2013-07-04

Genetic and environmental determinants for disease risk in subsets of rheumatoid arthritis defined by the anticitrullinated protein/peptide antibody fine specificity profile

Lundberg, Karin; Bengtsson, Camilla; Kharlamova, Nastya; Reed, Evan; Jiang, Xia; Källberg, Henrik; Pollak-Dorocic, Iskra; Israelsson, Lena; Kessel, Christoph; Padyukov, Leonid; Holmdahl, Rikard; Alfredsson, Lars; Klareskog, Lars

Ann Rheum Dis. 2013 May;72(5):652-8.
http://doi.org/10.1136/annrheumdis-2012-201484
http://hdl.handle.net/10616/41652

Genetic and environmental determinants for disease risk in subsets of RA, defined by the ACPA fine-specificity profile

Corresponding author

Name: Karin Lundberg

Postal address: Rheumatology Unit, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Solna, CMM L8:04, 171 76 Stockholm, Sweden

Email: Karin.Lundberg@ki.se

Phone number: +46 8 517 765 60

Fax number: +46 8 517 755 62

Authors and affiliations:

Karin Lundberg[*1], Camilla Bengtsson[*2], Nastya Kharlamova[1], Evan Reed[1], Xia Jiang[2], Henrik Källberg[2], Iskra Pollak-Dorocic[1], Lena Israelsson[1], Christoph Kessel[3], Leonid Padyukov[1], Rikard Holmdahl[3], Lars Alfredsson[#2] and Lars Klareskog[#1]

[*] *Shared first authors*

[#] *Shared last authors*

[1] *Rheumatology Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden*

[2] *Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden*

[3] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*

Keywords:

Rheumatoid arthritis (RA), anti-citrullinated protein/peptide antibody (ACPA), cyclic citrullinated peptide (CCP), *HLA-DRB1* shared epitope, smoking

Word count:

(excluding title page, abstract, references, figures and tables): 3000 words

ABSTRACT

Objectives In order to increase our understanding of the aetiology and pathogenesis in rheumatoid arthritis (RA), we have investigated genetic and environmental risk factors for RA subsets, defined by the presence or absence of different anti-citrullinated protein/peptide antibodies (ACPAs), targeting citrullinated peptides from α-enolase, vimentin, fibrinogen and collagen type II.

Methods RA cases (n=1985) and matched controls (n=2252) from the EIRA case-control cohort were used in this study. Serum samples were assayed by ELISA for presence of anti-CCP antibodies and four different ACPA fine-specificities. Cross-reactivity between ACPAs was examined by peptide-absorption experiments. Genotyping was performed for *HLA-DRB1* 'shared epitope' (SE) alleles and the *PTPN22* gene, while information regarding smoking was obtained by questionnaire. Association of genetic and environmental risk factors with different subsets of RA was calculated by means of logistic regression analysis.

Results Limited cross-reactivity was observed between different ACPA fine-specificities. In total, 17 RA subsets could be identified based on their different ACPA fine-specificity profiles. Large differences in association with genetic and environmental determinants were observed between subsets. Strongest association of *HLA-DRB1* SE, *PTPN22* and smoking was identified for the RA subset which was defined by the presence of antibodies to citrullinated α-enolase and vimentin.

Conclusion Our study provides the most comprehensive picture to this date, of how *HLA-DRB1* SE, *PTPN22* and smoking associate with the presence of specific ACPA-reactivities, rather than anti-CCP levels. The new data will form a basis for molecular studies aimed at understanding disease development in serologically distinct subsets of RA.

Keywords:

Rheumatoid arthritis (RA), anti-citrullinated protein/peptide antibody (ACPA), cyclic citrullinated peptide (CCP), *HLA-DRB1* shared epitope, smoking

INTRODUCTION

Rheumatoid arthritis (RA) has for many years been considered a prototype for immune-mediated diseases, due to the strong genetic association with certain MHC class II alleles, i.e. *HLA-DRB1* shared epitope (SE),[1-3]. More recently, the MHC class II association in RA was shown to be confined to the subset of patients which is characterised by the presence of antibodies to synthetic cyclic citrullinated peptides (CCP),[4, 5]. The concomitant recognition, that the best-known environmental risk factor for RA, namely smoking, constitutes a risk only for the development of CCP positive disease, together with the finding that smoking induces the exposure of citrullinated proteins in the lungs,[4, 6, 7], suggest an aetiology for RA where *HLA-DRB1* SE-restricted immune reactions to citrullinated epitopes trigger and drive disease pathogenesis,[8]. Subsequently, it has been demonstrated that the CCP assays, such as the most frequently used CCP2 ELISA, is able to capture anti-citrullinated protein/peptide antibodies (ACPAs) with a rather broad spectrum of fine-specificities, including naturally occurring citrullinated autoantigens,[9, 10].

In order to increase our understanding of RA aetiology and pathogenesis, it has thus become an important task to define which ACPA fine-specificities associate with the different *HLA-DRB1* SE alleles. Such studies have been performed for some ACPA fine-specificities,[11-15], and differences within the CCP positive population have been described. However, no comprehensive picture has emerged so far, partly because only single target antigens have been studied, and partly due to small study populations, and lack of information on appropriate controls. Hence, in the present study we have used the large and well-characterised EIRA (Epidemiological Investigation of Rheumatoid Arthritis) case-control cohort,[16], to investigate *HLA-DRB1* SE, in combination with *PTPN22* polymorphism - the second most important genetic risk factor for CCP positive RA,[17] - and cigarette smoking, in relation to the presence of antibodies targeting four well-defined citrullinated autoantigens (citrullinated α-enolase, -vimentin, -fibrinogen and -collagen type II).

METHODS

Subjects

The Epidemiological Investigation of RA (EIRA) is an on-going Swedish population-based case-control study, described in detail elsewhere,[16]. In the present study, we analysed 1985 incident cases of RA, included in the EIRA study between May 1996 - May 2006, and 2252 controls, randomly selected from the national population register, to match RA cases in terms of age, gender and residential area. Cases were diagnosed with RA according to the 1987 ACR criteria,[18]. Cases and controls donated blood at the time of inclusion and completed a self-administered questionnaire, relating to life style and environmental exposures.

ELISAs

Anti-CCP antibody status was determined in sera from 1985 EIRA cases and 1210 controls, using the CCP2 assay (Immunoscan CCPlus, Euro-Diagnostica), according the manufacturer's instructions. Cut-off for positivity was 25AU/ml. Antibody responses to citrullinated peptides from α-enolase (CEP-1), vimentin (Cit-vim), fibrinogen (Cit-fib) and collagen type II (Cit-C1), or the arginine-containing equivalents, were assayed in sera from 1985 EIRA cases and 150 randomly selected

EIRA controls, using in-house ELISAs, as previously described,[11, 19]. Cut-off for positivity was 10AU/ml for all ACPA fine-specificities. See data supplement online, for a detailed description of the ELISA method.

Cross-reactivity assay
Ten serum samples with high anti-CCP levels (>800 AU/ml), that also displayed reactivity to CEP-1 (n=10), Cit-vim (n= 8), Cit-fib (n=9) and Cit-C1 (n=8), were selected from EIRA for cross-reactivity experiments. Each serum (diluted 1:100) was incubated with each of the four peptides (CEP-1, Cit-vim, Cit-fib or Cit-C1) at 10μg/ml, or in the presence of buffer alone. Pre-absorption was performed in liquid-phase during constant agitation for 2h, at room temperature. Following centrifugation (15 minutes, 1000g), supernatants were transferred to the four peptide ELISA plates, and assayed as described above. Inhibition was determined based on the differences in AU/ml values between the absorbing peptide and buffer alone.

