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Anti-carbamylated protein and peptide antibodies as potential inflammatory joint biomarkers in the relatives of rheumatoid arthritis patients

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Abstract

Objective: Antibodies against carbamylated proteins/peptide (CarP) have been associated with severity in rheumatoid arthritis (RA) patients. However, their role in risk groups, specific targets and relation with periodontal disease (PD) is uncertain yet. The aim of this study was evaluated the association between the levels of anti-CarP with clinical manifestation, human leukocyte antigen (HLA) alleles, periodontal activity markers, PD diagnosis, PD severity, and presence of *Porphyromonas gingivalis (P gingivalis)* in relatives of patients with RA.

Methods: One hundred and twenty-four individuals with a family history of RA in first-degree relatives (FDR) and 124 healthy individuals gender- and age-matched, RA activity was assessed. Antibodies against carbamylated protein anti-FCS-Carp and 2 carbamylated peptides of fibrinogen were selected (anti-Ca-Fib2, anti-Ca-Fib3).

Results: Anti-FCS-Carp-positive, anti-Ca-Fib2 and anti-Ca-Fib3 were more frequent in FDR than controls (25.0% vs 14.5%, 34.7% vs 15.3% and 33.1% vs 11.3%, respectively). Anti-FCS-CarP were associated with the HLA-DRB1-SE* 1402 allele ($P = .035$) and highly sensitive C-reactive protein levels ($P = .016$), the anti-Ca-Fib2 antibodies were associated with the HLA-DRB1-SE* 1501 allele ($P = .03$), with non-SE* 0901 allele ($P = .01$), the anti-Ca-Fib3 was associated with positive rheumatoid factor ($P = .0012$). The FDR condition was associated with the presence of anti-Ca-Fib3 (odds ratio [OR] =4.7; 95% CI = 1.8-11.7; $P = .001$) and painful joints (OR = 2.2; 95% CI = 1.01-4.68; $P = .045$); we also detected an important trend toward the presence of *P gingivalis* (OR = 1.9; 95% CI = 0.9-3.7; $P = .062$).

Conclusion: The presence of anti-FCS-Carp, anti-Ca-Fib3 and anti-Ca-Fib2 antibodies may have a role for these antibodies as early biomarkers in the development of RA, probably including additional mechanisms related with other non-SE alleles; the anti-peptide antibodies proposed in the present study may represent a simpler way to identify antibodies directed to a specific target.

KEYWORDS

antibodies, carbamylation, rheumatoid arthritis

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and systemic disease that leads to the inflammation of the joints.[1,2] The etiology of RA remains uncertain.[3] However, the following conditions associated with the disease have been reported: environmental factors (eg, smoking), periodontal disease (PD)[4,5] and genetics (eg, alleles of the major histocompatibility complex of class II and others).[6-9]

The stages prior to RA diagnosis generally refer to individuals presenting with clinical symptoms and/or environmental, hormonal or genetic risk factors without meeting the RA classification criteria.[10-12] Recently, a study of risk factors for RA was implemented by the European League Against Rheumatism (EULAR) to facilitate research in the pre-clinical and early phases of RA through the identification of a risk group of individuals with a family history of RA (FDR).[11]

Many studies have focused on these early stages of the disease and aimed to describe autoantibodies, such as rheumatoid factor (RF) and the anti-cyclic citrullinated peptide (anti-CCP).[12-14] Recently, in an attempt to identify additional biomarkers that have a potential to be useful to identify the progression of early stages in high-risk individuals, antibodies directed against proteins that have undergone post-translational modification (PTM) by carbamylation were identified. Carbamylation is a non-enzymatic PTM in which cyanate reacts with the primary amino or thiol (*N*-carbamylation or *S*-carbamylation, respectively). The amino acids lysine and arginine contain side chains that can react with cyanate,[15] thus creating a homologous citrulline structure that extends over a single carbon residue and is also known as homocitrulline.[16]

Currently, very few studies report the presence of anti-CarP (anti-carbamylated proteins/peptides) in FDR.[17,18] Several previous studies that focused on patients with established RA proposed these antibodies as a possible biomarker of the early stages of this disease,[14,19,20] preceding the onset of symptoms and diagnosis.[21] Moreover, those studies did not identify specifically the target of anti-CarP. Previous work has mainly focused on fibrinogen or the mixture of proteins from carbamylated fetal bovine serum (FBS) and, more recently, antibodies against vimentin and α-enolase.[22,23]

Therefore, the aim of this study was to evaluate the association between the levels of anti-CarP antibodies and variables associated with rheumatologic and PD activity in the FDR.

