors from the same cities were included as healthy controls. Controls were matched to patients by age and sex. More characteristics of the Spanish dataset have been presented in previous reports \(^{30}\).

**Genotyping**

The genotyping procedures of the HLA-DRB1 alleles have been described in previous publications \(^{14,20,30,31}\). High-resolution, 4-digit typing was available for the entire Norwegian and Spanish cohorts. For the Dutch cohort, low-resolution typing was complemented by 4-digit typing of the DRB1*04 alleles, and by specific probes to detect the presence of the SE or DERAA sequence in individuals carrying DRB1*01, DRB1*10, DRB1*11 or DRB1*13 alleles. DRB1*1301 and *1302 were differentiated in part by 4-digit typing, and in part on the basis of their known, specific associations with HLA-DRB3 and -DQB1 alleles which were determined in the entire dataset.

Similarly, for the Swedish dataset high-resolution typing of all DRB1*04 alleles was performed. All alleles containing a DERAA sequence in the Swedish cohort were identified by help of an interpretation table for the HLA-DRB1 low resolution kit. This allowed
the ascertainment of the following allelic groups in this cohort: DRB1*0103, DRB1*0402, DRB1*11-DERAA (*1102 or *1103) and DRB1*13-DERAA (*1301 or *1302). Unfortunately, this did not allow the differentiation of DRB1*1301 from DRB1*1302.

Shared epitope alleles and protective classifications were defined according to Table 1.

Serological measurements
ACPA were determined by the measurement of antibodies against cyclic citrullinated peptides by a second-generation ELISA (anti-CCP2) (for the ORAR: DIASTAT; Axis-Shield Diagnostics, Dundee, UK. For the EURIDISS: INOVA, San Diego, CA, USA. For the Swedish, Dutch and Spanish cohorts: Immunoscan RA Mark 2; Eurodiagnostica, Malmö/Arnhem, Sweden/The Netherlands). These different anti-CCP2-assays have been shown to provide very similar results. Samples with a value above the cut-off as specified by the manufacturer were considered positive.

Statistical analysis
For each of the cohorts, we used logistic regression analysis to calculate the odds ratio's (ORs) and 95% confidence intervals of developing ACPA-positive and ACPA-negative RA in association with the different HLA-DRB1 alleles. To take into account the matching of cases and controls in the study design, the analyses in the Swedish cohort were corrected for residential area, since age and gender have been shown to have no effect on the distribution of HLA alleles. A dominant genetic model which estimates the effect of the presence of a certain allele, irrespective of the presence of 1 or 2 copies, was used for all analyses. This model provided a better fit of the data than an additive allele model which assumes the effect in homozygotes to be considerably larger than the effect in heterozygote individuals.

Due to the strong predominance of SE alleles among ACPA-positive patients, all other alleles are inherently less prevalent in patients than in controls. This results in seemingly protective effects of these alleles, which are in fact merely caused by skewing due to the large difference in the prevalence of SE alleles. In order to obtain an accurate estimate of the effect of the non-SE alleles, the analyses for these alleles in ACPA-positive patients were therefore stratified for the presence of SE in the following manner. For each non-SE allele (e.g. DRB1*03) the six possible combinations of this allele with SE alleles were investigated:

A) DRB1*03/DRB1*03  B) DRB1*03/x  C) x/x
D) SE/SE  E) SE/x  F) SE/DRB1*03

For the SE-negative stratum, the presence of DRB1*03 was compared to the absence of DRB1*03, hence the effect in group A and B was investigated using group C as the reference category. This corresponds to the dominant genetic model as described above. For the SE-positive stratum, the risk associated with the genotype F (SE/DRB1*03) was
analyzed using group E (SE/x) as the reference category, to adjust for the risk associated with the presence of 1 SE allele.

Subsequently, we performed a meta-analysis using the effect sizes (β) and standard errors of the different cohorts. To account for the fact that there was significant statistical heterogeneity (Q-statistic, p-value<0.10) in a small number of the analyses, a random effect approach was applied for all comparisons. This method allows between-study heterogeneity and incorporates it in the calculations. The data were analyzed per cohort with the Statistical Package for the Social Sciences (SPSS) 16.0. For the meta-analysis, we used the freely available software environment for statistical computing R.