Peptides
Freeze-dried synthetic peptides (>95% purity), corresponding to sequences from human α-enolase (amino acid 5-21), vimentin (amino acid 60-75) and fibrinogen (amino acid 36-52), in its 'native' form or with arginine-to-citrulline substitutions (Innovagen, Sweden), were dissolved at 10mg/ml (Milli-Q water, 10% DMSO) and stored at -20°C before used. The homotrimeric triple-helical collagen type II peptides, C1 and Cit-C1, were synthesized as previously described,[20, 21]. See supplementary Table 1, for a detailed description of the peptide sequences.

Genotyping
HLA-DRB1 subtyping was performed on DNA samples from 1961 RA cases and 1278 controls, by sequence-specific primer polymerase chain reaction, as previously described,[11]. *DRB1*01* (except *DRB1*0103*), *DRB1*04* and *DRB1*10* were classified as 'shared epitope' (SE) alleles of *HLA-DRB1*. The protein tyrosine phosphatase gene (*PTPN22* rs2476601) was genotyped on 1943 RA cases and 1284 controls, as previously described,[17].

Cigarette smoking
Information regarding cigarette smoking, antedating symptoms of arthritis in 1727 RA cases, and the corresponding time points among 1913 controls, were obtained by questionnaire. Individuals were categorized as ever smokers (current/past) or never smokers,[16].

Statistical analyses
To determine the association of *HLA-DRB1* SE (alone or in combination with *PTPN22* and/or smoking) with different subsets of RA, odds ratios (OR), together with 95% confidence intervals (CI), were calculated using unconditional logistic regression analyses, with cases and controls that were negative for the risk factors as referent category. All analyses were performed with adjustment for design variables (age, residential area and gender).

Interaction, as defined by departure from additivity of effects,[22], was evaluated between *HLA-DRB1* SE and smoking, and between *HLA-DRB1* SE and *PTPN22*. The attributable proportion due to interaction (AP) was calculated together with 95% CI, as previously described,[11, 23]. The AP between two interacting factors reflects the

joint effect beyond the sum of the independent effects. All analyses were performed using the Statistical Analysis System (SAS), version 9.1.

Differences in ACPA levels, between different RA subsets, or between absorbing peptides and buffer in the cross-reactivity experiment, were examined using Mann-Whitney *U* test for independent groups.

RESULTS

Identification of different RA subsets, based on ACPA fine-specificity profiles

Sixty three per cent of RA cases in EIRA were anti-CCP antibody positive; 35% anti-CEP-1 positive; 37% anti-Cit-vim positive; 28% anti-Cit-fib positive; and 37% were positive for anti-Cit-C1 antibodies. Low-level antibody responses to the arginine-containing control peptides from α-enolase, vimentin and fibrinogen were detected in less than 2.4% of patients, while reactivity to the arginine-containing C1 peptide,[20, 21, 24] of collagen type II was detected in 9% (data not shown). The presence of different ACPA fine-specificities overlapped to a large extent, and by analysing the different patterns, 17 serologically distinct subsets could be identified (figure 1a). A great majority of patients, which were positive for the specific ACPAs, were confined to the CCP positive population, although 18% of the CCP negative patients were positive for at least one ACPA fine-specificity (figure 1b). Conversely, 8.7% of all patients were CCP positive but negative for the four ACPA fine-specificities.

In the CCP negative population, ACPA positive patients typically showed reactivity to only one citrullinated antigen, where anti-Cit-C1 antibodies were dominant (9.45%). In the CCP positive subset on the other hand, presence of two (16.6%), three (13.4%) or four (11%) ACPA fine-specificities were common. ACPA fine-specificity levels were generally lower in the CCP negative, compared to the CCP positive population (data not shown). Moreover, there was a strong correlation between number of ACPAs present and anti-CCP antibody levels, with a significant ($p<0.0001$) increase of anti-CCP antibody levels for every additional ACPA fine-specificity (supplementary figure 1).

ACPAs show limited cross-reactivity

In order to analyse the extent of cross-reactivity between antibodies reactive with the different citrullinated peptides, serum samples from 10 CCP positive RA patients, with antibodies to CEP-1 (n=10), Cit-vim (n=8), Cit-fib (n=9) and Cit-C1 (n=8), were pre-absorbed against each of the four peptides, before re-tested for ACPA-reactivities. All four ACPAs were efficiently absorbed by pre-incubation with its homologous peptide, while only limited cross-reactivity was observed between specificities. Anti-CEP-1 IgG showed low-degree cross-reactivity with Cit-fib in some sera ($p = 0.043$), but no cross-reactivity with Cit-vim or Cit-C1 (figure 2a). Anti-Cit-vim IgG showed cross-reactivity with Cit-fib in two serum samples (figure 2b). Anti-Cit-fib IgG showed some degree of cross-reactivity with CEP-1 in some sera ($p = 0.050$) (figure 2c), and anti-Cit-C1 antibodies showed cross-reactivity mainly with Cit-fib, in approximately half of the serum samples ($p < 0.001$) (figure 2d).

***HLA-DRB1* SE, in combination with *PTPN22* and smoking, associate mainly with CEP-1 and Cit-vim positive RA**

To investigate associations of genetic and environmental risk factors with different subsets of RA, we first defined RA subsets by the presence or absence of antibodies to single citrullinated peptides, i.e. without taking into account which additional ACPA fine-specificities may also be present in the subset. As seen in figure 3a, being positive for *HLA-DRB1* SE, *PTPN22* and smoking associated mainly with CEP-1 positive RA (OR=39.1), compared to CEP-1 negative/CCP positive RA (OR=7.9). A similar pattern was observed for antibodies to Cit-vim, where the association of *HLA-DRB1* SE, in combination with *PTPN22* and smoking, was stronger with Cit-vim positive (OR=25.6) compared to Cit-vim negative/CCP positive disease (OR=8.9) (figure 3b). Subsets defined by antibodies to Cit-fib or Cit-C1, on the other hand, did not demonstrate such specific associations, beyond the effect of CCP (OR=15.6 *versus* 12.9 for Cit-fib, and 10.0 *versus* 13.8 for Cit-C1) (figure 3c and d).

Impact of *HLA-DRB1* SE on disease risk in different subsets of RA

The results described in the previous paragraph may be influenced by the co-existence of multiple ACPA fine-specificities. Hence, we next investigated the four RA subsets which were defined by the sole presence of one ACPA-fine-specificity, i.e. the subsets that were 'single positive' for anti-CEP-1, anti-Cit-vim, anti-Cit-fib or anti-Cit-C1 antibodies. In this analysis we focused on *HLA-DRB1* SE, the risk factor with strongest impact. We found a relatively strong association of *HLA-DRB1* SE with CEP-1 positive (OR=5.1), as well as with Cit-vim positive disease (OR=6.0), but not with Cit-fib positive disease (OR=1.4), and the association with Cit-C1 positive RA was weak (OR=1.6) (table 1). A significant association of *HLA-DRB1* SE (OR=2.5; 95% CI=1.8-3.6) could also be observed with the subset which was positive for CCP but negative for all four ACPA fine-specificities (data not shown).

