Anti-carbamylated protein antibodies in unaffected first-degree relatives of rheumatoid arthritis patients: lack of correlation with anti-cyclic citrullinated protein antibodies and rheumatoid factor

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Abstract

Objective

To investigate the prevalence of anti-carbamylated protein antibodies (anti-CarP) in the healthy first-degree relatives (HFDRs) of patients with rheumatoid arthritis (RA).

Methods

We enrolled 141 HFDRs of 63 patients with RA diagnosed accordingly to the 2010 ACR/EULAR criteria. Fifty-six normal healthy subjects (NHS), sex- and age-matched, served as controls. Anti-CarP IgG, anti-cyclic citrullinated peptide antibody (anti-CCP) IgG and rheumatoid factors (RF) isotypes (IgG, IgA, IgM) were assessed by solid-phase ELISA.

Results

Anti-CarP were detectable in 13 HFDRs (9.2%), anti-CCP in 9 (6.3%), IgG-RF in 10 (7%), IgA-RF in 17 (12%), and IgM-RF in 13 (9.2%) HFDRs. Twenty-nine (46%) RA patients were positive for anti-CarP, 31 (49.2%) for anti-CCP, and 34 (53.9%) for RF. One NHS (1.7%) resulted positive for anti-CarP, none for anti-CCP and RF. Anti-CarP showed significantly higher serum levels in RA and HFDRs than in NHS ($p<0.0001$ and $p=0.0012$, respectively).
A significant correlation between anti-CCP and RF were found among RA patients ($p=0.0002$), whereas no correlations were reported between autoantibodies tested in the HFDRs.

Conclusion

Anti-CarP can be found in the sera of HFDRs of RA patients and their prevalence is significantly higher than in NHS. No correlation of anti-CarP with anti-CCP and RF antibodies in RA HFDRs was found.

Key words

anti-CarP, anti-CCP, HFDRs, rheumatoid factor, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is the most commonly diagnosed autoimmune disease. It affects approximately 0.5–1% of the world's population, causing persistent pain, synovitis, joint destruction, and functional disability (1). The disease is diagnosed primarily on the basis of clinical manifestations and laboratory tests. The latter are usually restricted to the determination of rheumatoid factor (RF) (2) and antibodies directed against anti-citrullinated protein/peptide (ACPA), mainly anti-cyclic citrullinated peptide antibodies (anti-CCP), which are currently considered the most specific and sensitive serological markers for RA (3). These autoantibodies are detectable in the early stages of the disease, as well as several years before the onset of clinical symptoms (4, 5).

Recently, a new group of autoantibodies directed against carbamylated proteins (anti-carbamylated protein antibodies, anti-CarP) have been discovered in RA patients (6). Carbamylation is a post-translational process in which lysines are converted into homocitrullines under the influence of cyanate, which is in equilibrium with urea. Increased urea concentrations, smoking, and inflammation are able to shift this equilibrium toward cyanate and, hence, enhanced carbamylation process (7). Homocitrulline-containing proteins are present in the arthritic joint, thus possibly affecting T-cell triggering and autoantibody production in rodents (8, 9). Anti-CarP have been detected in both ACPA-positive and ACPA-negative RA patients and seem to be a prognostic factor for a higher likelihood of joint destruction independently of ACPA status. Moreover, anti-CarP have been found in patients with arthralgia and demonstrated to predict the development of RA, independently of ACPA (10).

A number of autoantibodies can be detected in the sera of healthy individuals. Such findings may represent an immunological abnormality that will never be associated with symptoms or a very early serological manifestation of a specific disease that will have clinical evidence months or even years later (11). Healthy first-degree relatives (HFDRs) of patients suffering from autoimmune diseases have been reported to have autoantibodies in their sera. Indeed, autoimmune diseases cluster within families because of a shared genetic basis and, possibly, environmental factors, thus HFDRs could represent a good model to study the preclinical phases of autoimmune diseases. During the last years HFDRs have been investigated in order to better understand predisposing factors and discover new reliable markers for disease developing. Of note, a genetic predisposition has been seen also for autoantibodies production (12-15). Recently, the presence of ACPA in HFDRs has been revealed as well: a rate of 48% in HFDRs of RA patients with a high prevalence of the shared epitope and smoking habit has been demonstrated (16). In addition, increasing ACPA in HFDRs may be associated with signs of joint inflammation (17).

