Arthritis & Rheumatology
Vol. 73, No. 6, June 2021, pp 963–969
DOI 10.1002/art.41630

American College of Rheumatology
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BRIEF REPORT

*HLA–B*08* Identified as the Most Prominently Associated Major Histocompatibility Complex Locus for Anti–Carbamylated Protein Antibody–Positive/Anti–Cyclic Citrullinated Peptide–Negative Rheumatoid Arthritis

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Objective. Previously, only the *HLA–DRB1* alleles have been assessed in rheumatoid arthritis (RA). The aim of the present study was to identify the key major histocompatibility complex (MHC) susceptibility factors showing a significant association with anti–carbamylated protein antibody–positive (anti-CarP+) RA.

Methods. Analyses were restricted to RA patients who were anti–cyclic citrullinated peptide antibody negative (anti-CCP–), because the anti-CCP status dominated the results otherwise. Therefore, we studied samples from 1,821 anti-CCP– RA patients and 6,821 population controls from Spain, Sweden, and the Netherlands. The genotypes for ~8,000 MHC biallelic variants were assessed by dense genotyping and imputation. Their association with the anti-CarP status in RA patients was tested with logistic regression and combined with inverse-variance meta-analysis. Significance of the associations was assessed according to a study-specific threshold of $P < 2.0 \times 10^{-5}$.

Results. The *HLA–B*08* allele and its correlated amino acid variant Asp-9 showed a significant association with anti-CarP+/anti-CCP– RA ($P < 3.78 \times 10^{-7}$; $I^2 = 0$). This association was specific when assessed relative to 3 comparator groups: population controls, anti-CarP–/anti-CCP– RA patients, and anti-CCP– RA patients who were positive for other anti–citrullinated protein antibodies. Based on these findings, anti-CarP+/anti-CCP– RA patients could be separated from other antibody-defined subsets of RA patients in whom an association with the *HLA–B*08* allele has been previously demonstrated. No other MHC variant remained associated with anti-CarP+/anti-CCP– RA after accounting for the presence of the *HLA–B*08* allele. Specifically, the reported association of *HLA–DRB1*03* was observed at a level comparable to that reported previously, but it was attributable to linkage disequilibrium.

Conclusion. These results identify *HLA–B*08* carrying Asp-9 as the MHC locus showing the strongest association with anti-CarP+/anti-CCP– RA. This knowledge may help clarify the role of the HLA in susceptibility to specific subsets of RA, by shaping the spectrum of RA autoantibodies.

Supported in part by the Instituto de Salud Carlos III (grant PI16/00113 to Dr. Rodriguez-Carrio, grant RD16/0012/0015 to Dr. Lopez-Pedrera, grant RD16/0012/0012 to Dr. Balsa, grant RD16/0012/0011 to Dr. Gonzalez-Alvaro, grant RD16/0012/0004 to Dr. Fernandez-Gutierrez, grant RD16/0012/0009 to Dr. Gonzalez-Gay, grant RD16/0012/0013 to Dr. Martin, and grants PI17/01606 and RD16/0012/0014 to Dr. Gonzalez), which are partially financed by the European Regional Development Fund of the EU (FEDER), and by the Innovative Medicines Initiative (BTCure grant 831434). Dr. Regueiro's work was supported by Ministerio de Educacion Cultura y Deporte (grant FPU15/03434). Dr. Acosta-Herrera's work was supported by a Juan de la Cierva Fellowship (grant IJC2018-035131-I).

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INTRODUCTION

The major histocompatibility complex (MHC) accounts for a large fraction (30–50%) of rheumatoid arthritis (RA) heritability (1) (background details are provided in the Supplementary Overview, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract). This notable contribution of the MHC involves 5 independent RA risk loci, *HLA–DRB1*, *HLA–B*, *HLA–DP*, *HLA–A,* and *HLA–DOA*, except in the Han Chinese, in whom a recently discovered risk locus in *HLA–DQA1* was found to predominate (2). The mentioned HLA loci show differential association with RA phenotypes defined by the presence of specific autoantibodies. For example, the *HLA–DRB1* alleles expressing valine at position 11 (Val-11) are strongly associated with anti–cyclic citrullinated peptide antibody–positive (anti-CCP+) RA but not with anti-CCP– RA (1,3). In contrast, *HLA–DRB1* alleles bearing other amino acid combinations are associated with anti-CCP– RA (4,5), including *HLA–DRB1*03*, which expresses serine at position 11 (Ser-11). This association could be partly explained by the presence of anti–carbamylated protein antibodies (anti-CarP) in anti-CCP– RA patients (6,7). The anti-CarP antibodies are RA autoantibodies targeting another posttranslational protein modification and revealing additional aspects of the pathogenesis and natural history of RA (8).