Table 1. Odds ratios for disease risk in different subsets of RA - in carriers of *HLA-DRB1* SE, compared to non-carriers

ACPAs	CEP-1/Cit-vim/Cit-fib/Cit-C1	*HLA-DRB1* SE		OR*	95% CI
		any	none		
control group	na	643	635	1.0	ref.
case groups	+/-/-/-	59	12	5.1	2.7-9.5
	-/+/-/-	108	18	6.0	3.6-10.0
	-/-/+/-	28	21	1.4	0.75-2.5
	-/-/-/+	91	57	1.6	1.11-2.3
	+/+/-/-	97	2	49.6	12.1-202.5
	+/-/+/-	21	7	3.2	1.3-7.6
	+/-/-/+	43	3	15.0	4.6-49.0
	-/+/+/-	18	8	2.3	1.0-5.4
	-/+/-/+	69	10	6.9	3.5-13.6
	-/-/+/+	36	14	2.5	1.3-4.7

* adjusted for age, gender, and residence area

We then studied the effect of being positive for different combinations of two ACPA fine-specificities, as the impact of *HLA-DRB1* SE may differ between subsets defined by specific combinations of non-cross reactive ACPAs. Strongest influence of *HLA-DRB1* SE was identified for CEP-1/Cit-vim double positive disease (OR=49.6), while the odds ratio for the association with Cit-fib/Cit-C1 double positive RA was merely 2.5. Interestingly, anti-CCP antibody levels were higher in the Cit-fib/Cit-C1 double positive subset than in the CEP-1/Cit-vim double positive subset, implicating that *HLA-DRB1* SE mainly influence the fine-specificity of the ACPA response, rather than anti-CCP levels (supplementary figure 2).

Impact of smoking, in combination with *HLA-DRB1* SE, on disease risk in different subsets of RA

Since the data presented above do not support an association of *HLH-DRB1* SE with the presence of anti-Cit-fib antibodies, we restricted the next analysis to RA subgroups defined by the presence or absence of antibodies to CEP-1, Cit-vim and Cit-C1. Here, we had enough power to investigate the combined effect of *HLA-DRB1* SE and smoking. As shown in table 2, smoking in the absence of *HLA-DRB1* SE did not significantly associate with any RA subset, but strengthened the *HLA-DRB1* SE association when present. This effect was most pronounced for 'double positive' and 'triple positive' subsets, compared to 'single positive' subsets, but only when anti-CEP-1 antibodies were present. Adding Cit-vim reactivity to the CEP-1 single positive subset, increased the odds ratio for the combined *HLA-DRB1* SE and smoking association from 12.5 to 18.4. A similar effect was seen when adding Cit-C1 reactivity to the CEP-1 single positive subset (from OR=12.5 to 17.2). However, in the absence of anti-CEP-1 antibodies, addition of Cit-C1 reactivity to the Cit-vim single positive subgroup did not increase the odds ratios further (7.3 *versus* 5.6). Highest odds ratio (23.3), for the combined *HLA-DRB1* SE and smoking association, was observed for the 'triple positive' subset (CEP-1+/Cit-vim+/Cit-C1+).

Table 2. Odds ratios for disease risk in different subsets of RA - in subjects exposed to different combinations of *HLA-DRB1* SE and smoking, compared to non-exposed subjects

CEP-1/Cit-vim/Cit-C1	*HLA-DBR1* SE	Smoking	Cases/controls	OR*	95% CI
-/-/-	-	-	115/228	1	Ref.
-/-/-	+	-	176/244	1.4	1.1-1.9
-/-/-	-	+	173/316	1.1	0.8-1.5
-/-/-	+	+	233/315	1.6	1.2-2.1
+/-/-	-	-	3/228	1	Ref.
+/-/-	+	-	25/244	8	2.4-27.0
+/-/-	-	+	13/316	3.3	0.9-11.9
+/-/-	+	+	46/315	12.5	3.8-40.9
-/+/-	-	-	7/228	1	Ref.
-/+/-	+	-	42/244	5.8	2.5-13.3
-/+/-	-	+	15/316	1.7	0.7-4.3
-/+/-	+	+	64/315	7.3	3.2-16.4
-/-/+	-	-	35/228	1	Ref.
-/-/+	+	-	41/244	1.1	0.7-1.8
-/-/+	-	+	31/316	0.6	0.4-1.1
-/-/+	+	+	68/315	1.4	0.9-2.3
+/+/-	-	-	5/228	1	Ref.
+/+/-	+	-	29/244	5.5	2.1-14.5
+/+/-	-	+	7/316	1	0.3-3.3
+/+/-	+	+	118/315	18.4	7.3-46.1
+/-/+	-	-	3/228	1	Ref.
+/-/+	+	-	25/244	7.7	2.3-26.1
+/-/+	-	+	11/316	2.7	0.7-10.0
+/-/+	+	+	65/315	17.2	5.3-55.8
-/+/+	-	-	7/228	1	Ref.
-/+/+	+	-	27/244	3.5	1.5-8.4
-/+/+	-	+	10/316	1	0.4-2.8
-/+/+	+	+	52/315	5.6	2.5-12.7
+/+/+	-	-	6/228	1	Ref.
+/+/+	+	-	54/244	8.5	3.6-20.1
+/+/+	-	+	14/316	1.6	0.6-4.3
+/+/+	+	+	189/315	23.3	10.1-53.7

*:Adjusted for age, gender and residential area

Impact of *PTPN22*, in combination with *HLA-DRB1* SE, on disease risk in different subsets of RA

We then investigated the combined effect of *HLA-DRB1* SE and *PTPN22* in subsets defined by anti-CEP-1, anti-Cit-vim and anti-Cit-C1 antibody status. As seen with smoking, *PTPN22* also mainly had an effect in the presence of *HLA-DRB1* SE, and also this association was most pronounce for 'double positive' and 'triple positive' subsets, encompassing anti-CEP-1 antibodies. Addition of Cit-vim reactivity to the CEP-1 single positive subset increased the odds ratio for the combined *HLA-DRB1* SE and *PTPN22* association from 8.7 to 29.2, while addition of Cit-C1 to the CEP-1 single positive subset changed the odds ratio from 8.7 to 17.1. However, similar odds ratios were observed for the Cit-vim single positive and the Cit-vim/Cit-C1 double positive subsets (8.4 *versus* 8.7). Strongest association (OR=34.8), of the *HLA-DRB1* SE and *PTPN22* combination, was seen with the 'triple positive' subset (CEP-1+/Cit-vim+/Cit-C1+) (supplementary table 2).

The combined effect of *HLA-DRB1* SE, *PTPN22* and smoking on disease risk in subsets of RA defined by anti-CEP-1 and anti-Cit-vim antibody status

In the final analysis we investigated the combined effect of all three risk factors (*HLA-DRB1* SE, *PTPN22* and smoking), and calculated gene-environment and gene-gene interactions. Here we focused on what appears to be the two most important ACPA fine-specificities in this context, and defined RA subgroups based on CEP-1 and Cit-vim reactivity alone (table 3). Presence of *HLA-DRB1* SE alleles, *PTPN22* polymorphism and cigarette smoking conferred a rather modest risk for the development of CEP-1 negative/Cit-vim negative disease (OR=2.4), while the risk was high for CEP-1 positive/Cit-vim negative RA (OR=24.4) and for CEP-1 negative/Cit-vim positive RA (OR=11.5). However, highest risk was identified for the CEP-1/Cit-vim double positive subset (OR=50.1).

