EXTENDED REPORT

Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies

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Handling editor Tore K Kvien

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2013-205109).

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Received 20 December 2013
Accepted 15 April 2014
Published Online First
8 May 2014

▶ http://dx.doi.org/10.1136/annrheumdis-2014-205307

To cite: Jiang X, Trouw LA, van Wesemael TJ, *et al*. *Ann Rheum Dis* 2014;**73**:1761–1768.

ABSTRACT

Introduction In rheumatoid arthritis (RA), several genetic risk factors and smoking are strongly associated with the presence of anticitrullinated protein antibodies (ACPA), while much less is known about risk factors for ACPA-negative RA. Antibodies against carbamylated proteins (anti-CarP) have been described in both ACPA-positive and ACPA-negative RA patients. In this study, we have analysed the relationships among anti-CarP antibodies, ACPA, genetic risk factors (*HLA-DRB1* alleles and *PTPN22*) and smoking in RA.

Methods Presence of antibodies to carbamylated fetal calf serum (CarP-FCS) and fibrinogen (CarP-Fib) was determined by inhouse ELISAs among RA cases in the Leiden Early Arthritis Clinic (n=846) and in the Swedish Epidemiological Investigation of Rheumatoid Arthritis (n=1985) cohorts. ORs for associations with different *HLA-DRB1* alleles, *PTPN22* genotypes and smoking were calculated separately for each cohort as well as in meta-analysis in RA subsets defined by the presence/absence of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies.

Results In both cohorts, anti-CarP antibody positivity was mainly detected in the anti-CCP-positive population (49%–73%), but also in the anti-CCP-negative population (8%–14%). No associations between anti-CarP antibodies and *HLA-DRB1* shared epitope alleles could be identified, while there were data to support an association between anti-CarP-FCS and *HLA-DRB1**03. Further analyses did not reveal any specific associations of anti-CarP antibodies with other *HLA-DRB1* alleles, *PTPN22* genotypes or smoking.

Conclusions Anti-CarP antibodies were present in both ACPA-positive and ACPA-negative RA. There were no significant associations among anti-CarP antibodies and *HLA-DRB1* alleles, *PTPN22* or smoking. These data suggest that different biological mechanisms may underlie anti-CarP versus anti-CCP antibody formation.

INTRODUCTION

The presence of autoantibodies is a distinctive feature of rheumatoid arthritis (RA). The two autoantibody systems, which are most commonly used as an aid for diagnosing/classifying RA, are rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPA).[1] These autoantibodies have good diagnostic properties and are associated with a more severe disease course.[2 3] Furthermore, they can predict future disease development and have been implicated in the pathogenesis of the disease.[4 5] The hypothesis that autoantibodies may be of pathophysiological importance has been fuelled by the discovery of strong associations among the most important genetic risk factors for RA (the *HLA-DRB1* shared epitope (SE) alleles and *PTPN22*), the best characterised environmental risk factor smoking and the presence of autoantibodies, in particular ACPA.[6 7] These epidemiological data form the basis of an aetiological hypothesis concerning the triggering of ACPA-positive RA, according to which smoking causes protein citrullination in the lungs, followed by presentation of citrullinated antigens by *HLA-DRB1*-SE molecules and, subsequently, in the context of a *PTPN22* polymorphism, activation of auto-reactive T and B cells and ultimately the production of ACPA by B cells.[8] In contrast, much less is known (and hypothesised) about the pathogenesis of ACPA-negative RA in terms of risk factors and biomarkers such as autoantibodies.

Recently, a new autoantibody system with antibodies directed against carbamylated proteins (anti-CarP antibodies) has been described in RA. These antibodies are present in both anticyclic citrullinated peptide (anti-CCP)-positive and -negative patients.[9] Anti-CarP antibodies have been found to associate with joint destruction, measured as radiological progression, in anti-CCP-negative RA;[9] and in an arthralgia cohort, anti-CarP antibodies were shown to predict the development of RA, independent of anti-CCP antibodies.[10] These findings raised questions concerning the relationship among anti-CarP antibodies, anti-CCP antibodies and known genetic and environmental risk factors. If risk factors for the development of anti-CarP antibodies could be identified, this may also shed more light on the pathogenesis of ACPA-negative RA in general. Hence, in the present study, we have analysed the presence of anti-CarP antibodies in two large well-characterised early RA cohorts (one Swedish and one Dutch), and further investigated the association

among anti-CarP status, anti-CCP status, *HLA-DRB1* alleles, *PTPN22* and smoking.

METHODS

Study population

This study is based on data from two European case-control cohorts, one Swedish and one Dutch. All participants provided written informed consent, and ethical permission was obtained from local ethical committees.

The Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA), an ongoing population-based case-control study, comprises incident cases and controls aged 18–70 years, from a geographically defined area in the south/central part of Sweden. A case is defined as a person who was given a first time RA diagnosis, according to the 1987 American College of Rheumatology (ACR) criteria,[11] by a rheumatologist. Controls were randomly selected from the national population register, matching cases on age, gender and residential area. Details of the study design have been reported elsewhere.[12] Subjects completed a self-administrated questionnaire relating to life style and environmental exposures and donated blood at inclusion. The present study included 1985 RA cases and 2252 controls recruited between 1996 and 2006.