RESULTS

The study cohort consisted of 2806 RA patients and 3772 controls from four different European populations. The distribution of cases and controls across the cohorts was: Norway: 788 cases and 898 controls, Sweden: 827 cases and 934 controls, the Netherlands: 844 cases and 1213 controls, and Spain: 347 cases and 727 controls. All patients fulfilled the 1987 ACR criteria for RA. The proportion of patients which was ACPA-positive was very similar in all cohorts and ranged from 58% to 62%.

The classifications of predisposing and protective HLA-DRB1 alleles, which have been described to be associated with RA, are listed in Table 1. The Shared Epitope (SE) classification incorporates several HLA-DRB1 alleles which confer a high risk for ACPA-positive disease, with reported odds ratio’s (ORs) ranging from 4.6 to 11.3.

The DERAA, D70 and I67 alleles have been claimed to be associated with protection from RA, with ORs of 0.50, 0.23 and 0.14 (for DERAA presence, D70 homozygosity and I67 homozygosity respectively). As shown in Table 1 there is considerable overlap between the protective classifications. The frequencies of the presence of the separate HLA-DRB1 alleles in the four control populations are also presented in Table 1, as well as the frequencies of the allele-classifications.

Associations between HLA-DRB1 alleles and ACPA-negative RA

Table 2 shows the results of the meta-analysis for ACPA-negative RA. Although the data show a predisposing effect of DRB1*03 and DRB1*04, as well as a possible protective effect of DRB1*07 and DRB1*15 on ACPA-negative RA, these associations were only weakly significant. With regard to the effects of DRB1*03 and DRB1*07 there were marked geographical differences. The previously reported association between DRB1*03 and susceptibility was present in the two Scandinavian cohorts and in the Dutch cohort, but was absent in Spain. However, a recent extensive study in Sweden did not reveal a predisposing effect of DRB1*03 on ACPA-negative RA, indicating that more studies will be
Table 1. Frequencies and classifications of HLA-DRB1 alleles according to predisposition and protection in RA

<table>
<thead>
<tr>
<th>Allele</th>
<th>Shared epitope</th>
<th>DERAA</th>
<th>D70</th>
<th>I67</th>
<th>Frequency in controls in %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>*0101</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>*0102</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>*0103</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>2.1</td>
</tr>
<tr>
<td>*03</td>
<td>-</td>
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<td>25</td>
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<td></td>
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<td></td>
<td>22</td>
</tr>
<tr>
<td>*0402</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>*0403</td>
<td>-</td>
<td>-</td>
<td></td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
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</tr>
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<td>-</td>
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<td>0.6</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
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<td>X</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>*08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.5</td>
</tr>
<tr>
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<td>-</td>
<td></td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td>*1001</td>
<td>X</td>
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<td></td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>*1101</td>
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<td></td>
<td></td>
<td></td>
<td>6.3</td>
</tr>
<tr>
<td>*1102</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>*1103</td>
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<td>*1104</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td>*12</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>*1301</td>
<td>X</td>
<td>X</td>
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<td>9.2</td>
</tr>
<tr>
<td>*1302</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>*1303</td>
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<td>X</td>
<td></td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>*1454</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>*15</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
</tbody>
</table>

Rare alleles with a median prevalence in controls < 0.5% were not listed.

No = Norway, Sw = Sweden, Nl = Netherlands, Sp = Spain.

* In the Swedish and Dutch cohorts, four-digit typing was not available for all alleles as described in the Methods section. The frequencies were therefore listed in the following manner: sum of DRB1*0101 and DRB1*0102, sum of all DRB1*10 alleles (in the row of *1001), sum of DRB1*1101 and DRB1*1104 (in the row of *1101), sum of DRB1*1102 and DRB1*1103, (for the Swedish cohort: sum of DRB1*1301 and DRB1*1302) and sum of all DRB1*14 alleles (in the row of *1454).