Table 3. Odds ratios for disease risk in different subsets of RA - in subjects exposed to different combinations of *HLA-DRB1* SE, smoking and *PTPN22*, compared to non-exposed subjects

CEP-1/Cit-vim	*HLA-DRB1* SE	Smoking	*PTPN22*	Cases/controls	OR*	95% CI
-/-	-	-	-	106/178	1	Ref.
-/-	-	-	+	41/49	1.4	0.9-2.3
-/-	+	-	-	168/200	1.4	1.0-1.9
-/-	+	-	+	47/43	2	1.2-3.2
-/-	-	+	-	140/233	1	0.8-1.4
-/-	-	+	+	61/81	1.4	0.9-2.1
-/-	+	+	-	223/252	1.6	1.1-2.1
-/-	+	+	+	76/61	2.4	1.5-3.6
+/-	-	-	-	4/178	1	Ref.
+/-	-	-	+	2/49	1.9	0.3-10.7
+/-	+	-	-	32/200	7	2.4-20.3
+/-	+	-	+	18/43	20.1	6.4-63.2
+/-	-	+	-	13/233	2.6	0.8-8.2
+/-	-	+	+	10/81	5.7	1.7-18.8
+/-	+	+	-	80/252	15.4	5.5-43.1
+/-	+	+	+	29/61	24.4	8.2-73.1
-/+	-	-	-	10/178	1	Ref.
-/+	-	-	+	4/49	1.7	0.5-5.7
-/+	+	-	-	47/200	4.3	2.1-8.8
-/+	+	-	+	22/43	9.9	4.3-22.8
-/+	-	+	-	21/233	1.7	0.8-3.8
-/+	-	+	+	4/81	1	0.3-3.3
-/+	+	+	-	83/252	6.3	3.2-12.7
-/+	+	+	+	33/61	11.5	5.3-25.3
+/+	-	-	-	6/178	1	Ref.
+/+	-	-	+	5/49	3.4	1.0-11.7
+/+	+	-	-	43/200	6.4	2.7-15.6
+/+	+	-	+	40/43	30.4	12.0-76.9
+/+	-	+	-	14/233	1.8	0.7-4.8
+/+	-	+	+	7/81	2.7	0.9-8.2
+/+	+	+	-	212/252	26.6	11.5-61.7
+/+	+	+	+	92/61	50.1	20.6-121.8

*:Adjusted for age, gender and residential area

Analyses of interaction, calculated as the attributable proportion due to interaction (AP),[22-23], demonstrated significant interactions between *HLA-DRB1* SE and smoking in the CEP-1 positive/Cit-vim negative as well as in the CEP-1/Cit-vim double positive subsets, with AP values of 0.33 and 0.64, respectively. Interactions were also observed between *HLA-DRB1* SE and *PTPN22* in the CEP-1 positive/Cit-vim negative (AP=0.44), the CEP-1 negative/Cit-vim positive (AP=0.56) and in the CEP-1/Cit-vim double positive subsets (AP=0.53) (supplementary table 3).

DISCUSSION

The present study provides novel data concerning the relationships between specific autoimmune reactions and genetic and environmental determinants for the risk of developing RA, with the overall conclusion that *HLA-DRB1* SE genes, the *PTPN22* risk allele and cigarette smoking associate with the presence of specific ACPA-reactivities, rather than total levels of anti-CCP antibodies.

We have focused our analyses on antibody responses to well-defined citrullinated epitopes on α-enolase,[25, 26], vimentin,[13, 27], fibrinogen,[28, 29] and collagen type II,[21], representing physiological antigens present in the rheumatoid joint. A vast majority of patients that were positive for these ACPA fine-specificities were also positive in the CCP2 ELISA, the current standard assay for ACPA-detection. Though important to note, 18% of the CCP negative patients were positive for at least one ACPA fine-specificity. Conversely, nearly 14% of the CCP positive patients were negative for the four ACPA fine-specificities, and this subset also showed some association with *HLA-DRB1* SE, clearly indicating that other ACPA targets exist, which has also been reported,[25, 30, 31], and should be considered in future studies.

Our peptide-absorption experiments showed that the ACPAs were mainly non-cross-reactive. Although some degree of cross-reactivity was observed in some sera, the majority did not demonstrate cross-reactivity, in line with previous reports,[9, 14]. Hence we consider these ACPAs as four distinct fine-specificities, and subsequently we base our RA subsets on the presence or absence of anti-CEP-1, anti-Cit-vim, anti-Cit-fib and anti-Cit-C1 antibodies, rather than CCP status. The fact that the ACPAs occur together in some patients, but on their own in others, imply that their production may be governed by partly different mechanisms. It is therefore very interesting to note the specific relationship between *HLA-DRB1* SE and certain ACPA fine-specificities (in particular CEP-1 and Cit-vim), but not others (mainly Cit-fib).

The estimated risks in some of the analyses should be viewed in the context of small sample sizes, with large confidence intervals. Still, our data collectively points to clear differences between ACPA fine-specificities, thus confirming and extending previous reports,[11-13]. Importantly, the present study answer the previously unanswered question,[32], concerning the influence of *HLA-DRB1* SE on the specificity *versus* the magnitude of the ACPA response. Notable is also the fact that the effects of smoking and *PTPN22*, in the context of *HLA-DRB1* SE, appear to be most pronounced in subsets defined by multiple ACPAs where reactivity to CEP-1 is present. These data may suggest that smoking is involved in citrullinating and exposing some, but not other, autoantigens to the immune system, and that *PTPN22* exerts its effects only when certain MHC class II dependent immune reactions are involved. These data will form the basis for future experimental studies, on molecular pathways of relevance for disease development in distinct subsets of RA.

In summary, our data highlight the complexity of the gene-environment influence on ACPA positive RA. A particularly interesting finding is that *HLA-DRB1* SE together with *PTPN22* and smoking, associate so strongly with the presence of anti-CEP-1 antibodies on one hand, and anti-Cit-vim antibodies on the other hand, but mainly with the simultaneous presence of both ACPAs. Since the ‘shared epitope’ comprise several different alleles, and since a dose-effect has been demonstrated for the *HLA-*

DRB1 SE association,[4, 5, 11], one may speculate that peptides from citrullinated α-enolase may be preferentially presented by one specific SE allele and subsequently trigger the production of anti-CEP-1 antibodies, while peptides from citrullinated vimentin are preferentially presented by another SE allele, giving rise to anti-Cit-vim antibodies. Hence, in such a scenario, presence of two different SE alleles would drive the simultaneous production of two different ACPAs, which may tentatively synergize in contributing to the development of RA. Detailed studies of the SE alleles, other MHC class II alleles, as well as other genetic polymorphisms and environmental exposures, in the context of different ACPA fine-specificities, will increase our understanding of the etiology in different subsets of ACPA positive RA. Results from such studies may also help to develop future personalised therapies for patients with rheumatoid artrhritis, and preventive strategies for individuals at risk of developing the disease.

ACKNOWLEDGEMENTS

The authors wish to thank investigators and study participants from the EIRA study. This work was supported by grants from the Swedish Research Council, Vinnova, the Swedish Council for Working Life and Social Research, King Gustaf V's 80-year foundation, the Swedish Rheumatic Foundation, the EU-funded projects Gums&Joints and MasterSwitch, and the IMI program BTCure.

COMPETING INTERESTS

KL is inventor of patent US12/524,465, describing diagnostic use of the CEP-1 epitope. RH is inventor of patent US7/148,020B2, describing diagnostic use of the C1 epitope.

REFERENCES

1 Stastny P. Mixed lymphocyte cultures in rheumatoid arthritis. *J Clin Invest* 1976; **57**:1148-1157.

2 Klareskog L, Forsum U, Scheynius A, et al. Evidence in support of a self-perpetuating HLA-DR-dependent delayed-type cell reaction in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 1982;**79**:3632-3636.

3 Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum* 1987;**30**:1205-1213.

4 Klareskog L, Stolt P, Lundberg K, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted

immune reactions to autoantigens modified by citrullination. *Arthritis Rheum* 2006;**54**:38-46.

5 Huizinga TW, Amos CI, van der Helm-van Mil AH, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. *Arthritis Rheum* 2005;**52**:3433-3438.

6 Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. *Ann Rheum Dis* 2006;**65**:366-371.

7 Makrygiannakis D, Hermansson M, Ulfgren AK, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann Rheum Dis* 2008;**67**:1488-1492.

8 Klareskog L, Ronnelid J, Lundberg K, et al. Immunity to citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol* 2008;**26**:651-675.

9 Snir O, Widhe M, von Spee C, et al. Multiple antibody reactivities to citrullinated antigens in sera from patients with rheumatoid arthritis: association with HLA-DRB1 alleles. *Ann Rheum Dis* 2009;**68**:736-743.