2 | MATERIALS AND METHODS

A cross-sectional study was conducted in individuals at FDR (with blood relatives with RA) >18 years of age. Two group of subjects were included in this study: 124 with FDR (subjects with first degree of consanguinity of patients with RA) selected according to the 2012 EULAR recommendations[11] and 124 healthy individuals gender- and age-matched. This study was approved by an ethics committee (Hospital Militar Central, Bogotá, Colombia 2015-047) and all individuals signed an informed consent for their participation.

2.1 | Exclusion criteria

Individuals with ongoing infectious process, diagnosis of neoplasia, autoimmune disease, type II diabetes mellitus or who were undergoing antibiotic treatment in the last 3 months, periodontal therapy in the last 6 months, had orthodontic appliances, were breastfeeding or pregnant were excluded.

The FDR had the EULAR criteria being part of the risk group of individuals with a family history of RA and the controls had no family history of RA.

Blood samples were collected for evaluation of RF, highly sensitive C-reactive protein (hs-CRP), and anti-CCP antibodies. Erythrocyte sedimentation rate was measured by photometry and human leukocyte antigen (HLA)-DRB1 allele typing (Appendix S1).

2.2 | Prediction and selection of the beta chain peptides of carbamylated fibrinogen

In brief, 3 peptides of the fibrinogen beta chain were synthesized, the first was a native peptide and the 2 remaining peptides exhibited a modified structure with homocitrulline residues in different positions. A more detailed description is in Appendix S1.

2.3 | Detection and quantification of antibodies against CarP

In brief, immunoglobulin G (IgG) antibodies against serum CarP were measured quantitatively using a modified in-house indirect enzyme-linked immunosorbent assay (ELISA) system based on the protocol established by Shi et al[19] Similarly, detection and quantification of antibodies against CarP (Anti-FCS-Carp) were assessed by ELISA. A more detailed description is in Appendix S1.

2.4 | Periodontal examination

All patients were evaluated by 2 calibrated periodontist, who performed the periodontal examination. A full-mouth examination including the selected sites on each permanent tooth was performed, excluding third molars. All patients were classified for periodontitis according to the Centers for Disease Control and Prevention (CDC) criteria.[24]

2.5 | Detection of P gingivalis and quantification of antibodies against it

The presence of the periodontopathogenic bacterium *P gingivalis* (ATCC 33 277) was assessed by quantitative polymerase chain reaction (qPCR). IgG1 and IgG2 antibodies against *P gingivalis* were assessed by indirect in-house ELISA.[4] A more detailed description of the ELISA is in Appendix S1.

2.6 | Environmental factors

We considered the epidemiological and environmental factors associated with RA: age, smoking status, body mass index (BMI), education and comorbidities.

2.7 | Evaluation of joint status in FDR

For the group of relatives of FDR, painful and inflamed joint counting was performed for all individuals by a rheumatologist.[25,26]

2.8 | Statistical analysis

The sample size of the FDR and healthy groups was calculated based on a design of pairs using the software TM® with statistical power of 80%, an alpha error of 0.05 in a 1:1 ratio and 30% discordant pairs (according to a pilot study conducted in the first phase of this study) adjusting to 110 cases and 110 paired controls.

The comparisons of the periodontal and rheumatologic variables between the individuals in the FDR group vs those in the paired control group were performed using the McNemar test and the Wilcoxon sign test. The Chi-squared and Fisher's exact tests were used for the analysis of categorical variables, such as demographic, rheumatologic and periodontal data. The associations were evaluated by the Mann–Whitney *U* test or *t* test. Conditional logistic regressions were performed to adjust for possible confounding variables (age and cigarette consumption), to establish the real association between anti-FSC-Carp or anti-Fib-Carp and the FDR condition. A link test model was used to validate the models.