Another example of differential association concerns the *HLA–B*08* allele, which encodes aspartic acid at position 9 (Asp-9). *HLA–B*08* is the MHC locus showing the second most prominent association with anti-CCP+ RA (3), and is the most strongly associated with anti-CCP– RA (4,5). According to a recent study by Terao et al (9), this complex association could be explained by a subset of anti–citrullinated protein antibodies (ACPAs). In effect, in that study (9), analysis of multiple ACPA fine specificities revealed that ACPAs could be grouped into 2 subsets, the canonical ACPAs and the noncanonical ACPAs, which were either correlated with the presence of anti-CCP2 antibodies (canonical ACPAs) or not correlated with the presence of anti-CCP2 (noncanonical ACPAs). The noncanonical ACPAs accounted for the association of *HLA–B*08* within the anti-CCP+ and anti-CCP– RA patient subsets (9). However, this interpretation is still uncertain, because the distinction between canonical and noncanonical ACPAs was only made in the Terao et al study.

As mentioned, the *HLA–DRB1*03* allele increases the risk of anti-CarP+ RA, but this association was identified in studies addressing only the *HLA–DRB1* alleles (6,7). We do not know if other MHC loci are also associated with anti-CarP+ RA. We therefore undertook the present study to analyze the whole MHC in 3 cohorts of RA patients, with the combined use of dense genotyping and imputation, to elucidate the risk variants for susceptibility to anti-CarP+ RA.

MATERIALS AND METHODS

Sample collection. We obtained blood samples from RA patients and population controls from cohorts in Spain, Sweden, and the Netherlands. Except in a preliminary analysis done with the samples from Spanish subjects, only the anti-CCP– subset of RA patients was considered in our analyses. Additional details and sample sizes are provided in Supplementary Materials and Methods and Supplementary Table 1, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract.

Laboratory determinations. The anti-CCP antibody status was established in each collection of blood samples using commercial anti-CCP2 kits. The anti-CarP antibody testing was done with a homemade enzyme-linked immunosorbent assay against carbamylated fetal calf serum, following an established protocol (8). Genotype data were obtained from Illumina InmunoChip and a minor contribution from genome-wide arrays. Information on the genotypes was enriched by the imputation of a rich set of 7,893 MHC binary markers comprising classic HLA alleles, polymorphic amino acids, and single-nucleotide polymorphisms (SNPs).

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Dr. Balsa has received consulting fees, speaking fees, and/or honoraria from AbbVie, Pfizer, Novartis, Bristol Myers Squibb, Nordic, Sanofi, Sandoz, UCB, and Lilly (less than $10,000 each) and research support from AbbVie, Pfizer, Novartis, Bristol Myers Squibb, Nordic, and Sanofi. Dr. Gonzalez-Alvaro has received consulting fees, speaking fees, and/or honoraria from Lilly, Sanofi, Bristol Myers Squibb, AbbVie, and Roche Laboratories (less than $10,000 each). Dr. Gonzalez-Gay has received consulting fees, speaking fees, and/or honoraria from AbbVie, Pfizer, Roche, Sanofi, Lilly, Celgene, Sobi and MSD (less than $10,000 each) and research support from AbbVie, MSD, Jansen, and Roche. Dr. Trouw is a coinventor on the patent EP2671078B1 relating to the detection of anti-CarP antibodies. Dr. Gonzalez has received speaking fees from Bristol Myers Squibb (less than $10,000) and research support from Bristol Myers Squibb. No other disclosures relevant to this article were reported.

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Submitted for publication September 15, 2020; accepted in revised form December 23, 2020.

Statistical analysis. As a first step, we used logistic regression analysis to test the association between the MHC binary markers and anti-CarP+/anti-CCP– RA among the samples from each collection. Summary-level statistics from each data set were combined using fixed-effects meta-analysis, weighting the contribution of each population with the inverse variance method. The random-effects meta-analysis method of DerSimonian and Laird was selected only when the heterogeneity of the data was notable ($I^2 > 60$). To control for the SNPs showing the strongest associations with anti-CarP+/anti-CCP– RA, we performed a conditional stepwise regression analysis.