Data on Dutch RA cases were collected from the Leiden Early Arthritis Clinic (EAC),[13] initiated in 1993, which includes patients with recent-onset arthritis (less than 2 years of symptoms) treated at the Leiden University Medical Center (LUMC). Further details have been described elsewhere.[13] For the current analyses only baseline samples were used. Dutch controls for genetics were randomly selected from the collection of the section of Immunogenetics and Transplantation Immunology, at the Department of Immunohematology and Blood Transfusion, LUMC. The present study included 846 RA cases (diagnosed according to the 1987 ACR criteria[11]) and 1211 controls.

Detection of anti-CarP antibodies and anti-CCP2 antibodies

Antibodies directed against CarP were detected using two carbamylated antigens, either carbamylated fetal calf serum (FCS) or carbamylated fibrinogen (Fib) using inhouse ELISAs as described before (EAC)[9] or with some modifications (EIRA); Nunc high-binding plates (Thermo Scientific) were coated with 10 μg/mL of CarP-Fib, CarP-FCS, or the non-carbamylated versions, Fib and FCS, diluted in phosphate buffered saline (PBS) (pH 9), at 4°C overnight. Plates were blocked with 2% bovine serum albumin (BSA) (Sigma) in PBS (pH 9) for 1 h at 4°C, and further incubated for 4 h at 4°C with serum diluted 1:50 in radioimmunoprecipitation assay (RIA) buffer (10 mM Tris, 1% BSA, 350 mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS). Bound antibodies were detected after incubation for 2 h at 4°C with horseradish peroxidase-conjugated goat antihuman IgG (Jacksson), diluted 1:10 000 in RIA buffer, followed by the addition of tetramethylbenzidine (TMB) substrate (Sigma). The colour reaction was stopped by the addition of 0.5 M H_2SO_4, and absorbance was measured at 450 nm. Optical density was transformed into arbitrary units (AU/mL) using the titration curve of a serum pool from three anti-CarP antibody-positive serum samples. Background signals of Fib and FCS were subtracted from the CarP-Fib and CarP-FCS signals, respectively. Cut-off for positivity was equivalent to 2 SDs above the mean of 316 EIRA controls.

Anti-CCP IgG was measured using the second-generation ELISA (anti-CCP2) (Immunoscan CCPlus, Euro-Diagnostica) according to the manufacturer's instructions, with a cut-off for positivity set to 25 AU/mL.

Genotyping

The genotyping procedures for *HLA-DRB1* alleles have been described previously.[14 15] In short, genotyping of DNA samples from both EIRA (1348 cases/976 controls) and EAC (846 cases/1211 controls) subjects was performed by sequence-specific primer PCR. An interpretation table for the *HLA-DRB1* low-resolution kit was used to determine the specific genotype according to manufacturer's recommendations. The *HLA-DRB1* allelic subgroups analysed in the present study were: *DRB1**01, *03, *04, *07, *08, *09, *10, *11, *12, *13, *14, *15 and *16. In EIRA, *HLA-DRB1**01 (except *DRB1**0103), *04 and*10 were classified as SE alleles. In EAC, a specific probe was used to detect the presence of the SE sequence, thereby classifying *DRB1**01:01, *01:02, *04:01, *04:04, *04:05, *04:08 and *10:01 as SE alleles.

The protein tyrosine phosphatase gene (*PTPN22* rs2476601) was genotyped on 3227 EIRA subjects (1943 cases/1284 controls) and 1062 EAC subjects (651 cases/411 controls). Detailed information regarding the typing procedure is described elsewhere.[16 17] Within both datasets, the information on genotyping (*HLA*, *PTPN22*) was not available for the exact same number of individuals as indicated above, without any evidence for a systematic bias.

Cigarette smoking

Information regarding smoking in the EIRA study was collected via questionnaires at inclusion.[18] Subjects were categorised as 'ever smokers' or 'never smokers', where 'ever smokers' comprise former and current cigarette smokers. In total, concomitant genetic and smoking data from 1727 EIRA cases and 1913 EIRA controls were available for analyses.

Information on cigarette smoking habits was collected at inclusion in the EAC study through interviews conducted by trained research nurses.[19] Patients were asked whether they had ever smoked or not. Data were available on 852 RA cases.

Statistical analysis

First, EIRA and EAC data were analysed separately, using unconditional logistic regression models, to calculate ORs with 95% CIs, for the development of RA in association with different *HLA-DRB1* alleles, SE alleles, *PTPN22* and smoking. We categorised RA cases into four subsets based on the presence/absence of anti-CCP IgG and anti-CarP (CarP-Fib or CarP-FCS) IgG. All analyses in EIRA were performed with adjustment for the matching variables (age, gender and residential area). A dominant genetic model that estimates the effect of the presence of a certain allele (irrespective of one or two copies) was used for all analyses, with subjects who were non-carriers as reference category. Since smoking data were not available in EAC controls, double-negative RA cases (CCP−/CarP−) were used as the reference category, and then compared with the other RA subgroups (ie, CCP−/CarP+, CCP+/CarP− and CCP+/CarP+) when analysing the association with smoking. In addition, in EIRA, we also performed the association analysis of smoking using EIRA controls as a referent.