Three subgroups were analyzed: the first was positive subjects for anti-FCS-Carp and anti-citrullinated peptide antibody (ACPA) (n = 7) compared with 21 subjects who did not meet the condition randomly in a 3:1 ratio; the second was positive subjects for anti-Ca-Fib2 antibodies and ACPA (n = 8) compared with 24 subjects who did not meet the condition randomly in a 3:1 ratio; and the third was positive subjects for anti-Ca-Fib3 antibodies and ACPA (n = 9) compared with 27 subjects who did not meet the condition randomly in a 3:1 ratio.

All analyses were performed by SPSS V24 and STATA for Windows. Significance was set at $P \leq .05$.

3 | RESULTS

3.1 | FDR and control group demographics

The FDR group had a mean age of 39.2 ± 12.2 years and a higher proportion of women (71.8%). The frequency of comorbidities was 41.1%: 4.8% of participants reported current smoking habits, 26.6% had a history of smoking and 14.5% were passive smokers; 36.3% of them were overweight and 4.8% were obese. The control group had comorbidities at a frequency of 32.3%, the most common being hypertension and hypothyroidism without significant differences between the 2 groups ($P = .147$). Overweight was observed in 27.4% of the control individuals and obesity was found in 4.8% of these individuals. In addition, a trend toward a lower current habit of smoking was observed in the FDR group compared with the healthy subjects; however, this difference was not significant ($P = .073$).

3.2 | Serological and articular variables in the FDR and control groups

A higher frequency of anti-CCP antibodies was observed in the FDR (19.3%) vs the control (6.4%) group, showing an association between the risk of having these antibodies and FDR (odds ratio [OR] =3.6, 95% CI = 21.4-11.0; $P = .002$). Similarly, the FDR group had a higher number of painful joints (37.9%) compared with the control group (22.58%; OR = 2.2, 95% CI = 1.1-4.2; $P = .007$) and a higher number of swollen joints (15.3% vs 4.0%; OR = 5.6, 95% CI = 1.6-30.1; $P = .002$). These findings indicate a risk association between presenting painful and swollen joints and FDR that was 2 and 5 times greater (Table 1).

3.3 | Description of periodontal variables in the FDR and control groups

The frequency of PD in the FDR group was similar to that of the control group (60.5% vs 59%, respectively). The presence of *P gingivalis* was more frequent in the FDR (62.1%) vs the control (42.7%) group (OR = 2.1, 95% CI = 1.2-3.7; $P = .003$). Inversely, anti-*P gingivalis* IgG1 and IgG2 antibodies were more frequent in controls than they were in the FDR group ($P = .003$ and $P = .001$, respectively). Variables such as gingival inflammation and sites with pocket depths ≥ 4 mm were more frequent in the FDR vs the control group. Gingival inflammation had a median value of 0.26 (interquartile range [IQR], 0.13-0.47) in FDR vs 0.44 in the control (IQR, 0.26-0.68; $P = .001$), and sites with pocket depths ≥ 4 mm exhibited a median value of 1.72 in the FDR (IQR, 0.00-6.90) vs 2.38 in the control (IQR, 0.68-9.48), with a statistically significant difference ($P = .034$).

3.4 | Distribution of HLA-DRB1 SE alleles in FDR individuals

The analysis of the genetic heritability related to RA revealed the presence of HLA-DRβ1 alleles, which were considered shared epitopes (SE), in 53.2% of FDR individuals. The most frequent alleles were HLA-DRβ1 *0405 (26.3%), *0404 (19.7%), *0101 (17.1%) and *1402 (15.8%). Among the individuals who were positive for SE,

TABLE 1 Serological variables and joint involvement in first-degree relative (FDR) compared with control individuals

	Control		FDR		
	N	%	N	%	*P*
ESR					
Negative	103	83.06%	107	86.29%	.492
Positive	21	16.94%	17	13.71%	
hsCRP					
Negative	84	67.74%	75	60.48%	.711
Positive	28	22.58%	33	26.61%	
High positive	12	9.68%	16	12.90%	
RF					
Negative	121	97.58%	119	95.97%	.479
Positive	2	1.61%	4	3.23%	
High positive	1	0.81%	1	0.81%	
ACPA[a]					
Negative	116	93.55%	100	80.65%	**.002**
Positive	8	6.45%	24	19.35%	
Painful joints[a]					
Any	96	77.42%	77	62.10%	**.007**
At least 1	28	22.58%	47	37.90%	
Swollen joints[a]					
Any	119	95.97%	105	84.68%	**.002**
At least 1	5	4.03%	19	15.32%	

Abbreviations: ESR, erythrocyte sedimentation rate; hsCRP, highly sensitive C-reactive protein; RF, rheumatoid factor.