Additionally, we performed analyses aimed at determining the anti-CarP specificity of the associations relative to other RA subgroups (4,9). A *P* value (corrected for multiple testing) of less than 2.03×10^{-5} was used as the significance threshold for interpretation.

RESULTS

Preliminary analysis of the Spanish anti-CarP+ RA patients. The previous studies that analyzed *HLA–DRB1* alleles showed that the anti-CCP status constitutes an obstacle for detecting anti-CarP–specific associations (6,7). In the current study, a preliminary exploration in samples from Spanish RA patients showed that the same applied to the entire MHC. In effect, the same top variant (AA_DRB1_13_HF) was found associated with anti-CarP+ RA and with anti-CCP+ RA (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract). The only difference between the results of the 2 analyses was that the association of this variant with anti-CarP+ RA had a lower level of significance (odds ratio [OR] 2.6, 95% confidence interval [95% CI] 2.3–2.9, $P = 9.0 \times 10^{-52}$ in anti-CarP+ RA patients versus OR 2.8, 95% CI 2.5–3.0, $P = 7.8 \times 10^{-84}$ in anti-CCP+ RA patients). This pattern was also observed in subsequent conditional analyses (for association with variant AA_DRB1_11_SGL in the first conditional analysis, $P = 1.8 \times 10^{-12}$ in anti-CarP+ RA patients versus $P = 5.1 \times 10^{-15}$ in anti-CCP+ RA patients and for association with SNP rs3130544 in the second conditional analysis, $P = 7.5 \times 10^{-10}$ in anti-CarP+ RA patients versus $P = 8.1 \times 10^{-12}$ in anti-CCP+ RA patients) (Supplementary Figure 1).

These findings suggest that the MHC associations were specific for anti-CCP+ RA, but not for anti-CarP+ RA. The specificity of anti-CCP+ RA was demonstrated by analyzing antibody-discordant patients. These analyses showed that in anti-CarP–/anti-CCP+ RA patients, a significant association with the top variant (AA_DRB1_13_HF) was found (OR 2.6, 95% CI 2.3–3.0, $P = 2.9 \times 10^{-50}$), whereas in anti-CarP+/anti-CCP– RA patients, no association was found (OR 1.1, 95% CI 0.8–1.6, $P = 0.47$) (see Supplementary Figure 2, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract). Therefore, all subsequent analyses were restricted to the anti-CCP– RA patients.

***HLA–B*08* or Asp-9 at *HLA–B* identified by meta-analysis as the major MHC risk variants in anti-CarP+/anti-CCP– RA patients.** The 3 case–control sample collections included a total of 1,821 anti-CCP– RA patients, 195 of whom were anti-CarP+/anti-CCP–, and 6,824 population controls (see sample sizes in Supplementary Table 1 [http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract]). Most binary markers showed a low level of heterogeneity between the collections. A total of 165 of those markers showed frequency differences that were significant at the study-specific threshold ($P < 2.03 \times 10^{-5}$) (Figure 1A and Supplementary Table 2, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract). The *HLA–B*08* allele was the HLA allele showing the strongest association with anti-CarP+/anti-CCP– RA ($P = 3.70 \times 10^{-7}$; $I^2 = 0$). Only 3 SNPs were ranked higher (according to *P* value) than *HLA–B*08* (see Supplementary Table 2). The high ranking of these 3 SNPs was attributed to the presence of *HLA–B*08* because 1) they were in strong linkage disequilibrium with *HLA–B*08* ($r^2 = 0.62$–0.81), 2) their association with anti-CarP+/anti-CCP– RA did not reach a higher level of significance than that of *HLA–B*08* ($P = 0.89$–0.95 in the pairwise comparison), and 3) they lack known relevance in RA.