Due to the strong effect of SE alleles among anti-CCP-positive RA cases, we also analysed the association with non-SE *HLA-DRB1* alleles after stratification for the presence of SE alleles. For each non-SE allele (eg, *DRB1**03), all six possible genotypes were taken into account: group A, *DRB1**03/*DRB1**03; group B, *DRB1**03/x; group C, x/x; group D, SE/SE; group E, SE/x; and group F, SE/*DRB1**03 (with x denoting alleles that were neither SE nor *DRB1**03). For the SE-negative stratum, the

effect in groups A and B was investigated by using group C as reference category. For the SE-positive stratum, the effect of group F was analysed by using group E as the reference category.

Subsequently, we performed a meta-analysis by using the effect size (β) and SEs from the two separate analyses (EIRA and EAC). Here, we applied a random-effect approach for all the comparisons to allow for inbetween-study heterogeneity to be incorporated into the calculations.

All analyses were performed using software SAS (V.9.3; Cary, North Carolina, USA) and Stata (V.11.0; STATA Corp, College Station, Texas, USA). All p values were two-sided.

RESULTS

Frequency of anti-CarP antibodies, in relation to anti-CCP IgG, in RA

Anti-CarP-FCS antibodies could be detected in 35.6% of EIRA patients and 44.9% of EAC patients; while anti-CarP-Fib antibodies were detected in 42.6% and 38.0%, respectively, in EIRA and EAC (figure 1A and B). Most anti-CarP antibody-positive RA patients were found in the anti-CCP antibody-positive population (49%–73%), while a smaller subset was found in the anti-CCP antibody-negative populations (8%–14%) of both cohorts. The differences were statistically significant ($p<0.0001$ for both EAC and EIRA). Hence, double-positive and double-negative as well as anti-CarP antibody single positive and anti-CCP antibody single positive subsets of RA could be identified in both cohorts (figure 1A and B).

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

We then proceeded to investigate associations of genetic and environmental risk factors with the presence of anti-CarP antibodies, in relation to anti-CCP IgG status. We first analysed associations of different RA subsets with *HLA-DRB1*-SE alleles, and found a significant strong risk effect in anti-CCP-positive RA, regardless of anti-CarP antibody status, in both EIRA and EAC, as well as in the meta-analysis. When analysing the difference between CCP+/CarP− and CCP+/CarP+ patients, the strongest association was found in double-positive (CCP+/CarP+) RA in both cohorts, with the exception of anti-CarP-FCS in EAC (data not shown). In contrast, we could not find statistically significant associations of SE specifically with anti-CarP antibodies (ie, with CCP−/CarP+ RA subset) (table 1). Consistent with previous reports, all three SE alleles (*HLA-DRB1**01, *04 and *10) contributed to an increased risk specifically for anti-CCP antibody-positive RA (rather than anti-CarP antibody-positive RA), with *HLA-DRB1**04 displaying the strongest association (see online supplementary tables S1A,B and S2A,B).

Association of different *HLA-DRB1* alleles and disease risk in different RA subsets

Since we could not identify any specific associations of SE alleles with anti-CarP antibodies, we next explored the relationship between other *HLA-DRB1* alleles and anti-CarP antibody-positive RA. In unstratified analyses, where presence/absence of SE was not taken into account, *HLA-DRB1**03, *07, *08, *13, *14 and *15 were all associated with protection against anti-CCP-positive RA, with a more pronounced effect seen in the double-positive (CCP+/CarP+) subgroups. However, after stratification for SE, only *HLA-DRB1**13 alleles continued to be associated with protection against anti-CCP-positive RA, while the protective effects of all other alleles were lost (see online supplementary tables S1A,B and S2A,B).

For *HLA-DRB1*03*, we observed a consistent risk association with the CCP−/CarP-FCS+ subgroup in both cohorts, with a meta-analysis OR of approximately 2.5 in both SE-positive and