[a]and boldface indicate significance at $P < .01$, as assessed using the McNemar test.

45.2% had at least 1 and 8.1% expressed 2 HLA-DRB1 SE alleles (Figure S1).

3.5 | Presence of anti-carbamylated protein and anti-carbamylated peptide antibodies

The presence of antibodies directed against carbamylated anti-FCS-Carp-positive proteins was more frequent in the FDR group compared with the control group (25.0% vs 14.5%; OR = 2.6; 95% CI = 1.1-6.8; $P = .015$). The analysis of the anti-carbamylated peptide antibodies revealed that anti-Ca-Fib2 antibodies were more frequent in the FDR group compared with the control group (34.7% vs 15.3%; OR = 4.0; 95% CI = 1.8-10.0; $P = .001$), as were the anti-Ca-Fib3 antibodies (33.1% vs 11.3%; OR = 4.8; 95% CI = 2.1-12.9; $P = .001$). These findings suggest that the FDR group had a higher risk of having anti-carbamylated peptide antibodies than did individuals who were not related to patients with RA (Figure 1).

The presence of antibodies directed against a fibrinogen peptide without modifications was also measured, to assess the autoimmune response in this group of individuals. We did not find differences in the frequency of anti-fibrinogen antibodies between the FDR and control groups ($P = .878$).

3.6 | Association between anti-carbamylated protein antibodies and anti-carbamylated peptide antibodies with HLA-DRB1 alleles and joint parameters in FDR

The anti-FCS-Carp antibodies were associated with the presence of the SE *1402 allele ($P = .035$; Figure S2). In addition, an association was detected between the presence of these antibodies and the serum hsCRP levels ($P = .016$; Figure 2), as well as a weak correlation with RF ($r = 0.26$, $P = .0034$) and the presence of painful joints ($r = 0.18$, $P = .0425$). There was no association with any of the periodontal variables.

The anti-Ca-Fib2 antibodies were associated with the presence of the HLA-DRB1 SE *1501 allele ($P = .03$) and with the non-SE *0901 allele ($P = .01$; Figure 3). We also found an association between the anti-Ca-Fib3 antibody and a positive RF ($P = .0012$). Moreover, we observed a trend toward the presence of the EC *0405 allele ($P = .061$). However, these antibodies were not associated with any of the periodontal variables (Table S1).

To assess the association between the presence of anti-carbamylated protein or peptide antibodies and variables associated with the FDR condition, 2 conditional logistic regression models were developed: one of them adjusted for age and current smoking exposure and the second adjusted for age and previous smoking exposure.

In the first regression, we chose the model that included anti-Ca-Fib3, the presence of painful joints, titers of IgG2 antibodies against *P gingivalis* ≥ 1:100 and the presence of *P gingivalis* adjusted to age and previous exposure to smoking, based on the values of the BIC validation (described above). We found that the FDR condition was associated with the presence of anti-Ca-Fib3 (OR = 4.7; 95% CI = 1.8-11.7; $P = .001$) and painful joints (OR = 2.2; 95% CI = 1.01-4.68); $P = .045$); we also detected an important trend toward the presence of *P gingivalis* (OR = 1.9; 95% CI = 0.9-3.7; $P = .062$). A multivariate regression including ACPA with age and current smoking exposure as a confounding variable was performed and is described (Table S2) showing that even so the model holds similar results as described above.

In the second regression model, anti-Ca-Fib2 anti-Ca-Fib3 antibodies, the presence of painful joints, titers of IgG1 and IgG2 antibodies against *P gingivalis* ≥ 1:100 and the presence of *P gingivalis* were included as variables of interest. We observed results that were similar to those of the first model. The FDR condition was associated with the presence of anti-Ca-Fib3 antibodies (OR = 3.0; 95% CI = 1.0-8.1; $P = .034$) and with the presence of painful joints (OR = 2.3; 95% CI = 1.0-4.9; $P = .038$). No association was found between FDR and the presence of IgG1 and IgG2 antibodies against *P gingivalis* (Tables 2 and 3).