The presence of Asp in the HLA–B amino acid position 9 (Asp-9) showed a very similar level of association with anti-CarP+/anti-CCP– RA ($P = 3.78 \times 10^{-7}$; $I^2 = 0$). This similarity is expected because of the tight correlation between Asp at HLA–B position 9 and the *HLA–B*08* allele ($r^2 = 0.995$ in our subjects). Both the *HLA–B*08* allele and the Asp-9 amino acid were consistently associated with anti-CarP+/anti-CCP– RA among the patients in the 3 cohorts, with a summary OR of 2.00 (95% CI 1.53–2.61) (see Supplementary Figure 3, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract). There was no other classic HLA allele or polymorphic amino acid with a significant association (see Supplementary Table 2). Furthermore, no other genetic marker was significantly associated with the subset of anti-CarP+/anti-CCP– RA in the conditional meta-analysis accounting for the presence of the *HLA–B*08* allele (Figure 1B).

The *HLA–DRB1*03* allele, which has previously been identified as a specific risk factor for anti-CarP+/anti-CCP– RA (6,7), showed the strongest association among the *HLA–DRB1* alleles. However, this association disappeared after accounting for the *HLA–B*08* allele in the conditional meta-analysis (see Supplementary Table 3, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract).

Predominance of the *HLA–B*08* association with anti-CarP+ RA relative to other anti-CCP– RA subsets. We next wished to distinguish the association of *HLA–B*08* (or Asp-9 at HLA–B) with anti-CarP+/anti-CCP– RA from previously reported *HLA–B*08* associations. Specifically, we assessed the 2 previously described associations of *HLA–B*08* in patients with anti-CCP– RA (4) and in RA patients with noncanonical ACPAs (9).

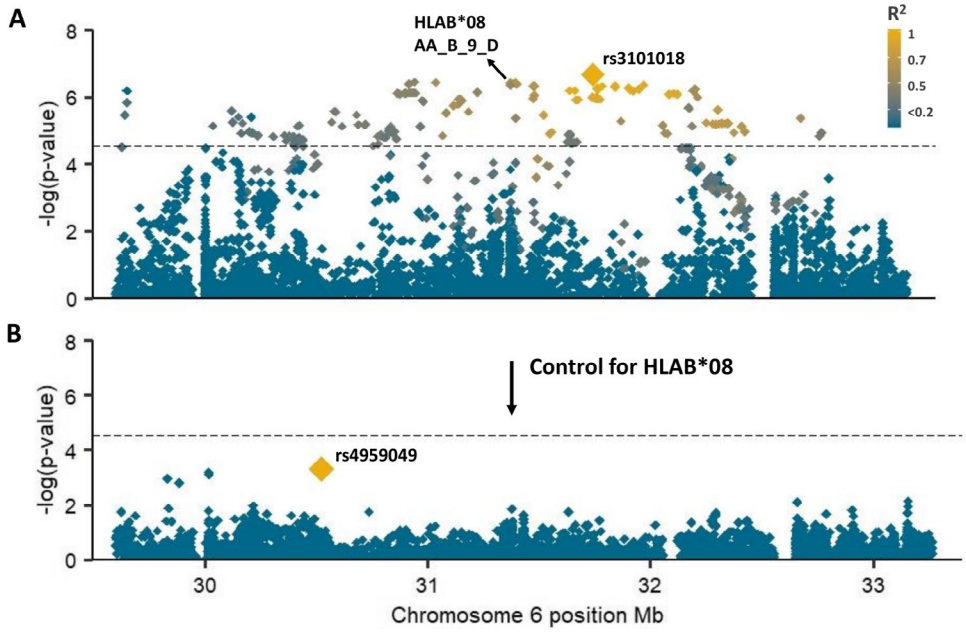

Figure 1. Association of major histocompatibility complex (MHC) variants with the risk of anti–carbamylated protein antibody–positive (anti-CarP+)/anti–cyclic citrullinated peptide antibody negative (anti-CCP–) rheumatoid arthritis (RA). Manhattan plots show the findings from the meta-analysis of MHC associations in anti-CarP+/anti-CCP– RA patients compared with population controls (**A**) and from the conditional meta-analysis accounting for the presence of the *HLA–B*08* allele (**B**). Each diamond represents a variant according to its chromosomal position (abscises) and $-\log_{10}$(*P* value) (ordinates). The color gradient indicates the linkage disequilibrium (r^2) with the top associated marker. The broken horizontal line represents the study-specific significance threshold ($P = 2.03 \times 10^{-5}$).