No data are currently available on the presence of anti-CarP in RA HFDRs. Thus, given the familiar clustering of RA and the genetic predisposition for autoantibody production, the aim of this study was to investigate the prevalence of anti-CarP and their correlation with anti-CCP and RF in the HFDRs of RA patients.

Methods

Patients and relatives

Patients with RA (probands) were consecutively recruited from the Rheumatology Outpatient Clinic of the Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma. All patients were diagnosed according to the 2010 ACR/EULAR criteria (1).

Each patient was asked to put us in touch with HFDRs of theirs who were willing to participate in the study. The probands' relatives who signed an informed consent underwent a complete rheumatologic evaluation, including both clinical and serological measurements. Their healthy status was assessed by two different rheumatologists; in the cases of suspected synovitis, ultrasound evaluation was performed to rule out this possibility. A questionnaire about their smoking habits was also administered. Fifty-six normal healthy subjects (NHS), mean age 45 years (range 22–65), 25 (44.6 %) female, served as con-

trols. RA probands, HFDRs and NHS live in the same geographical area. The study protocol was approved by the local ethics committee.

Anti-CarP antibody assays

In RA, HFDRs and NHS anti-CarP were detected by ELISA using carbamylated foetal calf serum (Ca-FCS) and non modified FCS as antigens, according to Shi J *et al*. with some modifications. (6) In brief, Nunc Maxisorp plates (Thermo Scientific) were coated overnight at + 4°C with non-modified FCS and Ca-FCS (10 ug/ml in carbonate bicarbonate buffer). After washing plates were blocked with phosphate buffered saline (PBS) 1% bovine serum albumin (BSA) (Sigma) for 6 h at + 4°C. Subsequently, the wells were incubated with patients serum diluted 1/50 in PBS/0.05% tween/BSA 1% overnight at + 4°C. After four washes, plates were incubated for 2 h at room temperature (RT) with goat polyclonal antihuman IgG alkaline phosphatase conjugated antibodies (Sigma) diluted at 1:1000 in PBS/0.05% tween/BSA 1%. After four washes, a solution of paranitrophenyl phosphate tablets in ethanolamine was used for the enzyme reaction and the plates were read at a 405 nm wavelength after 30 minutes at RT. All assays were performed in duplicate and the absorbance of control wells (non modified FCS) was subtracted to account for non-specific binding.

Intraassay variations were determined by assaying two RA samples 16 times (*i.e*. each of these two sera sample in 16 different wells of the same microplate). For the interassay, the same two RA samples were tested in quadruplicate (*i.e*. each of these two sera sample in 4 different wells of the same microplate), repeating this test for nine consecutive times. The coefficients of intraassay and interassay variation of the two RA samples assayed were 4%, 5.2% and 11.3%, 12.8%, respectively.

A titration curve of two positive reference sera with medium–high ELISA immunoreactivity for anti-Ca-FCS was performed to show the performance of the tests and to transform the absorbance of Ca-FCS to arbitrary units per milliliter (aU/mL). The cut-off was established as the mean OD + 3 SD of fifty-six age- and sex-matched healthy subjects (blood donors) and then the obtained value was converted into aU/mL (corresponding to 501 aU/mL), accordingly to Shi *et al*. (6).

Anti-CCP and RF antibody assays

All autoantibody assays were carried out in duplicate, commercial ELISA kits were used and the results were evaluated according to the manufacturers' instructions.

The autoantibody status of the probands was obtained from the outpatient medical records: anti-CCP titers were determined using the ImmunoCAP instrument (ELiA CCP2 assay, Phadia, Milan, Italy), whereas immunonephelometry (Behering, Marburg, Germany) was performed for RF, as routinely used in clinical practice.

In HFDRs and NHS, anti-CCP2 IgG antibodies were assessed with commercial ELISA kits (Immunoscan CCP Euro-Diagnostica, Malmo, Sweden). According to manufactures anti-CCP2 IgG levels exceeding 25 U/mL were considered positive. A solid-phase ELISA kit was performed in order to determine IgG, IgA, and IgM isotypes of RF (Diamedix, Miami, USA).