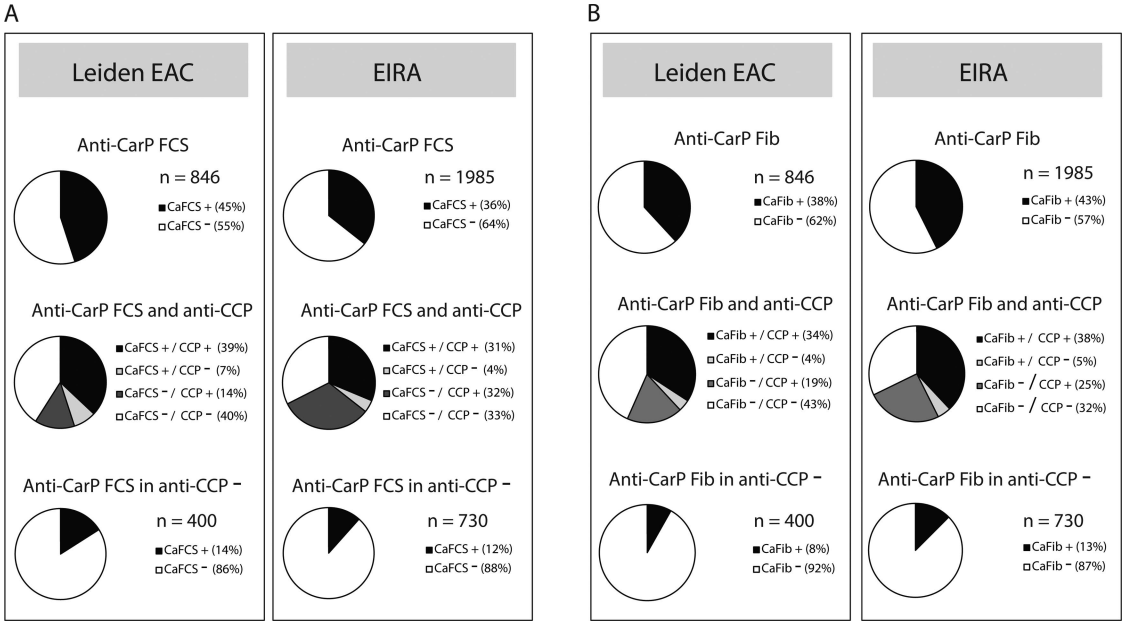

Figure 1 Disease subsets based on presence (+)/absence (−) of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies, as measured by ELISA, in two early rheumatoid arthritis (RA) cohorts, the Leiden Early Arthritis Clinic (EAC) (n=846) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) (n=1985). (A) anti-CarP-FCS data in Leiden EAC (left panel) and Swedish EIRA (right panel); (B) anti-CarP-Fib data in Leiden EAC (left panel) and Swedish EIRA (right panel). (Top panel) Only anti-CarP IgG data; (middle panel) anti-CarP IgG and anti-CCP IgG data; (bottom panel) anti-CarP IgG data in the anti-CCP-negative subsets. Percentages (%) of patients in each subset are shown in brackets. In the panels, anti-CarP-FCS antibodies are abbreviated as CaFCS, anti-CarP-Fib antibodies as CaFib and anti-CCP antibodies as CCP. FCS, fetal calf serum.

Table 1 Meta-analysis for disease risk in different rheumatoid arthritis (RA) subgroups divided by the presence/absence of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies in subjects exposed to any *HLA-DRB1* SE alleles compared with non-exposed subjects

Group	Cohort	SE		OR*	95% CI	p Value
		None N (%)	Any N (%)			
CarP-FCS						
Controls	Leiden EAC	674 (55.7)	537 (44.3)	1.0	Ref.	–
	Swedish EIRA	635 (49.7)	643 (50.3)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-FCS−	Leiden EAC	147 (52.5)	133 (47.5)	1.14	0.88 to 1.47	0.34
	Swedish EIRA	286 (45.0)	350 (55.0)	**1.22**	**1.01 to 1.49**	**0.043**
	Meta-analysis			**1.19**	**1.02 to 1.39**	**0.028**
CCP−/CarP-FCS+	Leiden EAC	29 (56.9)	22 (43.1)	0.95	0.54 to 1.68	0.87
	Swedish EIRA	43 (50.6)	42 (49.4)	0.92	0.59 to 1.44	0.73
	Meta-analysis			0.93	0.66 to 1.32	0.69
CCP+/CarP-FCS−	Leiden EAC	20 (20.4)	78 (79.6)	**4.90**	**2.96 to 8.10**	**<0.001**
	Swedish EIRA	111 (17.7)	515 (82.3)	**4.63**	**3.65 to 5.86**	**<0.001**
	Meta-analysis			**4.68**	**3.78 to 5.80**	**<0.001**
CCP+/CarP-FCS+	Leiden EAC	57 (20.4)	222 (79.6)	**4.89**	**3.58 to 6.68**	**<0.001**
	Swedish EIRA	78 (12.7)	536 (87.3)	**6.99**	**5.37 to 9.10**	**<0.001**
	Meta-analysis			**5.91**	**4.16 to 8.38**	**<0.001**
CarP-Fib						
Controls	Leiden EAC	674 (55.7)	537 (44.3)	1.0	Ref.	–
	Swedish EIRA	635 (49.7)	643 (50.3)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-Fib−	Leiden EAC	168 (53.5)	146 (46.5)	1.09	0.85 to 1.40	0.49
	Swedish EIRA	290 (46.0)	340 (54.0)	1.16	0.96 to 1.41	0.14
	Meta-analysis			1.13	0.97 to 1.32	0.11
CCP−/CarP-Fib+	Leiden EAC	12 (41.4)	17 (58.6)	1.78	0.84 to 3.76	0.13
	Swedish EIRA	39 (42.9)	52 (57.1)	1.35	0.87 to 2.08	0.18
	Meta-analysis			1.45	0.99 to 2.11	0.05
CCP+/CarP-Fib−	Leiden EAC	35 (26.7)	96 (73.3)	**3.44**	**2.30 to 5.15**	**<0.001**
	Swedish EIRA	90 (18.3)	403 (81.7)	**4.50**	**3.48 to 5.82**	**<0.001**
	Meta-analysis			**4.12**	**3.22 to 5.28**	**<0.001**
CCP+/CarP-Fib+	Leiden EAC	44 (17.3)	210 (82.7)	**5.99**	**4.25 to 8.45**	**<0.001**
	Swedish EIRA	99 (13.3)	648 (86.7)	**6.58**	**5.17 to 8.37**	**<0.001**
	Meta-analysis			**6.38**	**5.24 to 7.77**	**<0.001**

Bold typeface indicates statistical significance.
*ORs in Sweden cohort adjusted for age, gender and residential areas.
EAC, Early Arthritis Clinic; EIRA, Epidemiological Investigation of Rheumatoid Arthritis; FCS, fetal calf serum; SE, shared epitope.