First, we compared the frequencies of *HLA–B*08* (and Asp-9 at HLA–B) between the anti-CarP+/anti-CCP– RA patients and the anti-CarP–/anti-CCP– RA patients. This comparison showed that *HLA–B*08* and Asp-9 at HLA–B were significantly more frequent in the anti-CarP+/anti-CCP– RA patients than in the anti-CarP–/anti-CCP– RA patients (OR 1.53, 95% CI 1.15–2.04, $P = 0.003$ in the meta-analyses with the 2 binary markers). The relative increase in frequency of *HLA–B*08* and Asp-9 at HLA–B in anti-CarP+/anti-CCP– RA patients was observed in all 3 cohorts (Figures 2A and B).

We next addressed the *HLA–B*08* association with noncanonical ACPA+ RA, using the patients from the Swedish Epidemiological Investigation of RA cohort, for whom information on canonical versus noncanonical ACPAs was uniquely available as part of the original report (9). That report differentiated 18 ACPA fine specificities into 2 groups: 6 noncanonical ACPAs, which did not correlate with anti-CCP+ RA nor did they correlate with other ACPAs, and 12 canonical ACPAs, which were tightly concordant with the anti-CCP status. Therefore, we stratified the anti-CCP– RA patients according to the presence of the canonical ACPAs, noncanonical ACPAs, and anti-CarP antibodies and assessed the *HLA–B*08* association with each of these subsets. The associations were stronger in the anti-CarP+ subsets than in the anti-CarP– subsets (Figure 3 and Supplementary Figure 4, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract).

These preferential associations were observed in the strata of patients positive for any ACPA (OR 2.75, 95% CI 1.63–4.63 in anti-CarP+ RA versus OR 1.52, 95% CI 1.15–2.01 in anti-CarP– RA), for canonical ACPAs (OR 2.38, 95% CI 1.32–4.30 in anti-CarP+ RA versus OR 1.38, 95% CI 0.98–1.88 in anti-CarP– RA), or, most notably, for noncanonical ACPAs (OR 3.14, 95% CI 1.54–6.41 in anti-CarP+ RA versus OR 1.88, 95% CI 1.23–2.88 in anti-CarP– RA). Similar results were obtained in assessing the association of Asp-9 at HLA–B (data not shown). However, it is important to note that sample sizes were small in these strata, and none of the

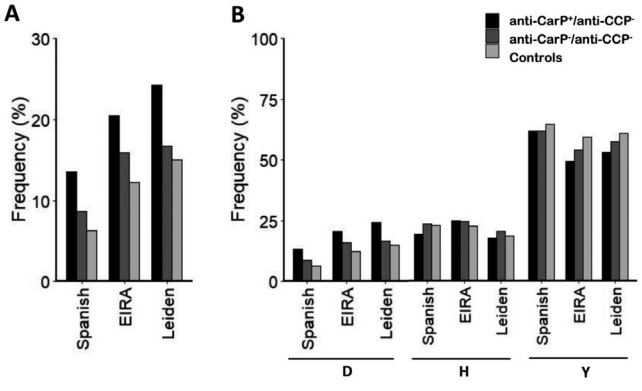

Figure 2. Analysis of the specificity of the association of *HLA–B*08* and Asp-9 at HLA–B with anti-CarP+/anti-CCP– RA. Individual frequencies of the *HLA–B*08* alleles (**A**) and the 3 possible amino acids at HLA–B position 9 (**B**) were plotted in anti-CarP+/anti-CCP– RA patients, anti-CarP–/anti-CCP– RA patients, and population controls in each cohort. EIRA = Epidemiological Investigation of RA; D = aspartic acid; H = histidine; Y = tyrosine (see Figure 1 for other definitions).