Statistical analysis

The statistical analysis was performed using Statistical Package for Social Sciences 13.0 (SPSS, Chicago, IL, USA) and GraphPad 5.0 (La Jolla, CA, USA). Qualitative differences between subgroups were analysed by the chi-square test or Fisher's exact test when appropriate. Differences between groups were analysed by Mann-Whitney U-test. The Spearman test was performed for the correlation analysis between serum titer antibodies. Probability (p) values of 0.05 were considered statistically significant.

Results

Sixty-three probands with RA were enrolled in the study. RA mean age was 41 years (range 19-70), mean duration of symptoms was 8 years (range 2–18.5), 36 (57.1%) were female, and 38 (60.3%) were smokers. A total of 204 HFDRs were identified analysing 63 probands, 141 (69.1%) of whom agreed to participate in the study. HFDRs mean age was 42 years (range 7–75), 77 (54.6%) were female, and 39 (27.6%) were smokers. Seventy-three were descendants of the probands, 36 were collaterals, and 27 were ascendants. The highest number of HFDRs per single family participating in the study was 8 (including the proband). At time of the enrollment, we found that thirty percent of HFDRs subjects complained arthralgias, however ultrasound of the joints were performed and nobody had synovitis. None of the controls experienced arthralgias and/or synovitis.

Anti-CarP, anti-CCP and RF in RA patients

The overall prevalence of anti-CarP, RF, and anti-CCP in RA patients is showed in Figure 1. Twenty-nine of the 63 RA patients (46%) resulted positive for anti-CarP. Thirty-one of the 63 RA patients (49.2%) were anti-CCP-positive. Thirty-four of the 63 RA patients (53.9%) resulted RF-positive.

The percentage of anti-CCP and RF positive in the RA probands is low compared to prior studies with established disease, probably because casually the percentage of patients with an erosive disease included in the study is lower than in other studies.

Of interest, 12 of the 63 RA patients (19%) resulted negative for all three assays, whereas 12 (19%) were positive for all the three. Anti-CarP identified 10 (15.8%) of RA patients negative for anti-CCP and RF, while the single anti-CCP positivity was depicted in 1 (1.5%) of RA subjects (Fig. 1). A significant correlation between anti-CCP and RF, but no between these antibodies and anti-CarP, were found among RA patients (p=0.0002).

Anti-CarP, anti-CCP and RF in HFDRs and NHS

The overall prevalence of anti-CarP, RF, and anti-CCP in HFDRs of RA patients is showed in Figure 2. Thirteen of the 141 HFDRs (9.2%) resulted positive for anti-CarP antibodies, whereas only one of the 56 NHS (1.7%) was positive. Nine of the 141 HFDRs (6.3%) were anti-CCP-positive. Thirty of the 141 HFDRs (21.2%) resulted RF-positive:

13 (9.2%) were positive for IgM-RF, 17 (12%) were positive for IgA-RF, and 10 (7%) were positive for IgG-RF. Simultaneous positivity for more than one RF isotype was observed in 8 HFDRs (5.6%); two (1.4%) were positive for all three RF isotypes. No correlation was found between autoantibodies tested. None NHS was positive for aCCP and RF. Of interest, 99 of the 141 HFDRs (70.2%) of patients resulted negative for all three assays. Anti-CarP identified 6 (4.2%) of HFDRs negative for anti-CCP and RF, while the single anti-CCP positivity was detectable in 4 (2.8%) of HFDRs.

The prevalence of antibodies was unrelated to the autoantibody status of the proband. Further, the autoantibody positivity rates and concentrations in HFDRs were not significantly related to the nature of their kinship with the proband (ascendants, collaterals, descendants).

Anti-CarP serum levels in RA, HFDRs and controls are shown in Figure 3. As expected serum levels of anti-CarP were significantly higher in RA patients than in NHS ($p<0.0001$). Notably, serum levels of anti-CarP were also significantly higher in HFDRs than in NHS ($p=0.0012$).