SE-negative strata (table 2). We observed a minor association of *HLA-DRB1**03 with anti-CCP/anti-CarP-FCS double-negative subset, albeit with lower ORs (approximately 1.5 in the meta-analysis, for both strata). When the analysis was performed using CCP−/CarP− patients as reference category, the differences between the two ORs were not statistically significant (see online supplementary table S3). Thus, although the effect observed in the CCP−/CarP-FCS+ group was considerably more pronounced, it was not statistically significantly higher than the effect observed in double-negative group.

No consistent associations between other *HLA-DRB1* alleles and the CCP−/CarP+ subgroup were found across cohorts or across anti-CarP antibody specificities.

Association between *PTPN22* gene and different RA subsets

We then performed similar association analyses for *PTPN22*, the second most important genetic risk factor identified for RA. The *PTPN22* polymorphism was not associated with anti-CarP-positive disease, although at first glance there might appear to be an association with CCP−/CarP-FCS+ RA in EIRA (table 3). However, the estimate was neither replicated in the EAC cohort nor in the meta-analysis. In the meta-analysis, there was a consistent association between *PTPN22* and the presence of anti-CCP, but not anti-CarP antibodies. In EIRA we could also observe a positive association of *PTPN22* with the double-negative subset (in line with previously published data on the marginally significant risk effect of *PTPN22* in anti-CCP-negative RA[20]), which could not be seen in EAC.

Association between smoking and different RA subsets

Finally, we explored the association between RA subgroups and cigarette smoking, the best known environmental risk factor for (ACPA-positive) RA. In accordance with previously published data, smoking associated with anti-CCP antibody-positive RA. There was no specific association with anti-CarP antibody-positive RA (table 4). In addition to the analyses presented in table 4, we performed a separate analysis, using only EIRA data, where we have detailed information on smoking habits also for controls (in addition to RA cases). Comparing different RA case subsets with the control group generated similar results on the association with smoking (data not shown), as did the first analysis, where we used the antibody-negative RA subset as referent.

DISCUSSION

The detection of autoantibodies in sera of RA patients has opened up possibilities of subgrouping the RA population to allow more precise prognosis and possibly therapeutic management. Next to the well-known IgM RF and ACPA, a number of new autoantibodies have been reported to be useful in this

Table 2 Meta-analysis for disease risk in different rheumatoid arthritis (RA) subgroups divided by the presence/absence of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies in subjects exposed to any *HLA-DRB1-03* alleles compared with non-exposed subjects, stratified by shared epitope status

		Shared epitope-positive stratum					Shared epitope-negative stratum				
		Any DRB1*03					Any DRB1*03				
Group	Cohort	None N (%)	Any N (%)	OR*	95% CI	p Value	None N (%)	Any N (%)	OR*	95%CI	p Value
CarP-FCS											
Controls	Leiden EAC	385 (83.7)	75 (16.3)	1.0	Ref.	–	480 (71.2)	194 (28.8)	1.0	Ref.	–
	Swedish EIRA	318 (83.0)	65 (17.0)	1.0	Ref.	–	338 (67.2)	165 (32.8)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–			1.0	Ref.	–
CCP−/CarP-FCS−	Leiden EAC	84 (75.0)	28 (25.0)	**1.71**	**1.04 to 2.81**	**0.030**	87 (59.2)	60 (40.8)	**1.71**	**1.18 to 2.47**	**0.005**
	Swedish EIRA	166 (78.3)	46 (21.7)	1.32	0.85 to 2.04	0.22	128 (60.4)	84 (39.6)	1.28	0.91 to 1.81	0.16
	Meta-analysis			**1.48**	**1.06 to 2.05**	**0.020**			**1.47**	**1.11 to 1.95**	**0.008**
CCP−/CarP-FCS+	Leiden EAC	14 (66.7)	7 (33.3)	**2.57**	**1.00 to 6.57**	**0.050**	13 (44.8)	16 (55.2)	**3.04**	**1.44 to 6.45**	**0.004**
	Swedish EIRA	18 (66.7)	9 (33.3)	**2.69**	**1.07 to 6.72**	**0.035**	15 (53.6)	13 (46.4)	1.95	0.88 to 4.35	0.10
	Meta-analysis			**2.63**	**1.36 to 5.08**	**0.004**			**2.47**	**1.43 to 4.27**	**0.001**
CCP+/CarP-FCS−	Leiden EAC	42 (77.8)	12 (22.2)	1.47	0.74 to 2.92	0.28	15 (75.0)	5 (25.0)	0.83	0.30 to 2.30	0.83
	Swedish EIRA	195 (81.3)	45 (18.7)	1.17	0.75 to 1.81	0.50	48 (61.5)	30 (38.5)	1.33	0.80 to 2.22	0.28
	Meta-analysis			1.25	0.86 to 1.81	0.24			1.21	0.77 to 1.90	0.41
CCP+/CarP-FCS+	Leiden EAC	126 (80.3)	31 (19.7)	1.26	0.79 to 2.01	0.32	38 (66.7)	19 (33.3)	1.24	0.70 to 2.20	0.47
	Swedish EIRA	158 (81.0)	37 (19.0)	1.31	0.82 to 2.09	0.26	35 (70.0)	15 (30.0)	0.84	0.43 to 1.63	0.60
	Meta-analysis			1.29	0.92 to 1.79	0.14			1.05	0.68 to 1.62	0.82
CarP-Fib											
Controls	Leiden EAC	385 (83.7)	75 (16.3)	1.0	Ref.	–	480 (71.2)	194 (28.8)	1.0	Ref.	–
	Swedish EIRA	318 (83.0)	65 (17.0)	1.0	Ref.	–	338 (67.2)	165 (32.8)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–			1.0	Ref.	–
CCP−/CarP-Fib−	Leiden EAC	96 (77.4)	28 (22.6)	1.50	0.92 to 2.44	0.11	95 (56.5)	73 (43.5)	**1.90**	**1.34 to 2.69**	**<0.001**
	Swedish EIRA	165 (77.5)	48 (22.5)	1.48	0.96 to 2.29	0.08	127 (59.1)	88 (40.9)	1.37	0.97 to 1.93	0.074
	Meta-analysis			**1.49**	**1.08 to 2.06**	**0.016**			**1.61**	**1.17 to 2.22**	**0.004**
CCP−/CarP-Fib+	Leiden EAC	8 (50.0)	8 (50.0)	**5.13**	**1.87 to 14.10**	**0.002**	7 (58.3)	5 (41.7)	1.77	0.55 to 5.64	0.34
	Swedish EIRA	19 (73.1)	7 (26.9)	1.27	0.48 to 3.38	0.63	16 (64.0)	9 (36.0)	1.21	0.51 to 2.88	0.67
	Meta-analysis			2.54	0.65 to 9.96	0.18			1.39	0.69 to 2.78	0.36
CCP+/CarP-Fib−	Leiden EAC	53 (76.8)	16 (23.2)	1.55	0.84 to 2.86	0.16	24 (68.6)	11 (31.4)	1.13	0.55 to 2.36	0.74
	Swedish EIRA	148 (75.1)	49 (24.9)	**1.65**	**1.07 to 2.55**	**0.024**	36 (55.4)	29 (44.6)	**1.74**	**1.01 to 3.01**	**0.046**
	Meta-analysis			**1.62**	**1.13 to 2.30**	**0.008**			1.49	0.96 to 2.31	0.07
CCP+/CarP-Fib+	Leiden EAC	119 (81.0)	28 (19.0)	1.21	0.75 to 1.95	0.44	31 (70.5)	13 (29.5)	1.04	0.53 to 2.03	0.91
	Swedish EIRA	205 (86.1)	33 (13.9)	0.90	0.56 to 1.44	0.65	47 (74.6)	16 (25.4)	0.68	0.36 to 1.27	0.22
	Meta-analysis			1.04	0.75 to 1.46	0.81			0.83	0.52 to 1.31	0.43