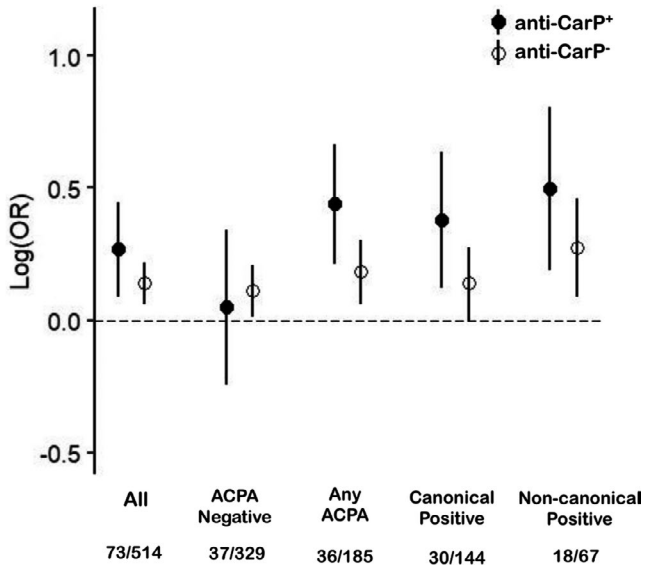

Figure 3. Preferential association of the *HLA–B*08* allele with anti-CarP+/anti-CCP– RA independently of anti–citrullinated protein antibodies (ACPAs), either noncanonical or canonical. The category of "All" refers to all anti-CCP– RA patients selected from the Epidemiological Investigation of RA (EIRA) cohort. The remaining groups are subsets of these patients from the EIRA cohort according to the absence or presence of ACPAs (not including anti-CCP2). For the ACPA-positive groups, ACPAs were stratified as any of the 18 analyzed, the 12 canonical ACPAs, or the 6 noncanonical ACPAs. Circles with whiskers represent the log(odds ratio [OR]) with 95% confidence interval (95% CI) for the association of *HLA–B*08* with anti-CarP+ RA versus anti-CarP– RA. The horizontal broken line indicates the null association (OR of 1). Values below the categories indicate the number/total number of patients per group. See Figure 1 for other definitions.

differences between anti-CarP+ and anti-CarP– RA patients were statistically significant.

Confirmation of HLA associations in additional analyses. We then performed additional analyses to complement our results, including a meta-analysis of principal components analysis–adjusted associations, a combined logistic regression analysis, and an omnibus test of all of the HLA–B polymorphic amino acid positions. The results of these 3 analyses were fully consistent with those already described above (see Supplementary Results, Supplementary Figures 5, 6, and 7, and Supplementary Tables 4 and 5, at http://onlinelibrary.wiley.com/doi/10.1002/art.41630/abstract).

DISCUSSION

Our results show that the *HLA–B*08* allele and the corresponding amino acid variant Asp-9 are the major susceptibility variants specifically associated with anti-CarP antibodies in patients with RA. The outcomes observed in this study, the first one to address the relationship between the entire MHC region and anti-CarP antibodies, complement our understanding of the role of the HLA in RA susceptibility. Our data indicate that the *HLA–B*08* allele contributes differentially to RA autoantibody phenotypes. No other MHC variant showed evidence of association with anti-CarP+/anti-CCP– RA, after accounting for the *HLA–B*08* association. This finding raises questions about the previously described association of *HLA–DRB1*03* with anti-CarP antibodies (6,7), as our current results indicate it was attributable to linkage disequilibrium. Therefore, the susceptibility to anti-CarP+ RA purportedly conferred by the MHC should be reassigned from *HLA–DRB1*03* to *HLA–B*08*. Moreover, our findings reinforce previous observations of the predisposing role of the HLA to the various serologic RA phenotypes.

The strength of the *HLA–B*08* association with anti-CarP+/anti-CCP– RA was remarkable, as shown by the OR of 2.0. This strength determined the notable level of significance obtained. Another contributing factor was the absence of heterogeneity in the *HLA–B*08* association. These characteristics indicate that replication studies would be feasible once more samples with the required information become available.

We demonstrated the specificity of the *HLA–B*08* association with anti-CarP+/anti-CCP– RA relative to 3 comparator groups: population controls, anti-CarP–/anti-CCP– RA patients, and RA patients with noncanonical ACPAs. Comparison with anti-CarP–/anti-CCP– RA patients was necessary because the *HLA–B*08* allele has been described as a risk factor for seronegative RA (4,5). The significant association with anti-CarP+/anti-CCP– RA observed in this comparison excluded the possibility of a mere bystander association as part of the anti-CCP– subgroup of RA patients. The other alternative, that the associations with anti-CarP+ RA could be attributable to the concordant presence of noncanonical ACPAs, was also reasonably excluded. The noncanonical ACPAs were recently identified as being specifically associated with the *HLA–B*08* allele, in particular in anti-CCP– RA patients (9). However, the fraction of our patients bearing noncanonical ACPAs was similar between the anti-CarP+/anti-CCP– and anti-CarP–/anti-CCP– RA subsets (6.8% versus 5.6%, respectively), making it very unlikely that antibody concordance accounted for the *HLA–B*08* association. The same interpretation was obtained from our observations of the more marked *HLA–B*08* association with anti-CarP+ RA than with anti-CarP– RA independently of the presence or absence of noncanonical ACPAs. It is important to note that the same pattern of association was observed in the presence of any ACPA, making our results immune to changes in the canonical/noncanonical ACPA classification, which has been described in only a single study (9). Therefore, it seems more likely that the *HLA–B*08* allele is independently associated with the 2 antibody types, anti-CarP antibodies and noncanonical ACPAs.