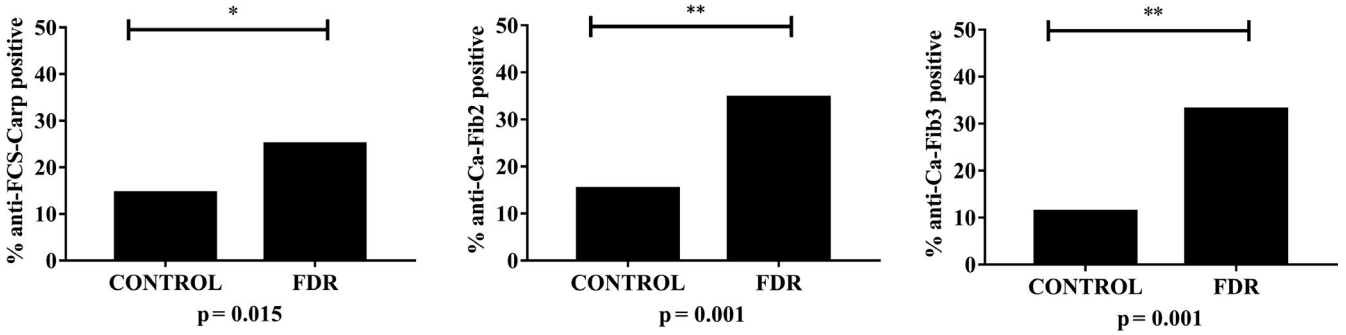

FIGURE 1 Frequency of anti-carbamylated protein antibodies (anti-FCS-Carp) and anti-carbamylated peptide antibodies in the first-degree relatives (FDR) compared with the control (CTRL) groups. Each bar represents the percentage of positivity of the respective antibodies. *Significant at $P < .05$ and **$P < .01$, as assessed by the McNemar test

3.7 | Association between positive groups of anti-carbamylated protein antibodies, anti-carbamylated peptide antibodies and ACPA with joint and serological parameters in FDR

In the first positive group for anti-FCS-Carp-positive and ACPA simultaneously, a significant statistical association was found between the presence of these 2 conditions with positive CRP values; 57.1% present CRP values greater than 9 mg/dL ($P = .019$) compared to 9.5% of those without any of the conditions mentioned above. Also, it was found that 57.1% of anti-FCS-Carp + and ACPA + present more swollen joints than those negative for these 2 variables 9.5% ($P = .021$).

In the positive group for anti-Ca-Fib2 antibodies and ACPA, no statistically significant association was found, and in the third group a significant statistical association was found between the presence of CRP values greater than 9 mg/dL compared with those without any of the conditions mentioned above (33.3% vs 3.7% $P = .04$). None were associated with periodontal or genetic variables.

4 | DISCUSSION

Recently, many research efforts have focused on the understanding of the "risk" phases of RA before the development of the clinical signs of joint inflammation. Some epidemiological studies have shown that having a family history of RA increases the risk of RA by approximately 3 to 5 times, which defines the FDR group as a population that has a high risk of RA because of the genetic and environmental factors that are shared with patients with RA. This is also explained by the high percentage of heritability of RA, which can reach 60% compared with healthy individuals with no family history of the disease.[18,19,27-34]

The findings in the literature are that smoking is an important and potentially modifiable risk factor during the pre-clinical transition phases of RA pathogenesis. In our study we only observed 26.6% of smokers and only 14.5% had smoking history; this is corroborated by Sparks et al[29] with a 12.0% current smoking habit and Simons et al[33] reporting that only a small proportion of FDRs were current smokers, which makes us think that relatives are becoming increasingly aware of the risk that smoking gives to the development of inflammatory joint signs and are employing changes in lifestyle to modify risk.[29,33,34]

Similar to citrullination, carbamylation is a kind of PTM of proteins, which provides a source of new epitopes that can be recognized as non-self-antigens,[35] the presence of these modified sequences of amino acids may provoke specific autoantibody production in RA. Anti-CarP seems associated with erosive disease; Montes et al described in an animal model, it was shown that carbamylated proteins can trigger primary immune responses inducing