Bold typeface indicates statistical significance.
*ORs in Sweden cohort adjusted for age, gender and residential areas.
EAC, Early Arthritis Clinic; EIRA, Epidemiological Investigation of Rheumatoid Arthritis; FCS, fetal calf serum.

respect, and we have focused on the anti-CarP antibodies.[21] These anti-CarP antibodies were initially identified in adult RA patients and patients suffering from arthralgia,[9 10] but have now also been described to occur in juvenile idiopathic arthritis, in both the ACPA-positive and the ACPA-negative subgroups.[22]

In the current analyses of two large early RA cohorts, the presence of anti-CarP antibodies in the anti-CCP-negative stratum, originally described in the Leiden EAC, was confirmed in the EIRA cohort with comparable proportions of presence. This finding lends further support to the current assumption that besides a cross-reactive component of the ACPA/anti-CarP antibody responses, there are also antibodies directed against only citrullinated or only carbamylated proteins.[23] Comparing the information obtained with the anti-CarP-Fib- and anti-CarP-FCS-assays thus far, the notion arises that the anti-CarP-FCS assay may provide more unique information than the anti-CarP-Fib assay as the latter shows a stronger association with the same risk factors as anti-CCP2.[9 23] The specificity of anti-CarP antibodies for RA in the setting of an outpatient rheumatology clinic/among patients with early arthritis has not been reported thus far and is the subject of further studies. Given the differences mentioned above, it is not unlikely that anti-CarP-FCS and anti-CarP-Fib will display (slightly) different specificity/sensitivity profiles in such settings.

Carbamylation is a chemical post-translational modification mediated via cyanate, which is present in the body in equilibrium with urea.[24] There are several factors that can shift the balance towards more cyanate and hence more carbamylation. Among these factors, renal dysfunction, inflammation and smoking are considered most important.[24] Therefore, in this study we have specifically analysed to which extent the smoking status of the RA patients is associated with the presence of anti-CarP antibodies. However, we did not obtain evidence to support the hypothesis that smoking would induce anti-CarP antibodies nor did we find a specific association between anti-CarP antibodies and the *PTPN22* polymorphism or *HLA-DRB1-SE* alleles. This lack of association might indicate a different biological mechanism leading to anti-CarP antibody formation compared with anti-CCP antibody formation.