Grouping HFDRs according to their smoking status we found no differences between anti-CarP and anti-CCP serum levels (Table I). Interestingly, only one of the 13 HFDRs who were positive for anti-CarP was a smoker whereas 4 of the 9 HFDRs positive for anti-CCP positive were smokers. The lack of association between anti-CCP in RA probands and smoking probably is due to the sample size.

HFDRs follow-up using ultrasonography

After a follow-up of 3 years, all the HFDRs were invited to perform ultrasonographic assessment at articular level. Sixteen of them accepted and the evaluation of bilateral radiocarpal, metacarpophalangeal (MCP), proximal interphalangeal (PIP), metatarsophalangeal (MTP) and knee joints was performed, in order to identify the presence of synovial hypertrophy (SH), power Doppler (pD) and bone erosions.

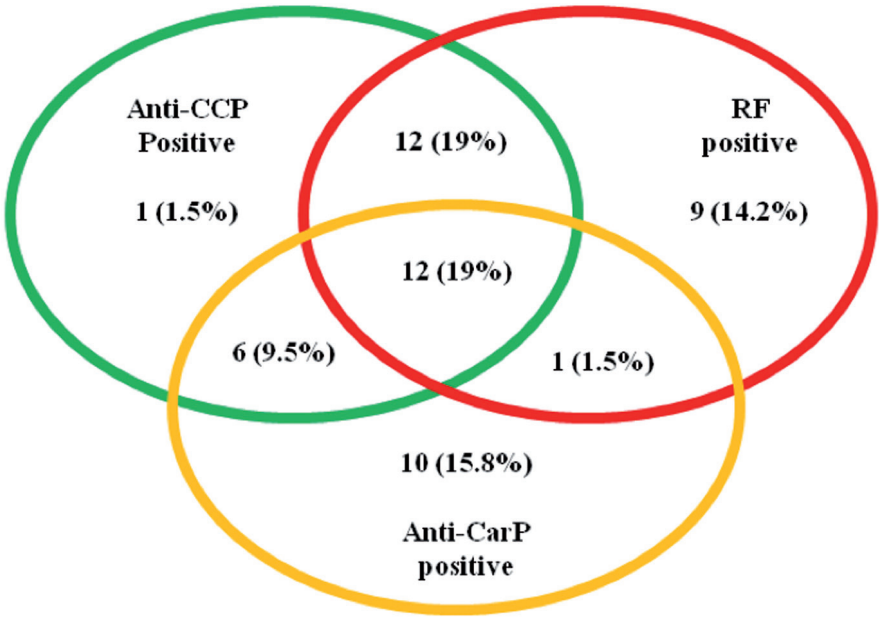

Fig. 1. Overall prevalence of anti-CarP, RF, and anti-CCP in 63 RA patients. The absolute numbers of single-positive, double-positive, triple-positive are listed.