It has been known for some time that the HLA contributions to anti-CCP+ RA and to anti-CarP+ RA are discordant. The shared epitope alleles of *HLA–DRB1* predisposing to anti-CCP+ RA are not associated with anti-CarP antibodies (6,7). In contrast,

the *HLA–DRB1*03* allele has been found to be associated with anti-CarP antibodies, but not with anti-CCP+ RA (6,7). Accordingly, *HLA–DRB1*03* was the *HLA–DRB1* allele with the strongest association with anti-CarP+/anti-CCP– RA in our patients, showing an OR similar to that previously reported (6,7). However, the conditional analysis, which was unavailable in the previous studies, showed that the *HLA–DRB1*03* association was attributable to linkage disequilibrium in our patients. This result is not surprising, because the *HLA–B*08* and *HLA–DRB1*03* alleles are part of the 8.1 ancestral haplotype. The 8.1 ancestral haplotype has been historically associated with a variety of autoimmune diseases. Recently, some of these associations have been disentangled, leading to the identification of the *HLA–B*08* allele as a prominently associated locus in early-onset myasthenia gravis (10,11), anti–Jo-1–positive myositis (12), and celiac disease (13), whereas the significant locus within the haplotype is either another one or still uncertain for other diseases.

The association with celiac disease could be informative, because gliadin peptides are presented to CD8 T cells on the *HLA–B*08* molecule in patients with celiac disease (14). A similar antigen-presentation mechanism can be proposed for its involvement in the susceptibility to anti-CarP+ RA. However, this hypothesis is incomplete. A missing element concerns the connection of peptide presentation on *HLA–B*08* to antibody production. Another is the identity of the endogenous peptides inducing the anti-CarP reactivity, which may be from carbamylated proteins or proteins with other posttranslational modifications, as observed in a recent mouse study (15). This uncertainty about the endogenous peptides and the fuzzy boundaries between RA autoantibody types could potentially be addressed by understanding the specific association of *HLA–B*08* with anti-CarP+/anti-CCP– RA. Moreover, the eventual development of antigen-specific therapies will require the identification of peptides and HLA alleles, as they increasingly rely on the delivery of peptide–HLA complexes as being more effective than the peptides on their own.

In summary, our results identify *HLA–B*08* carrying Asp-9 as the major MHC risk factor for anti-CarP+/anti-CCP– RA, instead of the previously reported *HLA–DRB1*03* allele. This knowledge contributes to clarification of the role of *HLA–B*08* in susceptibility to antibody-defined RA subsets and, more generally, the role of the HLA in shaping the spectrum of RA autoantibodies.

ACKNOWLEDGMENTS

We thank the patients for their generous participation in the study, and Yolanda Lopez-Golan and Carmen Pena for their help in patient recruitment and sample processing.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gonzalez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Regueiro, Casares-Marfil, Martin, Gonzalez.
Acquisition of data. Regueiro, Casares-Marfil, Lundberg, Knevel, Acosta-Herrera, Rodriguez-Rodriguez, Lopez-Mejias, Perez-Pampin, Triguero-Martinez, Nuño, Ferraz-Amaro, Rodriguez-Carrio, Lopez-Pedrera, Robustillo-Villarino, Castañeda, Remuzgo-Martinez, Alperi, Alegre-Sancho, Balsa, Gonzalez-Alvaro, Mera, Fernandez-Gutierrez, Gonzalez-Gay, Trouw, Grönwall, Padyukov, Martin, Gonzalez.
Analysis and interpretation of data. Regueiro, Casares-Marfil, Lundberg, Knevel, Acosta-Herrera.

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