With regard to *HLA-DRB1* alleles, we could not identify any specific association with anti-CarP autoantibodies, although we cannot exclude an association between *HLA-DRB1**03 and anti-CCP-negative anti-CarP-FCS-positive RA. Since we also

Table 3 Meta-analysis for disease risk in different rheumatoid arthritis (RA) subgroups divided by the presence/absence of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies, any *PTPN22* allele carriers compared with non-carriers

		PTPN22				
Group	**Cohort**	**None N (%)**	**Any N (%)**	**OR***	**95% CI**	**p Value**
CarP-FCS						
Controls	Leiden EAC	338 (82.2)	73 (17.8)	1.0	Ref.	–
	Swedish EIRA	1010 (78.7)	274 (21.3)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-FCS−	Leiden EAC	200 (80.0)	50 (20.0)	1.16	0.78 to 1.73	0.47
	Swedish EIRA	464 (74.0)	163 (26.0)	**1.35**	**1.07 to 1.69**	**0.011**
	Meta-analysis			**1.30**	**1.07 to 1.59**	**0.009**
CCP−/CarP-FCS+	Leiden EAC	38 (86.4)	6 (13.6)	0.73	0.30 to 1.79	0.49
	Swedish EIRA	54 (63.5)	31 (36.5)	**2.16**	**1.35 to 3.46**	**0.001**
	Meta-analysis			1.35	0.47 to 3.86	0.58
CCP+/CarP-FCS−	Leiden EAC	65 (73.9)	23 (26.1)	1.64	0.96 to 2.81	0.07
	Swedish EIRA	435 (69.8)	188 (30.2)	**1.63**	**1.31 to 2.04**	**<0.001**
	Meta-analysis			**1.63**	**1.33 to 2.00**	**<0.001**
CCP+/CarP-FCS+	Leiden EAC	182 (71.7)	72 (28.3)	**1.83**	**1.26 to 2.66**	**0.001**
	Swedish EIRA	414 (68.1)	194 (31.9)	**1.78**	**1.43 to 2.22**	**<0.001**
	Meta-analysis			**1.79**	**1.48 to 2.17**	**<0.001**
CarP-Fib						
Controls	Leiden EAC	338 (82.2)	73 (17.8)	1.0	Ref.	–
	Swedish EIRA	1010 (78.7)	274 (21.3)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-Fib−	Leiden EAC	225 (81.5)	51 (18.5)	1.05	0.71 to 1.56	0.81
	Swedish EIRA	457 (73.4)	166 (26.6)	**1.38**	**1.10 to 1.74**	**0.005**
	Meta-analysis			1.26	0.98 to 1.62	0.07
CCP−/CarP-Fib+	Leiden EAC	20 (76.9)	6 (23.1)	1.39	0.54 to 3.58	0.50
	Swedish EIRA	61 (68.5)	28 (31.5)	**1.80**	**1.12 to 2.90**	**0.016**
	Meta-analysis			**1.71**	**1.12 to 2.61**	**0.014**
CCP+/CarP-Fib−	Leiden EAC	89 (75.4)	29 (24.6)	1.51	0.93 to 2.46	0.10
	Swedish EIRA	354 (72.4)	135 (27.6)	**1.46**	**1.15 to 1.87**	**0.002**
	Meta-analysis			**1.47**	**1.18 to 1.83**	**0.001**
CCP+/CarP-Fib+	Leiden EAC	163 (70.6)	68 (29.4)	**1.93**	**1.32 to 2.82**	**0.001**
	Swedish EIRA	495 (66.7)	247 (33.3)	**1.87**	**1.52 to 2.30**	**<0.001**
	Meta-analysis			**1.88**	**1.57 to 2.26**	**<0.001**

Bold typeface indicates statistical significance.
*ORs in Sweden cohort adjusted for age, gender and residential areas.
EAC, Early Arthritis Clinic; EIRA, Epidemiological Investigation of Rheumatoid Arthritis; FCS, fetal calf serum.

observed an association between this HLA allele and the CCP/CarP-FCS double-negative RA subgroup, this precludes any definitive conclusions about whether the association is specific for anti-CarP-FCS-positive RA or not. The *HLA-DRB1*03* association was stronger with anti-CarP-FCS-positive subgroup than with the double-negative subgroup (ORs were approximately 2.5 for CCP−/CarP-FCS+ RA vs 1.5 for CCP−/CarP-FCS− RA), although this difference was not statistical significant, potentially due to lack of power as a consequence of the rather limited numbers of CCP−/CarP-FCS+ patients. Hence, whether *HLA-DRB1*03* is involved in the formation of anti-CarP antibodies needs further investigation.

There are several limitations to the current study, mainly concerning the breadth of autoantibodies and genetics risk factors that were investigated. With regard to autoantibodies, the scope was limited to anti-CCP2 antibodies, although ACPA reactive with citrullinated epitopes on α-enolase, vimentin, fibrinogen and collagen type II have been detected in anti-CCP-negative RA.[20] It is thus possible that the anti-CarP antibody-positive subset of the anti-CCP antibody-negative RA population in fact also contains ACPA fine specificities that were captured by the anti-CCP2 detection kit. Likewise, RFs could be present in the anti-CCP-negative/anti-CarP-positive subset. This is the subject of an ongoing investigation. Regarding the genetic and environmental risk factors, we cannot exclude that there are other genes, not investigated here, which may also be of importance for the development of anti-CarP-positive RA. Although the combination of two cohorts allowed us to include a large number of patients in our analyses, the smaller size of some of the subgroups may still have led to some power limitations as described above.