10 Ioan-Facsinay A, el-Bannoudi H, Scerer HU, et al. Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities. *Ann Rheum Dis* 2011;**70**:188-193.

11 Mahdi H, Fisher BA, Kallberg H, et al. Specific interaction between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis. *Nat Genet* 2009;**41**:1319-1324.

12 van der Woude D, Alemayehu WG, Verduijn W, et al. Gene-environment interaction influences the reactivity of autoantibodies to citrullinated antigens in rheumatoid arthritis. *Nat Genet* 2010;**42**:814-816; author reply 816.

13 Verpoort KN, Cheung K, Ioan-Facsinay A, et al. Fine specificity of the anti-citrullinated protein antibody response is influenced by the shared epitope alleles. *Arthritis Rheum* 2007;**56**:3949-3952.

14 van de Stadt LA, van der Horst AR, de Koning MH, et al. The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. *Ann Rheum Dis* 2011;**70**:128-133.

15 Willemze A, van der Woude D, Ghidey W, et al. The interaction between HLA shared epitope alleles and smoking and its contribution to autoimmunity against several citrullinated antigens. *Arthritis Rheum* 2011;**63**:1823-1832.

16 Stolt P, Bengtsson C, Nordmark B, et al. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. *Ann Rheum Dis* 2003;**62**:835-841.

17 Plenge RM, Padyukov L, Remmers EF, et al. Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. *Am J Hum Genet* 2005;**77**:1044-1060.

18 Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315-324.

19 Snir O, Widhe M, Hermansson M, et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. *Arthritis Rheum* 2010;**62**:44-52.

20 Burkhardt H, Koller T, Engstrlm A, et al. Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. *Arthritis Rheum* 2001;**46**:2339-2348.

21 Burkhardt H, Sehnert B, Bockermann R, et al. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. *Eur J Immunol* 2005;**35**:1643-1652.

22 Rothman KJ, Greenland S, Walker AM. Concepts of interaction. *Am J Epidemiol* 1980;**112**:467-470.

23 Hosmer DW, Lemeshow S. Confidence interval estimation of interaction. *Epidemiology* 1992;**3**:452-456.

24 Dobritzsch D, Lindh I, Uysal H, et al. Crystal structure of an arthritogenic anticollagen immune complex. *Arthritis Rheum* 2011;**63**:3740-3748.

25 Lundberg K, Kinloch A, Fisher BA, et al. Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase. *Arthritis Rheum* 2008;**58**:3009-3019.

26 Kinloch A, Lundberg K, Wait R, et al. Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis. *Arthritis Rheum* 2008;**58**:2287-2295.

27 Vossenaar ER, Despres N, Lapointe E, et al. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 2004;**6**:R142-150.

28 Masson-Bessiere C, Sebbag M, Girbal-Neuhauser E, et al. The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 2001;**166**:4177-4184.

29 Takizawa Y, Suzuki A, Sawada T, et al. Citrullinated fibrinogen detected as a soluble citrullinated autoantigen in rheumatoid arthritis synovial fluids. *Ann Rheum Dis* 2006;**65**:1013-1020.

30 Pratesi F, Tommasi C, Anzilotti C, et al. Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis. *Arthritis Rheum* 2006;**54**:733-741.

31 Scherer HU, van der Woude D, Willemze A, et al. Distinct ACPA fine specificities, formed under the influence of HLA shared epitope alleles, have no effect on radiographic joint damage in rheumatoid arthritis. *Ann Rheum Dis* 2011;**70**:1461-1464.

32 Lundberg K, Alfredsson L, Kallberg H, et al. Reply to "Gene-environment interaction influences the reactivity of autoantibodies to citrullinated antigens in rheumatoid arthritis". *Nat Genet* 2010;**42**:816.

FIGURES

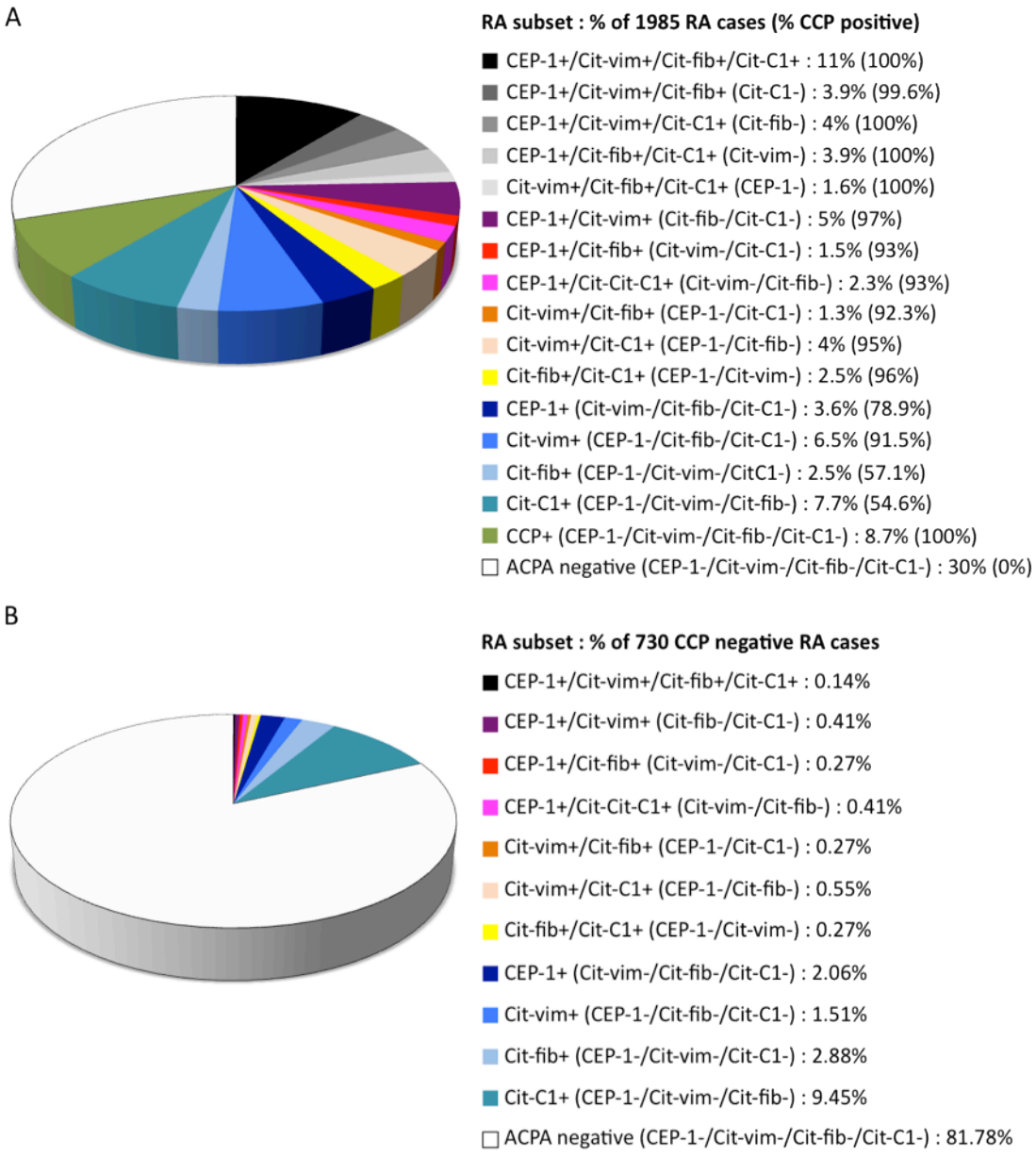


Figure 1 Subsets of RA, defined based on the presence (+) or absence (-) of four different ACPA fine-specificities (CEP-1, Cit-vim, Cit-fib and Cit-C1), as detected by ELISA. A) 1985 RA cases. B) 730 anti-CCP negative RA cases. In panel A, subsets may comprise CCP positive and CCP negative RA cases, with percentages of anti-CCP positive cases shown in brackets.