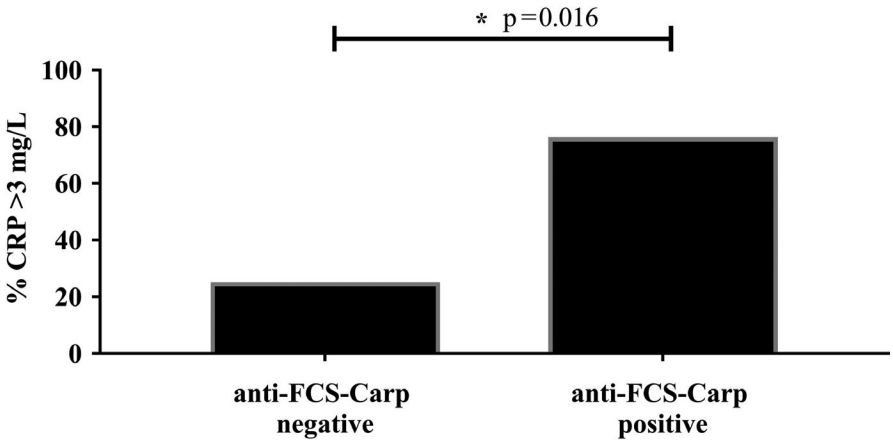

FIGURE 2 Association between anti-carbamylated protein antibodies (anti-FCS-Carp) antibodies and inflammatory variables in first-degree relatives (FDR). Each bar represents a level of C-reactive protein (CRP) greater than 3 mg/L in the groups of individuals who were positive and negative for antibodies against carbamylated proteins. *Significant at $P < .05$ as assessed by Fisher's test

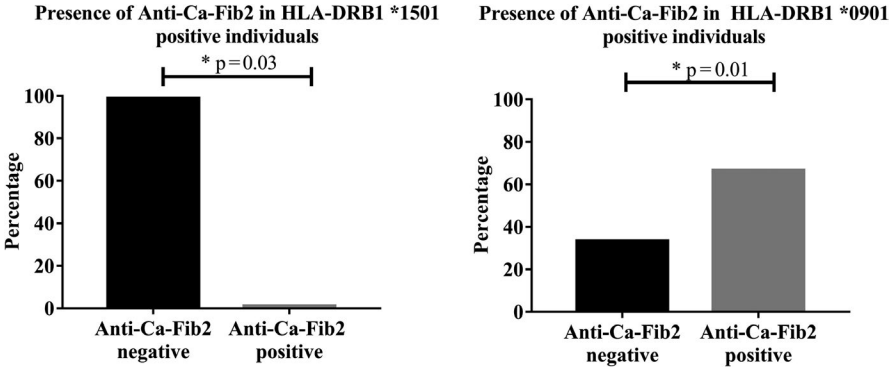

FIGURE 3 Association between anti-Ca-Fib2 antibodies and the human leukocyte antigen (HLA)-DRB1 *1501 and *0901 alleles in first-degree relatives (FDR). Each bar represents the percentage of positivity for anti-carbamylated peptide antibodies in the group of individuals who were positive for the respective allele. *Significant at $P < .05$ and **$P < .01$, as assessed by Chi-squared or Fisher's tests

TABLE 2 Conditional logistic regression model for indicators associated with the first-degree relative condition, adjusted for age and current cigarette exposure

	Adjusted model		
	OR	95% CI	*P*
Age	0.840	0.450-1.568	.584
Current cigarette exposure	0.618	0.169-2.264	.468
Anti-Ca-Fib3 antibodies*	4.716	1.892-11.750	**.001**
Presence of painful joints*	2.184	1.016-4.689	**.045**
IgG2 antibodies for *Phorphyromonas gingivalis**	0.107	0.035-0.324	**.010**
Presence of *P gingivalis*	1.903	0.967-3.742	.062

*Significant at $P < .05$.