Whereas most studies up to date have focused on ACPA-positive RA regarding investigation of genetic and environmental risk factors, with this study we wished to move the field forward by analysing a subset of ACPA-negative RA patients, those positive for anti-CarP antibodies, for specific associations regarding the major genetic and environmental risk factors described for RA. Interestingly, we did not obtain evidence for a specific association of anti-CarP antibody with *HLA-SE* alleles, *PTPN22* variants or smoking, but we did find an association between *HLA-DRB1*03* and anti-CarP-FCS antibodies. These data suggest that anti-CarP antibody induction may be facilitated by different mechanisms than the ones involved in the induction of ACPA.

In conclusion, anti-CarP antibodies are present in ACPA-positive and ACPA-negative RA patients in both EAC and EIRA cohorts. We did not find any association among anti-CarP antibodies and smoking, *PTPN22* or *HLA-DRB1* alleles, with the exception of the association identified between anti-CarP-FCS antibodies and *HLA-DRB1*03*.

Table 4 Meta-analysis for disease risk in different rheumatoid arthritis (RA) subgroups divided by the presence/absence of anti-CarP and anticyclic citrullinated peptide (anti-CCP) antibodies, ever smokers compared with non-smokers

Group	Cohort	Smoking		OR*	95% CI	p Value
		Never N (%)	Ever N (%)			
CarP-FCS						
CCP−/CarP-FCS− (as control group)	Leiden EAC	160 (52.0)	148 (48.0)	1.0	Ref.	–
	Swedish EIRA	247 (44.5)	308 (55.5)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-FCS+	Leiden EAC	31 (55.4)	25 (44.6)	0.87	0.49 to 1.55	0.64
	Swedish EIRA	24 (32.9)	49 (67.1)	1.67	0.96 to 2.89	0.07
	Meta-analysis			1.21	0.64 to 2.30	0.56
CCP+/CarP-FCS−	Leiden EAC	51 (51.5)	48 (48.5)	1.02	0.65 to 1.60	0.94
	Swedish EIRA	195 (35.2)	359 (64.8)	**1.58**	**1.22 to 2.04**	**<0.001**
	Meta-analysis			1.32	0.87 to 2.02	0.19
CCP+/CarP-FCS+	Leiden EAC	119 (41.0)	171 (59.0)	**1.55**	**1.12 to 2.15**	**0.008**
	Swedish EIRA	141 (25.9)	404 (74.1)	**2.27**	**1.73 to 2.97**	**<0.001**
	Meta-analysis			**1.90**	**1.31 to 2.76**	**<0.001**
CarP-Fib						
CCP−/CarP-Fib− (as control group)	Leiden EAC	193 (52.2)	177 (47.8)	1.0	Ref.	–
	Swedish EIRA	235 (43.2)	309 (56.8)	1.0	Ref.	–
	Meta-analysis			1.0	Ref.	–
CCP−/CarP-Fib+	Leiden EAC	17 (50.0)	17 (50.0)	1.09	0.54 to 2.20	0.81
	Swedish EIRA	36 (42.9)	48 (57.1)	0.99	0.61 to 1.61	0.96
	Meta-analysis			1.02	0.69 to 1.52	0.92
CCP+/CarP-Fib−	Leiden EAC	73 (45.6)	87 (54.4)	1.30	0.90 to 1.89	0.17
	Swedish EIRA	146 (33.4)	291 (66.6)	**1.60**	**1.21 to 2.11**	**<0.001**
	Meta-analysis			**1.49**	**1.19 to 1.86**	**<0.001**
CCP+/CarP-Fib+	Leiden EAC	118 (41.0)	170 (59.0)	**1.57**	**1.15 to 2.14**	**0.004**
	Swedish EIRA	190 (28.7)	472 (71.3)	**1.90**	**1.48 to 2.45**	**<0.001**
	Meta-analysis			**1.76**	**1.45 to 2.14**	**<0.001**

Bold typeface indicates statistical significance.
*ORs in Sweden cohort adjusted for age, gender and residential areas.
EAC, Early Arthritis Clinic; EIRA, Epidemiological Investigation of Rheumatoid Arthritis; FCS, fetal calf serum.

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Acknowledgements The authors thank the investigators and study participants from the EIRA study and the EAC study.

Contributors XJ, LAT, KL and DvdW had main responsibility for the study, including design, experimental set-up, analyses, tables, figures and writing the paper. TJvW, JS, LI and HH were involved in the planning and execution of the experiments. CB, HK, VM, LK, LA, TWJH and REMT were involved in supervising, data analyses and writing the paper. All authors contributed to and agreed with the final version of the paper.

Funding This work was supported with grants from the IMI JU funded project BTCure (contract No. 115142-2); the EU projects Masterswitch (contract No. 2.4.5-12), Gums&Joints (contract No. 261460) and Trigger (contract No. 306029); the Swedish Research Council, the Swedish Rheumatic Foundation, and the Swedish Medical Research Council for Health, Working Life and Welfare; the Swedish AFA insurance; the Netherlands Proteomics Center and the Center for Medical Systems Biology as part of The Netherlands Genomics Initiative. LT is supported by a NWO ZON-MW Vidi grant and by a fellowship from Janssen Biologics. DvdW is supported by a NWO ZON-MW Agiko grant.

Competing interests None.

Patient consent Obtained.

Ethics approval All aspects of this study were approved by the Leiden University Medical Center (LUMC) and Karolinska Institutet (KI) institutional review boards.

Provenance and peer review Not commissioned; externally peer reviewed.

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