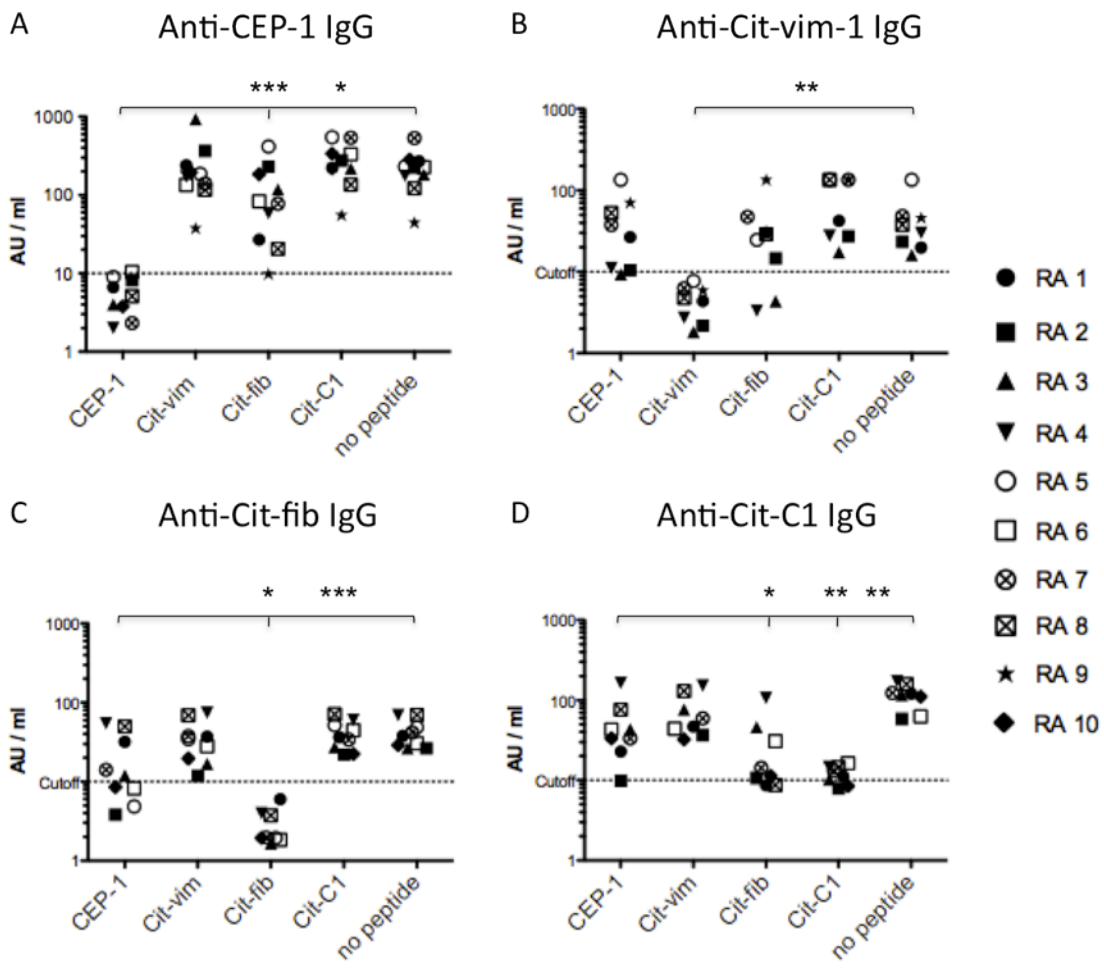


Figure 2 Cross-reactivity between different ACPA fine-specificities. Antibody responses to CEP-1, Cit-vim, Cit-fib and Cit-C1 was measured in serum samples from 10 CCP positive RA patients (RA1 - RA10), after pre-absorption with the different peptides. A) anti-CEP-1 IgG levels; B) anti-Cit-vim IgG levels; C) anti-Cit-fib IgG levels; D) anti-Cit-C1 IgG levels. Serum samples that were negative for one or several ACPA fine-specificities were removed from those specific analyses: RA6 and RA10 (B); RA9 (C); RA5 and RA9 (D). Log scales of AU/ml values are shown on the Y-axis. Dotted lines indicate cut-off for positivity (10AU/ml). X-axis indicate pre-absorbing peptides, or no peptide (buffer alone). Asterisks indicate significant differences in relation to 'no peptide'. *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$.