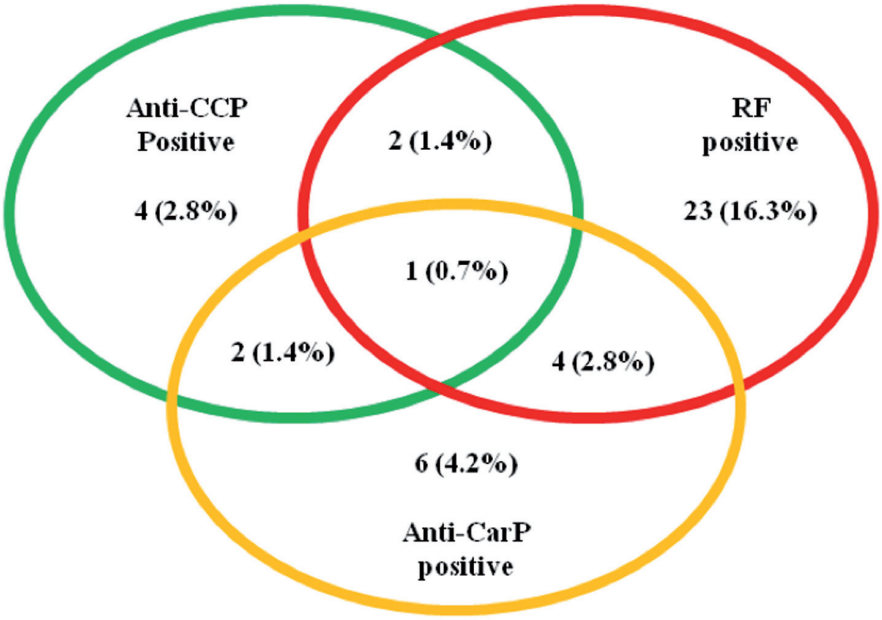

Fig. 2. Overall prevalence of anti-CarP, RF, and anti-CCP in 141 HFDRs of RA patients. The absolute numbers of single-positive, double-positive, triple-positive are listed.

SH was identified in at least one joint in 6 of 16 subjects (37.5%). In particular, SH was identified at level of MCP joints (2 subjects), knee (1 subject), MTP joints (3 subjects). No subjects showed the presence of pD signal or bone erosions. In Table II, we reported the autoantibody profile of 16 HFDRs. Five out of subjects with SH showed the positivity for at least one autoantibody and experienced arthralgias.

Due to the small size of this group, we did not perform a statistical analysis. Nonetheless, we identified a trend of association between the presence of SH and positivity for RF-IgM: indeed, the frequency of RF-IgM was higher in subjects with SH comparing with subjects without (50% *vs*. 0).

Discussion

Several studies have investigated bio-

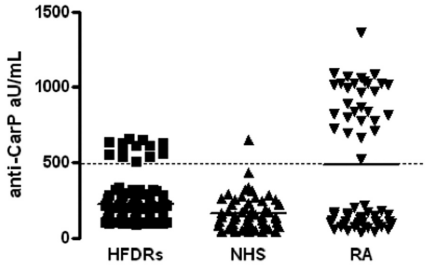

Fig. 3. Serum levels of anti-CarP in RA, HFDRs and controls. Plot of anti-CarP antibodies detected by means of an ELISA in HFDRs (n=141), NHS (n=56) and RA patients (n=63). Median values are shown. Dotted line represents cut-off. RA *vs*. NHS: $p<0.0001$; HFDRs *vs*. NHS: $p=0.0012$; RA *vs*. HFDRs: not significant. Differences between groups were analysed by Mann-Whitney U-test.

Table I. Anti-CarP, anti-CCP anf RF in HFDRs grouped according to smoker status.

	Smokers (39)	Non Smokers (102)
Anti-CarP (aU/mL; median, range)	178, 91-655	187, 94-632
N positive (%)	1 (2.5)	12 (11.7)
Anti-CCP (U/mL; median, range)	12, 9-110	11, 8-93
N positive (%)	4 (10.2)	5 (4.9)
IgM RF (U/mL; median, range)	9.7, 3-118	9.7, 2-159
N positive (%)	5 (12.8)	8 (7.8)
IgG RF (U/mL; median, range)	14, 6-45	14, 4-326
N positive (%)	3 (7.6)	7 (6.8)
IgA RF (U/mL; median, range)	8, 2-104	8, 1-225
N positive (%)	6 (15.3)	11 (10.7)

Table II. Ultrasonographic assessment and autoantibody profile of 16 HFDRs after a follow-up of 3 years.

Pt	anti-CCP	RF-IgG	RF-IgM	RF-IgA	anti-CarP	SH*
1	0	0	0	1	0	0
2	1	0	0	0	0	0
3	0	0	1	1	0	1
4	0	0	0	0	0	0
5	0	1	0	0	0	0
6	0	0	0	1	0	0
7	0	0	0	1	0	0
8	0	0	0	0	0	0
9	1	0	1	1	0	1
10	0	0	1	0	1	1
11	0	0	0	0	0	0
12	1	0	0	0	0	0
13	1	0	0	0	0	1
14	0	0	0	0	0	1
15	1	0	0	0	0	0
16	0	1	0	0	0	1

SH: synovial hypertrophy; *In at least one joint. 0: negative; 1: positive.