It has recently been shown that the majority of individuals previously genotyped as DRB1*1401 in fact carry the genotype DRB1*1454. In anticipation of probable genotyping revisions we have therefore listed DRB1*1454 as the most common DRB1*14 allele.
required in order to draw definitive conclusions about this association. With regard to the protection conferred by DRB1*07, there appeared to be a North-to-South gradient with the strongest protective effect in Norway and no observable effect in Spain.

To perform a more detailed analysis of possible protective alleles, we also investigated the effects of the three classifications proposed to be associated with protection: the DERAA-, D70- and I67- alleles. The DERAA alleles did not convey a protective effect for ACPA-negative RA, while both the D70- and I67- alleles showed a modest protective effect (OR (95% CI) 0.75 (0.59-0.96) and 0.70 (0.53-0.94) respectively).

Associations between HLA-DRB1 alleles and ACPA-positive RA

Table 3 displays the results of the meta-analysis for ACPA-positive RA. Since our aim was to specifically investigate protective effects, the table only lists the four-digit subtype analysis of alleles which have been reported to be associated with protection. Due to the preponderance of SE alleles among ACPA-positive patients, all other alleles are inherently less prevalent in patients than in controls. This leads to seemingly protective effects of these alleles, which are in fact merely the result of skewing caused by the large difference in the prevalence of SE alleles. In order to obtain an accurate estimate of the effect of the non-SE alleles, the analyses for these alleles were therefore stratified for the presence of SE.

The well-known association between SE alleles and susceptibility to ACPA-positive RA is confirmed by our data, as well as the hierarchy in the strength of this association, being DRB1*04>*10>*01. We also observed predisposing effects of DRB1*09, *15 and *16, although the effect of *09 and *16 alleles was limited to SE-negative individuals. Despite the fact that some of these associations were relatively weak, they nonetheless suggest that

<table>
<thead>
<tr>
<th>HLA-DRB1 allele</th>
<th>ACPA-negative patients</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>*01</td>
<td>1.06 (0.90-1.25)</td>
<td>0.46</td>
</tr>
<tr>
<td>*03</td>
<td>1.39 (1.01-1.93)</td>
<td>0.05</td>
</tr>
<tr>
<td>*04</td>
<td>1.17 (1.00-1.37)</td>
<td>0.05</td>
</tr>
<tr>
<td>*07</td>
<td>0.67 (0.48-0.95)</td>
<td>0.03</td>
</tr>
<tr>
<td>*08</td>
<td>0.97 (0.59-1.60)</td>
<td>0.90</td>
</tr>
<tr>
<td>*09</td>
<td>0.90 (0.54-1.50)</td>
<td>0.68</td>
</tr>
<tr>
<td>*10</td>
<td>1.04 (0.67-1.60)</td>
<td>0.86</td>
</tr>
<tr>
<td>*11</td>
<td>1.01 (0.79-1.29)</td>
<td>0.95</td>
</tr>
<tr>
<td>*12</td>
<td>0.84 (0.59-1.20)</td>
<td>0.34</td>
</tr>
<tr>
<td>*13</td>
<td>0.87 (0.73-1.03)</td>
<td>0.10</td>
</tr>
<tr>
<td>*14</td>
<td>1.09 (0.80-1.48)</td>
<td>0.58</td>
</tr>
<tr>
<td>*15</td>
<td>0.78 (0.65-0.94)</td>
<td>0.01</td>
</tr>
<tr>
<td>*16</td>
<td>1.04 (0.42-2.58)</td>
<td>0.94</td>
</tr>
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</table>
Table 3. HLA-DRB1 associations with ACPA-positive RA, meta-analysis results