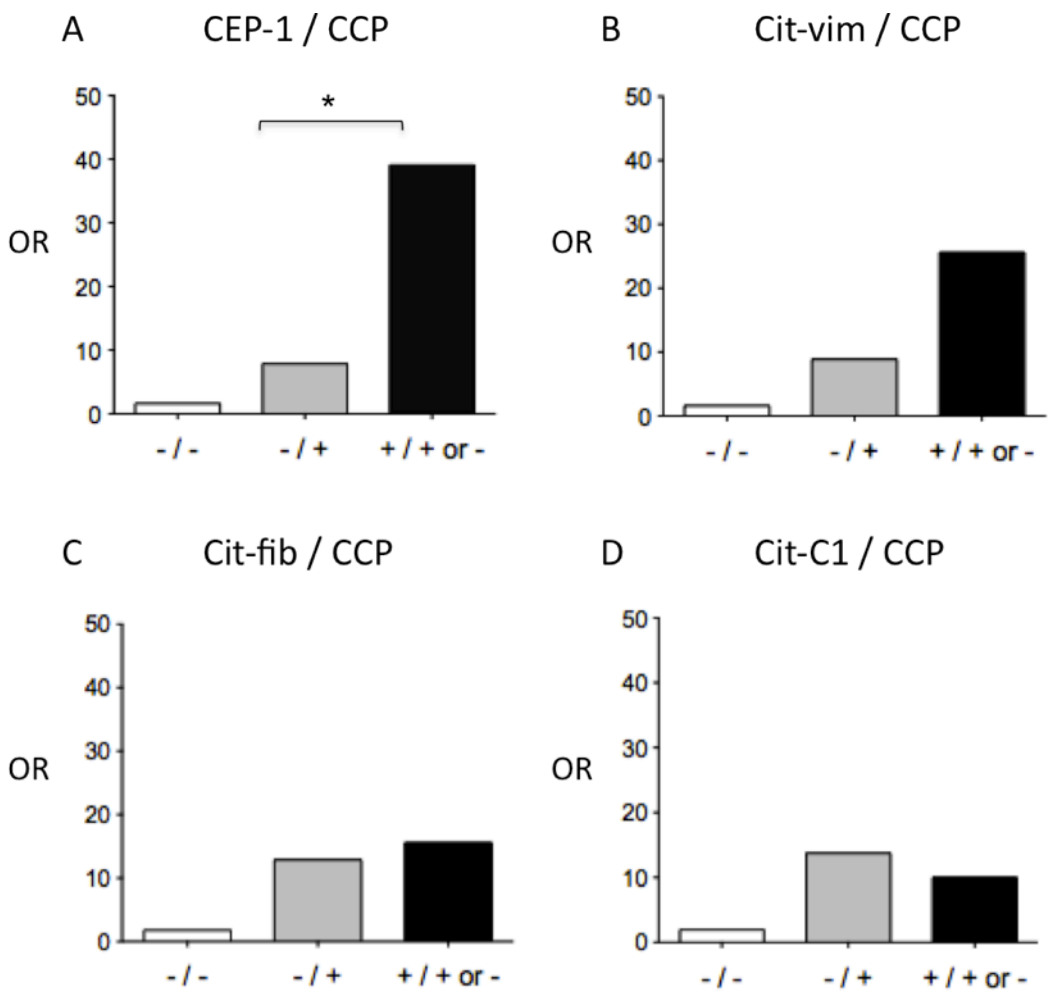


Figure 3 The combined effect of *HLA-DRB1* SE, *PTPN22* and smoking, on disease risk in different subsets of RA. Odds ratios (OR), with 95% confidence intervals (CI), were calculated using unconditional logistic regression analysis, by comparing individuals that were positive for the risk factors with individuals that were negative. RA subsets were defined based on the presence or absence of one ACPA fine-specificity, without taking into account which other ACPA fine-specificities may be present simultaneously. A) anti-CEP-1; B) anti-Cit-vim; C) anti-Cit-fib; D) anti-Cit-C1. White bars show subsets negative for the ACPA fine-specificity and negative for CCP (-/-); grey bars show subsets negative for the ACPA fine-specificity, but positive for CCP (-/+); black bars show subsets positive for the ACPA fine-specificity, but where the CCP status was not considered (+/ + or -). 95% CI for the (-/+) and the (+ / + or -) subsets are: 4.58-13.53 *versus* 19.07-79.97 (A); 5.03-15.68 *versus* 13.95-47.02 (B); 7.55-22.10 *versus* 8.51-28.64 (C); 7.55-24.49 *versus* 6.07-16.49 (D). The asterisk indicate non-overlapping 95% CI.

SUPPLEMENTARY METHODS

ELISAs

Antibody responses to citrullinated peptides from α-enolase (CEP-1), vimentin (Cit-vim), fibrinogen (Cit-fib) and collagen type II (Cit-C1), or the arginine-containing equivalents, were assayed in sera from 1985 EIRA cases and 150 randomly selected EIRA controls, using in-house ELISAs, as previously described,[11, 19]. Briefly, 96-well plates (MaxiSorp, Nunc) were coated with CEP-1 at 5µg/ml, or Cit-C1 at 10µg/ml, and incubated at 4°C over night. Alternatively, 96-well plates (Streptavidine, Pierce, Thermo Scientific) were washed in PBS, 0.05% Tween, prior to coating with biotinylated Cit-vim or Cit-fib peptides, at 1µg/ml, for 1h. Plates were washed and blocked with 1% BSA in PBS for 1h at room temperature (only CEP-1 and Cit-C1 plates), before adding serum samples, diluted 1:100 in RIA buffer (10mM Tris, 1% BSA, 350mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS) and incubated for 1h at room temperature. A standard curve, with serial dilutions of a positive serum pool, for each peptide ELISA, as well as a blank (RIA buffer), a positive and a negative control serum, were included on all plates. All samples were analysed in duplicates. Plates were subsequently washed and incubated for 1h at room temperature, with horse radish peroxidase (HRP)-conjugated goat anti-human IgG (Jacksson), diluted 1:10,000 in RIA buffer. After a final wash, TMB substrate (Sigma) was added for approximately 15 minutes (1h for Cit-C1), before the reaction was stopped by adding 1M H_2SO_4, and absorbance determined at 450nm. Optical density was expressed as arbitrary units (AU/ml), based on the standard curves. The cut-off values for positive samples were determined based on the 98^{th} percentile among 150 controls, and subsequently converted to 10AU/ml for all ACPA fine-specificities.

Each ELISA was optimized independently. The CEP-1 and the Cit-C1 peptides, which were synthesized to contain cysteins at the N and C terminus (CEP-1) or in a triple helical form (Cit-C1), and therefore likely to expose conformational epitopes, were coated directly onto MaxiSorp Nunc plates, while the linear peptides Cit-vim and Cit-fib were biotinylated and coated onto streptavidine plates, to enhance the likelihood of exposing conformational epitopes.

SUPPLEMENTARY TABLES

Supplementary Table 1. Citrullinated peptides

peptide	protein	amino acids	amino acid sequence
CEP-1	α-enolase	5-21	C-KIHA-cit-EIFDS-cit-GNPTVE-C
Cit-vim	vimentin	60-75	VYAT-cit-SSAV-cit-L-cit-SSVP-K-biotin
Cit-fib	fibrinogen, β-chain	36-52	biotin-NEEGFFSA-cit-GHRPLDKK
Cit-C1	collagen type II	359-369	(GPP*)5-GA-cit-GLTG-cit-P*-GDA-(GPP*)2-GKKYG

cit = citrulline
P* = hydroxyproline

Supplementary Table 2. Odds ratios for disease risk in different subsets of RA - in subjects exposed to different combinations of *HLA-DRB1* SE and *PTPN22*, compared to non-exposed subjects

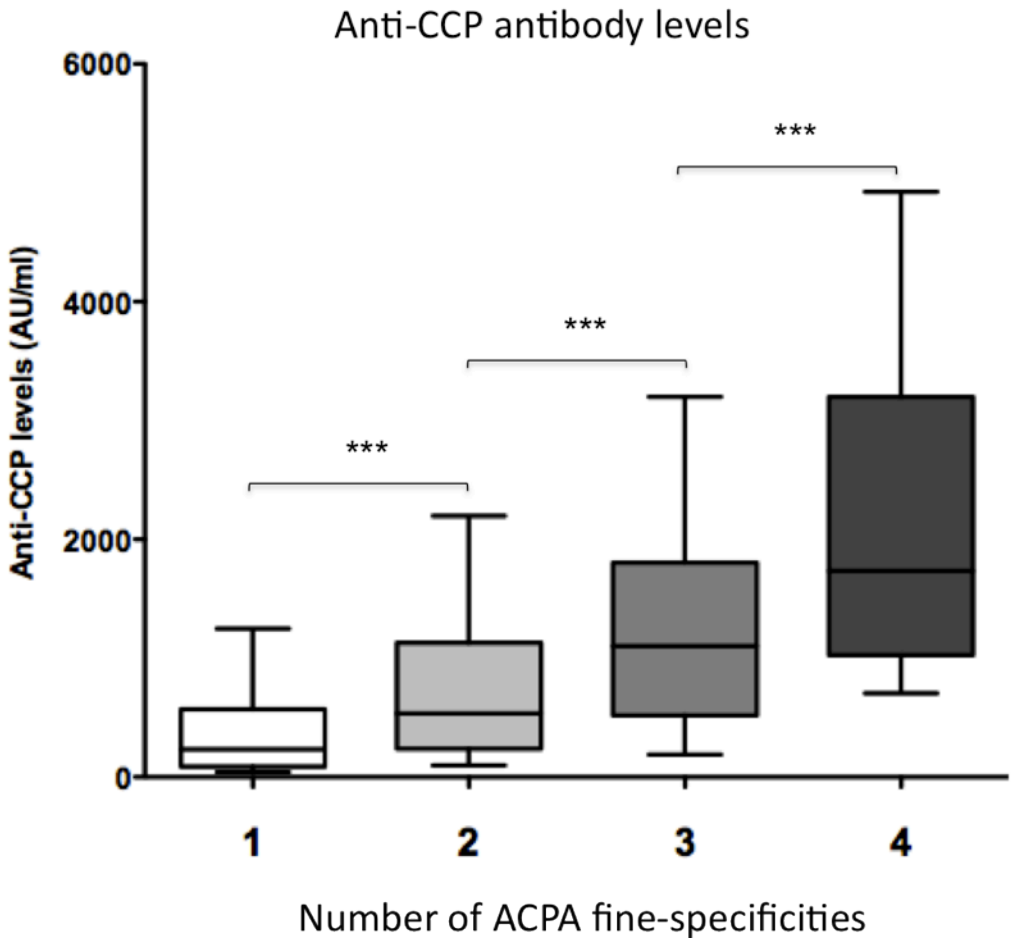
CEP-1/Cit-vim/Cit-C1	*HLA-DRB1* SE	*PTPN22*	Cases/controls	OR*	95% CI
-/-/-	-	-	241/477	1.0	Ref.
-/-/-	+	-	339/520	1.3	1.1-1.6
-/-/-	-	+	91/154	1.2	0.9-1.6
-/-/-	+	+	128/119	2.3	1.7-3.1
+/-/-	-	-	10/477	1.0	Ref.
+/-/-	+	-	59/520	5.5	2.8-10.9
+/-/-	-	+	8/154	2.4	0.9-6.2
+/-/-	+	+	20/119	8.7	3.9-19.2
-/+/-	-	-	21/477	1.0	Ref.
-/+/-	+	-	87/520	3.8	2.3-6.3
-/+/-	-	+	5/154	0.8	0.3-2.1
-/+/-	+	+	39/119	8.4	4.7-14.9
-/-/+	-	-	43/477	1.0	Ref.
-/-/+	+	-	102/520	2.2	1.5-3.2
-/-/+	-	+	25/154	1.9	1.1-3.2
-/-/+	+	+	24/119	2.3	1.4-4.0
+/+/-	-	-	8/477	1.0	Ref.
+/+/-	+	-	111/520	13.3	6.4-27.6
+/+/-	-	+	4/154	1.7	0.5-5.6
+/+/-	+	+	51/119	29.2	12.9-61.6
+/-/+	-	-	10/477	1.0	Ref.
+/-/+	+	-	66/520	6.2	3.2-12.3
+/-/+	-	+	6/154	2.0	0.7-5.6
+/-/+	+	+	38/119	17.1	8.2-35.8
-/+/+	-	-	14/477	1.0	Ref.
-/+/+	+	-	65/520	4.3	2.4-7.8
-/+/+	-	+	3/154	0.7	0.2-2.5
-/+/+	+	+	28/119	8.7	4.4-17.3
+/+/+	-	-	12/477	1.0	Ref.
+/+/+	+	-	178/520	14.0	7.7-25.6
+/+/+	-	+	9/154	2.4	1.0-5.7
+/+/+	+	+	96/119	34.8	18.4-65.9