markers predictive of development of RA among the relatives of RA patients (15, 16, 20, 21, 24, 25). Indeed, studying HFDRs of RA patients could be considered one way to investigate the preclinical phases of RA, since patients and relatives could share some of the genetic and environmental risks for RA. In particular, it is tempting to speculate over that phase of pre-RA in which the systemic autoimmune response is present without any other clinical manifestations. Exploring this preclinical phase with highly accurate tests at the individual level would mean to become able to state the possibility that a person might ever progress to develop RA or not. The development of such risk stratification is essential for designing preventive clinical trials and would provide further information on the pathogenesis of the disease (18). Aiming to this item, a number of studies investigating markers of this specific preclinical phase of RA has been conducted in the HFDRs of RA patients. Of note, most of the markers studied are the autoantibodies typically present in RA such as RF and anti-CCP. In our study we first demonstrated detectable levels of anti-CarP antibodies uncorrelated to anti-CCP and RF in HFDRs of RA patients.

Recently, a study conducted by Kim and coworkers has shown that in families with multiple cases of RA, beyond the increased prevalence of RF-positivity (15), the association with anti-CCP antibody seropositivity is present (19). Nonetheless, it has been documented that the fine specificity of ACPAs in patients with RA is at least partially different from that observed in their healthy relatives. Indeed, Barra and coworkers reported IgA ACPA in HFDRs and IgG ACPA in RA patients (16). Moreover, Ioan-Facsinay *et al*. have displayed the reactivity to citrullinated fibrinogen and citrullinated vimentin/Sa in over half of the patients examined but almost none of their healthy relatives (20).

A study conducted over the unaffected relatives of RA patients in Southern Brazil reported an overall prevalence of RF of 8%, which was not significantly different from that found in the general population of the same area (6%) (21); on the contrary, the prevalence of anti-CCP positivity (5.5%) was significantly higher than that found in the general population (1%). A Canadian study showed frequencies of IgM and IgA RF isotypes of 7.8% and 11.6%, in the indigenous populations of central Canada (Cree and Ojibway), whereas anti-CCP positivity was markedly higher (19%) than that observed in the general Canadian population (8.8%) (20). This discrepancy can be explained by the fact that native North American populations have a higher prevalence of shared epitope (SE) alleles (59%) (22), and ACPA positivity is known to be linked to the presence of SE alleles (23). It means that ACPA positivity has to be interpreted in accordance to the prevalence in the general population of the SE; on the other hand, pointing only on ACPA positivity would miss those cases persistently negative for anti-CCP who would develop a seronegative-RA in the future.

More recently, a new system of autoantibodies directed against carbamylated peptides, the anti-CarP, has been found

in RA patients. Previous studies have demonstrated that anti-CarP antibodies present in patients with arthralgia years before the onset (26) are associated with a higher risk of developing RA independently from the ACPA and RF status (10). Indeed, although anti-CarP antibodies might recognise different specificities from ACPA (8, 9, 27), anti-CarP response seems to be, at least in part, not cross-reactive with citrullinated proteins (28). In the other hand, in a study conducted in Hcit-immunised mice, Mydel *et al*. showed that carbamylated peptides active T and B cells and induce autoantibody production in mice that developed erosive arthritis (8). This might suggest that Hcit-modification could change immunologically inert peptides into autoantigens and enable the production of ACPA; thus, these authors supposed that carbamylation could be the missing link in the chain of pathogenesis of autoimmune arthritis. Taken together, these data would suggest that anti-CCP and anti-CarP antibodies are not necessarily cross-reactive.

These findings prompted us to study the prevalence of anti-CarP in a cohort of HFDRs of RA patients. For the first time we have demonstrated the presence of anti-CarP in the HFDRs of RA patients with a prevalence significantly higher than in the healthy controls. Our findings confirm the conclusions of previous studies which demonstrated that anti-CarP, and anti-CCP are distinct antibody systems (6). Indeed, in our study no obvious correlations were found between anti-CarP and anti-CCP, or between anti-CarP and RF, strengthening the statement that this new class of antibodies might be unrelated to the other ones. Nonetheless, the lack of correlation between anti-CarP and anti-CCP can also be the result of lack of power.