<table>
<thead>
<tr>
<th>HLA-DRB1 allele</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>Stratification, OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>*01</td>
<td>1.33 (1.10-1.66)</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*0101 and *0102</td>
<td>1.44 (1.18-1.74)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*0103</td>
<td>0.31 (0.13-0.75)*</td>
<td>0.009</td>
<td></td>
<td>#</td>
</tr>
<tr>
<td>*03</td>
<td>0.64 (0.55-0.75)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*04</td>
<td>3.76 (2.93-4.84)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*0402</td>
<td>1.48 (0.81-2.71)</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>other *04</td>
<td>3.74 (2.86-4.91)</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>*07</td>
<td>0.56 (0.47-0.67)</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>*08</td>
<td>0.50 (0.36-0.70)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*09</td>
<td>1.43 (0.94-2.16)</td>
<td>0.10</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>*10</td>
<td>2.37 (1.56-3.60)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>*11</td>
<td>0.56 (0.46-0.68)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1102 and *1103</td>
<td>1.01 (0.61-1.67)</td>
<td>0.97</td>
<td></td>
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</tr>
<tr>
<td>other *11</td>
<td>0.49 (0.39-0.62)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>*12</td>
<td>0.60 (0.42-0.84)</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*13</td>
<td>0.33 (0.25-0.45)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1301 and *1302</td>
<td>0.31 (0.22-0.45)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>other *13</td>
<td>1.01 (0.48-2.10)</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*14</td>
<td>0.48 (0.34-0.68)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*15</td>
<td>0.69 (0.57-0.82)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*16</td>
<td>1.21 (0.73-1.99)</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Printed in bold are effects with a p-value below 0.01. For the non-SE alleles, only those associations with a p-value ≤ 0.005 after stratification for SE, are printed bold.

* No cases in Leiden cohort.
# Stratification for SE led to absence of cases or controls in the different strata in two of the four cohorts, therefore not performed.
** No cases in Norwegian cohort.
## HLA-DRB1*1402 alleles have a SE motif and were exceedingly rare in all cohorts (prevalence below 0.3% in controls). Therefore the HLA-DRB1*14 effect was also stratified for SE, after exclusion of individuals with DRB1*1402 alleles.
the effect of HLA-DRB1 alleles on susceptibility to ACPA-positive RA may extend beyond the SE alleles.

Intriguingly, the DRB1*13 alleles appeared to be the only alleles associated with protection (Table 3). In both the SE-negative and SE-positive stratum, the protective effect of DRB1*13 not only remained present, but was also associated with a considerable effect size (OR 0.54 and 0.57 respectively) (Figure 1).

In the subtype analysis of some of the protective alleles, e.g. in the case of DRB1*0103 and DRB1*0402, stratification resulted in a lack of cases or controls in several cohorts,

Figure 1A: Unstratified analysis.
Figure 1B: SE-negative stratum.
Figure 1C: SE-positive stratum.

Figure 1: The effect of DRB1*13 on ACPA-positive RA.
Forest plots depicting the ORs and 95% CIs of the four separate cohorts and the combined estimate of the random effects meta-analysis.
Figure 1A: Unstratified analysis.
Figure 1B: SE-negative stratum.
Figure 1C: SE-positive stratum.
rendering a meta-analysis ineffective. We can therefore not formally exclude that these alleles may be associated with a protective effect, although in the case of *0402 this is unlikely based on the result of the non-stratified analysis.

The protective effect of DRB1*13 and protective classifications on ACPA-positive RA

All protective classifications described for RA include DRB1*13 or some of the DRB1*13 suballeles (Table 1). When analyzed according to the different protective classifications, the protective effect of the I67 alleles did not remain significant after stratification for SE. Both the D70 and the DERAA alleles were associated with a protective effect, which

![Figure 2: The effect of D70 alleles with and without DRB1*13 alleles in ACPA-positive RA.](image)

Figure 2A: Effect of all D70 alleles, SE-negative stratum.
Figure 2B: Effect of all D70 alleles, SE-positive stratum.
Figure 2C: Effect of D70 alleles after exclusion of DRB1*13, SE-negative stratum.
Figure 2D: Effect of D70 alleles after exclusion of DRB1*13, SE-positive stratum.
remained present after stratification for SE (Figure 2A and 2B), yet it was remarkable that, apart from DRB1*13, none of the alleles with a D at position 70 or a DERAA-motif appeared to confer protection by themselves (Table 3). We therefore investigated if the protection associated with the group of D70 or DERAA alleles as a whole, could be explained solely by the protective effect of the DRB1*13 alleles. To this end, we excluded the DRB1*13 alleles from the analysis and re-analyzed the effect of all alleles with a D at position 70 on ACPA-positive RA. As can be seen in Figure 2C and 2D, these alleles were not protective, despite the fact that they had a D at position 70. The same was the case for the DERAA alleles (data not shown). These data indicate that the presence of a D at position 70 or of

![Figure 3: The effect of DRB1*1301 and *1302 in ACPA-positive patients.](image)

Forest plots depicting the ORs and 95% CIs of the three separate cohorts with high-resolution typing of the DRB1*13 alleles, and the combined estimate of the random effects meta-analysis.