*:Adjusted for age, gender and residential area

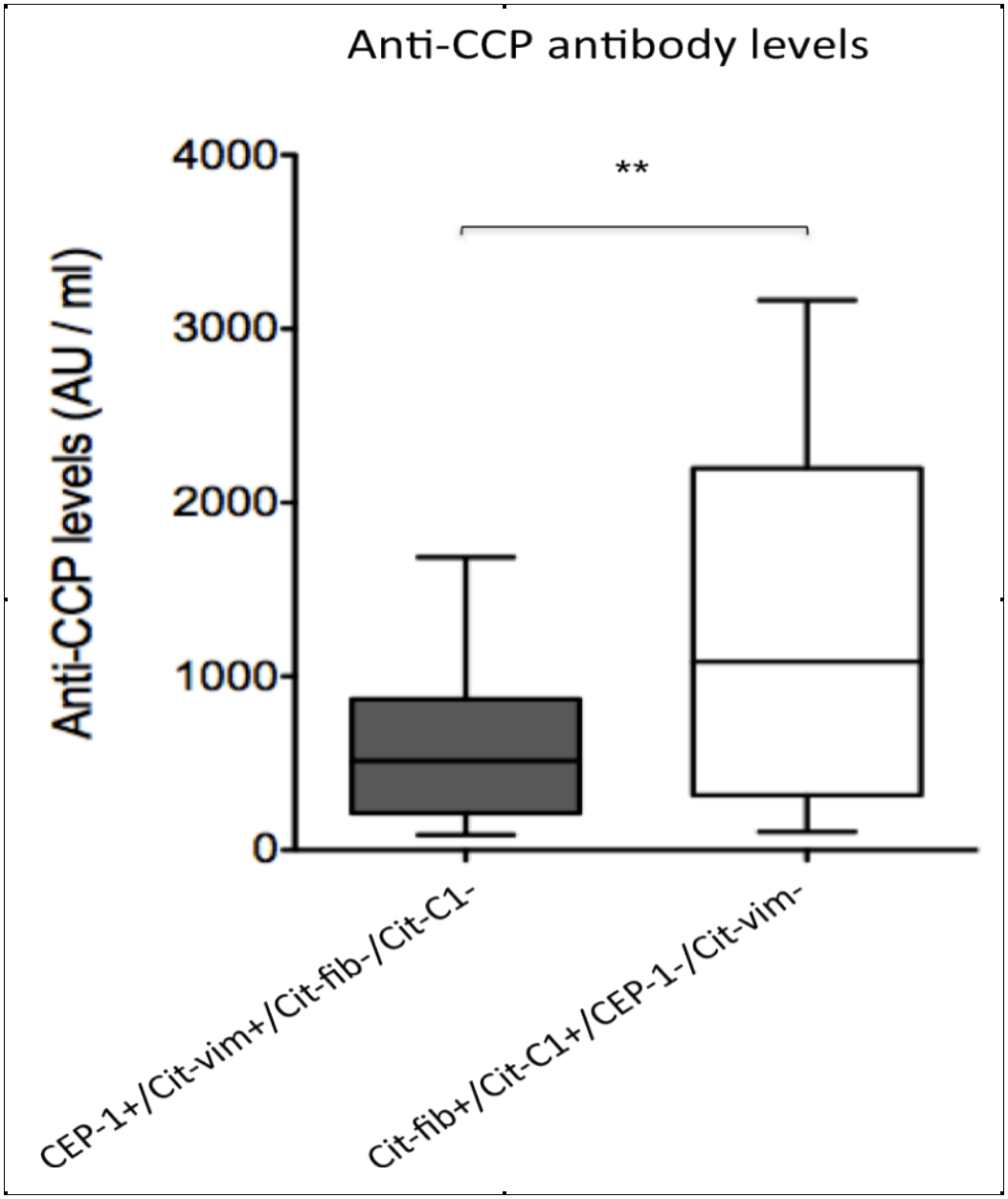
Supplementary Table 3. Interaction (calculated as the attributable proportion (AP) due to interaction) between *HLA-DRB1* SE and smoking, and between *HLA-DRB1* SE and *PTPN22*, in different subsets of RA

RA subset	*HLA-DRB1* SE - smoking		*HLA-DRB1* SE - *PTPN22*	
CEP-1/Cit-Vim	**AP**	**95% CI**	**AP**	**95% CI**
-/-	0.11	-0.17-0.39	0.24	-0.01-0.50
+/-	0.33	0.09-0.58	0.44	0.21-0.67
-/+	0.21	-0.08-0.51	0.56	0.38-0.74
+/+	0.64	0.53-0.76	0.53	0.40-0.67

SUPPLEMENTARY FIGURES



Supplementary figure 1 Anti-CCP antibody levels in different anti-CCP positive subsets of RA, defined based on the number of ACPA fine-specificities present (1, 2, 3 or 4). Box plot whiskers show anti-CCP levels (AU/ml) within the 10^{th}-90^{th} percentile. The median value for each subset is indicated as a line within the boxes. *** $p < 0.0001$.



Supplementary figure 2 Anti-CCP antibody levels in two different anti-CCP positive subsets of RA, each defined based on the sole presence of two ACPA fine-specificities: CEP-1 and Cit-vim (grey bar); Cit-fib and anti-Cit-C1 (white bar). Box plot whiskers show anti-CCP levels (AU/ml) within the 10^{th}-90^{th} percentile. The median value for each subset is indicated as a line within the boxes. ** p = 0.0025.