HFDRs and RA probands live in the same geographical area. Unfortunately, given the small number of the group, the genetic testing for HLA-DR4 containing the SE alleles was not performed. This is a limit of the study.

Almost all of the ACPA-positive HFDRs of our cohort were smokers. This finding corroborates a possible correlation between the use of tobacco and the presence of ACPA, which might be related to an increased expression of citrullinated antigens in smokers lungs (29). Besides citrullination, smoke can also induce carbamylation (30), thus, we have also investigated the presence of a correlation between anti-CarP antibodies and smoke, but no correlation was found in our cohort of HFDRs. This, probably due to the small size of our HFDRs cohort, too underpowered to detect an association between anti-CarP and smoking.

Finally, HFDRs performed an ultrasonographic evaluation in order to identify the presence of subclinical synovitis: at baseline, no pathological features were identified. After 36 months, the presence of mild SH was identified in 6 out of 16 HFDRs accepting to perform a re-evaluation. Interestingly, we did not identify any sign of active synovitis and/or erosions.

In conclusion, the results of this study demonstrate for the first time that detectable levels of anti-CarP antibodies can be found in HFDRs of RA patients. However, positivity for both anti-CarP and anti-CCP in HFDRs is much less common than RF and shows no relation to the probands' autoantibody status. Anti-CCP positivity in HFDRs seems to be correlated with smoking, confirming the important role of this environmental factor in anti-CCP antibodies production in predisposed subjects; on the contrary, we did not report any correlation between smoke and anti-CarP antibodies production in HFDRs, although it has been demonstrated that smoke can induce carbamylation. No correlation among anti-CarP, anti-CCP, and RF has been found. This, in agreement with the view that these are distinct antibodies systems, could give advantages for interpreting and intercepting ACPA-seronegative RA. However, since this study neither tests anti-CarP as a biomarker for predicting disease onset nor does it explore any potential mechanistic processes, it is an exploratory, hypothesis-driving study. No doubt, further studies over larger cohort of patients and *in vitro* experiments are necessary to better define the role of anti-CarP antibodies in the pathogenesis of the disease.

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References

1. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 Rheumatoid Arthritis Classification Criteria. *Arthritis Rheum* 2010; 9: 2569-81.
2. NEWKIRK MM: Rheumatoid factors: host resistance or autoimmunity? *Clin Immunol* 2002; 104: 1-13.
3. NISHIMURA K, SUGIYAMA D, KOGATA Y *et al*.: Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Ann Intern Med* 2007; 146: 797-808.
4. ATEŞ A, KARAASLAN Y, AKSARAY S: Predictive value of antibodies to cyclic citrullinated peptide in patients with early arthritis. *Clin Rheumatology* 2007; 26: 499-504.
5. NIELEN MM, VAN SCHAARDENBURG D, REESINK HW *et al*.: Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 2004; 50: 380-6.
6. SHI J, KNEVEL R, SUWANNALAI P, VAN DER LINDEN MP *et al*.: Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci USA* 2011; 42: 17372-7.
7. WANG Z, NICHOLLS SJ, RODRIGUEZ ER *et al*.: Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* 2007; 10: 1176-84.
8. MYDEL P, WANG Z, BRISSLERT M *et al*.: Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J Immunol* 2010; 12: 6882-90.
9. TURUNEN S, KOIVULA MK, RISTELI L, RISTELI J: Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 2010; 62: 3345-52.
10. SHI J, VAN DE STADT LA, LEVARHT EW *et al*.: Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. *Arthritis Rheum* 2013; 4: 911-5.
11. RANTAPÄÄ-DAHLQVIST S, DE JONG BA, BERGLIN E *et al*.: Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 2003; 48: 2741-9.
12. LAZARIDIS KN, JURAN BD, BOE GM *et al*.: Increased prevalence of antimitochondrial antibodies in first degree relatives of patients with primary biliary cirrosis. *Hepatology* 2007; 46: 785-92.
13. CLELAND LG, BELL DA, WILLIAMS M, SAURINO BC: Familial Lupus. Family studies of HLA and serologic findings. *Arthritis Rheum* 1978; 21: 183-91.
14. ARNETT FC, HAMILTON RG, REVEILLET JD, BIAS WB, HARLEY JB, REICHLIN M: Genetic studies of Ro (SS-A) and La (SS-B) autoantibodies in families with systemic lupus erythematosus and primary Sjögren's syndrome. *Arthritis Rheum* 1989; 32: 413-9.