Figure 3A: Effect of DRB1*1301, SE-negative stratum.
Figure 3B: Effect of DRB1*1301, SE-positive stratum.
Figure 3C: Effect of DRB1*1302, SE-negative stratum.
Figure 3D: Effect of DRB1*1302, SE-positive stratum.
the DERAA sequence as such does not result in protection from ACPA-positive RA, but rather, that DRB1*13 appears to be associated with protection.

In light of the strong protective effect conveyed by DRB1*13, we investigated if the presence of a DRB1*13 allele could annul the predisposition associated with a SE allele. The risk of SE/DRB1*13 heterozygote individuals compared to the risk of individuals carrying neither SE nor DRB1*13 alleles, was however still increased (OR (95%CI): 2.14 (1.64-2.80)). Although this effect will vary according to the difference in risk associated with the different SE alleles and in different cohorts, the presence of one DRB1*13 allele does not compensate the risk associated with the presence of one SE allele in meta-analysis.

Next, we analyzed whether the DRB1*13 association was confined to DRB1*13 alleles which contain a DERAA-sequence: DRB1*1301 and*1302 (DRB1*1304 was not present in the study populations). As can be seen in Table 3, the protective effect was limited to the DRB1*1301 and*1302 alleles, although the analysis for the other DRB1*13 alleles was possibly hampered by relatively small numbers of cases and controls. Complete four-digit typing of DRB1*13 was available for three of the four populations included in this meta-analysis (Norway, Netherlands and Spain). Subtype analysis revealed that the protective effect of DRB1*1302 was no longer present after stratification for the SE (Figure 3). DRB1*1301 therefore was the only allele which was consistently associated with protection from ACPA-positive RA.

DISCUSSION

HLA alleles contribute to the susceptibility of RA in various ways. As a consequence of the highly polymorphic nature of the HLA-region in the population, it has been difficult to dissect the contribution of the various HLA alleles to RA-susceptibility. Previously, several different classifications have been developed in order to summarize the predisposing and protective effects of the HLA alleles with regards to RA. Our data confirm the predisposing effect of the SE alleles and also corroborate the differential effect sizes with which different HLA SE alleles predispose to ACPA-positive disease. Furthermore, our results indicate a contribution of DRB1*09 and DRB1*15 to ACPA-positive disease. The relatively modest effect of DRB1*15 requires replication in further studies, before any firm conclusions can be drawn. The predisposing effect of DRB1*09 to ACPA-positive RA on the other hand, has been described in other populations as well. Therefore, it may be appropriate to include DRB1*09 in the list of susceptibility genes for ACPA-positive RA.

More importantly, however, our results confirm the association of HLA-DRB1 alleles with protection and considerably refine the definition of protective alleles. Our data indicate that the protective effect is only apparent for the DRB1*13 allelic group. Analysis of the different allele-classifications which have been developed to capture the protective effects...
of HLA-DRB1 alleles in RA\(^{15, 16, 18}\) revealed that the protective effect of the DERAA and D70 classification could largely be attributed to the DRB1*13 alleles. This underscores the relative importance of the protective effect mediated by DRB1*13 in comparison to other alleles, and also raises the question whether the classifications of protective effects may need to be reconsidered.

Further analysis of the DRB1*13 alleles showed that protection was only apparent for the DRB1*1301 allele. Although our study included over 2700 patients and 3000 controls from four large data-sets from four different European populations, we cannot exclude that smaller protective effects may also be present for other alleles than DRB1*1301 which could not be detected in the present investigation. Previous studies in other ethnicities have also reported protective effects of other HLA alleles such as DRB1*1302 and DRB1*14 for RA, although not all of these results were stratified for ACPA-status or corrected for the effect of the HLA SE alleles\(^ {37, 38}\). It would be interesting to know to what extent the protective effects differ among different populations.

The present study clearly confirms that the SE alleles are only associated with ACPA-positive RA. The same is true for the association between DRB1*13 or *1301 and protection. The present study thus demonstrates once more that the associations between HLA-DRB1 alleles and ACPA-positive versus ACPA-negative RA are very different both quantitatively and qualitatively. Regarding protective effects of HLA-DRB1 alleles in ACPA-negative RA, a recent study in a limited number of patients has reported that the DERAA alleles may be protective in this subset as well\(^ {39}\). We could not confirm this finding in the present study, although the weak protective effects we observed for DRB1*07 and DRB1*15 in ACPA-negative RA do not exclude the presence of HLA-mediated protection in ACPA-negative disease.

In the current investigation, the effect of the presence of the different HLA-DRB1 alleles was investigated separately. The risk for ACPA-positive RA in individuals heterozygous for the SE was assessed as part of the stratified analysis, but we cannot make conclusions about the risk associated with heterozygosity for the various other HLA-DRB1 alleles. It is conceivable that combinations of certain alleles may confer susceptibility or protection, as was described in a recent report, in which the combination of DRB1*03 and DRB1*13 alleles was found to be associated with an increased risk of ACPA-negative disease\(^ {14}\). A meta-analysis of heterozygosity effects may therefore yield very interesting results in the future.

For the statistical analysis, a dominant allele model was applied in the present study. An alternative would have been to use an additive allele model, which assumes substantially larger effects in homozygous versus heterozygous individuals. Although the dominant allele model provided the best fit to the data in the current analysis, other reports have favored an additive model\(^7\). Discrepancies between publications may therefore be partly attributable to differences in statistical methods. A meta-analysis such as presented here is
helpful in this respect because it overcomes these statistical differences and provides an overview of the results of four cohorts analyzed in the same way.

As can be seen from Figures 1A and 3B, there was a tendency towards a North-South gradient in the strength of the associations of several HLA-DRB1 alleles. Associations were often the strongest in Norway and Sweden, slightly less strong in the Netherlands and the weakest or sometimes even absent in Spain. This was the case for both predisposing and protective alleles in both ACPA-positive and ACPA-negative disease. For HLA-associated susceptibility, these same geographical differences can be observed in previous publications \(^\text{40, 41}\), but they have not been described for the protective effects of HLA in RA. If the existence of this gradient proves to be real, it may be a factor which needs to be taken into account when comparing data from different populations. It may also serve to reconcile some of the seemingly conflicting data which have been reported in different populations. Furthermore and perhaps most importantly, it may provide clues to candidate environmental factors that may be involved in the pathogenesis of the disease.

The main reason to perform studies such as the current meta-analysis, is to obtain mechanistic insight into the contribution of the HLA-region to RA. This has provided important new insights in the past, such as the realization that the HLA SE alleles do not contribute to RA as such, but rather to ACPA-positive disease \(^\text{8}\). More recently it was also shown that the presence of the HLA SE alleles influences both the magnitude and specificity of the ACPA-response \(^\text{9, 42}\). Together, these observations indicate that the HLA SE alleles are primarily involved in shaping of the ACPA-response presumably by facilitating T-cell help to ACPA-producing B-cells. Intriguingly, our data show that the protective effects associated with the presence of DRB1*13 are also most prominent in the ACPA-positive group of RA patients. These observations would be in line with the notion that the predisposing effect of the HLA SE alleles and the protective effect of DRB1*13 act within the same biological pathway. Indeed, the presence of DRB1*13 considerably lowered the predisposing effects of the HLA SE alleles in individuals heterozygote for both, although the predisposing effect of the HLA SE alleles was not annulled. In case these effects target the same biological pathway, the presence of DRB1*13 may perhaps also influence the specificity and magnitude of the ACPA-response.
REFERENCES

9. van der Helm-van Mil AH, Verpoort KN, Breedveld FC et al. The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. Arthritis Rheum 2006;54(4):1117-1121.