15. SILMAN AJ, OLLIER B, MAGEED RA: Rheumatoid factor detection in the unaffected first degree relatives in families with multicase rheumatoid arthritis. *J Rheumatol* 1991; 18: 512-5.
16. BARRA L, SCINOCCA M, SAUNDERS S *et al*.: Anti-citrullinated protein antibodies in unaffected first-degree relatives of rheumatoid arthritis patients. *Arthritis Rheum* 2013; 6: 1439-47.
17. YOUNG KA, DEANE KD, DERBER LA, HUGHES-AUSTIN JM, WAGNER CA, SOKOLOVE J: Relatives without rheumatoid arthritis show reactivity to anti-citrullinated protein/peptide antibodies which are associated with arthritis-related traits: Studies of the etiology of rheumatoid arthritis. *Arthritis Rheum* 2013; 8: 1995-2004.
18. VAN STEENBERGEN HW, HUIZINGA TW, VAN DER HELM-VAN MIL AH: Review: The preclinical phase of rheumatoid arthritis: what is acknowledged and what needs to be assessed? *Arthritis Rheum* 2013; 9: 2219-32.
19. KIM SK, BAE J, LEE H, KIM JH, PARK SH, CHOE JY: Greater prevalence of seropositivity for anti-cyclic citrullinated peptide antibody in unaffected first-degree relatives in multicase rheumatoid arthritis-affected families. *Korean J Intern Med* 2013; 1: 45-53.
20. IOAN-FACSINAY A, WILLEMZE A, ROBINSON DB *et al*.: Marked differences in fine specificity and isotype usage of the anti-citrullinated protein antibody in health and disease. *Arthritis Rheum* 2008; 58: 3000-8.
21. GOELDNER I, SKARE TL, DE MESSIAS REASON IT, NISIHARA RM, SILVA MB, UTIYAMA SR: Anti-cyclic citrullinated peptide antibodies and rheumatoid factor in rheumatoid arthritis patients and relatives from Brazil. *Rheumatology* 2010; 49: 1590-3.
22. LEE AN, BECK CE, HALL M: Rheumatoid factor and anti-CCP autoantibodies in rheumatoid arthritis: a review. *Clin Lab Sci* 2008; 21: 15-8.
23. BALSA A, CABEZÓN A, OROZCO G *et al*.: Influence of HLA DRB1 alleles in the susceptibility of rheumatoid arthritis and the regulation of antibodies against citrullinated proteins and rheumatoid factor. *Arthritis Res Ther* 2010; 12: R62.
24. CHARPIN C, MARTIN M, BALANDRAUD N, ROUDIER J, AUGER I: Autoantibodies to BRAF, a new family of autoantibodies associated with rheumatoid arthritis. *Arthritis Res Ther* 2010; 12: R194.
25. FERUCCI ED, DARRAH E, SMOLIK I *et al*.: Prevalence of anti-peptidylarginine deiminase type 4 antibodies in rheumatoid arthritis and unaffected first-degree relatives in indigenous North American populations. *J Rheumatol* 2013; 40: 1523-8.
26. SHI J, VAN DE STADT LA, LEVARHT EW *et al*.: Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. *Ann Rheum Dis* 2014; 73: 780-3.
27. SCINOCCA M, BELL DA, RACAPÉ M *et al*.: Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. *J Rheumatol* 2014: 41: 270-9.
28. SHI J, WILLEMZE A, JANSSEN GM *et al*.: Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the "AMC-Senshu" method. *Ann Rheum Dis* 2013; 72: 148-50.
29. VAN DER HELM-VAN MIL AH, VERPOORT KN, LE CESSIE S, HUIZINGA TW, DE VRIES RR, TOES RE: The HLA–DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide. *Arthritis Rheum* 2007; 56: 425-32.
30. ROBERTS JM, VERES PR, COCHRAN AK *et al*.: Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. *Proc Natl Acad Sci USA* 2011; 108: 8966-71.