TABLE 3 Conditional logistic regression model for indicators associated with the first-degree relative condition, adjusted for age and previous cigarette exposure

	Adjusted model		
	OR	95% CI	*P*
Age	0.770	0.402-1.473	.43
Previous exposure to cigarettes	0.919	0.397-2.124	.843
Anti-Ca-fib2 antibodies	2.800	0.931-8.411	.067
Anti-Ca-fib3 antibodies*	2.976	1.084-8.166	**.034**
Presence of painful joints*	2.282	1.047-4.971	**.038**
IgG2 antibodies for *Phorphyromonas gingivalis**	0.118	0.038-1.365	**.010**
IgG1 antibodies for *P gingivalis**	0.457	0.193-1.076	**.073**
Presence of *P gingivalis*	1.778	0.876-3.604	.111

*Significant at $P < .05$.

chemotaxis, T cell activation, antibody synthesis, and production of interferon-γ, interleukin (IL)-10, and IL-17, promoting inflammation.[36] Also authors such as Truchetet et al, identified the presence of anti-CarP antibodies in a cohort of early RA patients in approximately one-third of patients (32.6%) and in another study in which the objective was to analyze the presence of anti-FCS-Carp and its association with juxta-articular or systemic bone loss in a cohort of patients with arthritis.[37]

To our knowledge, this is one of the first studies describing the frequency of anti-carbamylated peptide antibodies in FDR individuals. The literature reports only the evaluation of anti-carbamylated antibodies using carbamylated FBS as an antigen. Alessandri and co-workers were the first to analyze the prevalence of anti-carbamylated protein antibodies without including anti-carbamylated peptide antibodies in FDR and RA compared with healthy subjects. Those authors found that these antibodies were significantly increased in FDR vs healthy subjects (9.2% vs 6.3%), thus confirming that anti-FCS-Carp occurs in family members.[18] Koppejan analyzed patients with RA, FDR individuals and Native-American controls and reported that anti-FCS-Carp antibodies were more frequent in FDR vs control subjects (18.3% vs 4.7%; OR = 4.5; 95% CI = 1.4-13.8; $P = .008$).[17] These results suggest that these antibodies may play a role in the onset of autoimmunity in these risk populations.[17] The comparison of the 2 studies described above with our study revealed that the frequencies reported previously in FDR individuals were lower than those found in our FDR population; however, the results of our study are consistent, as we reported a higher frequency of anti-FCS-Carp in the FDR vs control individuals and showed that the FDR population has a high risk of carrying these autoantibodies.

The findings discussed above support and confirm the hypothesis that FDR constitutes a sub-group of healthy individuals who are at high risk of having autoantibodies associated with RA. In addition, these findings may provide new insights into the evolution of autoimmunity that may be occurring in the pre-clinical phases in such individuals. Therefore, follow-up studies in this sub-group of individuals are important.

As mentioned previously, it should be noted that there are no studies describing the frequency of anti-carbamylated peptide antibodies. Our study reports important evidence pertaining to this group of genetically related individuals, suggesting that anti-carbamylated autoantibodies generated in FDR individuals are also mainly directed against joint proteins, such as fibrinogen, that undergo PTM in the early stages of disease development. Importantly, this new modification associated with RA is occurring in individuals with a high risk of developing RA. The presence of anti-Ca-Fib2 and anti-Ca-Fib3 antibodies and their association with joint parameters may indicate that, in these pre-clinical stages, the production of these autoantibodies against modified sequences may mark the beginning of joint inflammation. This result is important because we did not find antibodies against the fibrinogen peptide without any modification, suggesting that the peptides described above are possible early indicators of the development of RA.

The association between the anti-FCS-Carp antibodies, as well as the anti-Ca-Fib2 and anti-Ca-Fib3 antibodies, and the rheumatologic variables and the FDR condition reflects the manner in which the anti-FCS-Carp antibodies are associated with the presence of high levels of hsCRP and weakly correlated with high levels of RF and the presence of painful joints. The findings may confirm that this group exhibits many risk factors associated with RA and that the presence of autoantibodies in high-risk subjects, such as the FDR population, is involved in important initial inflammatory processes. Ultimately, this may indicate that these antibodies play an important role in the development of RA compared with healthy individuals without any genetic link.

We found relevant results regarding the distribution of autoantibodies related to the development of RA in this population of individuals. The presence of anti-CCP antibodies was detected in a high proportion of FDR individuals (19.3%) vs controls (6.45%), as was the presence of painful joints (37.9% vs 22.6%) and the number of swollen joints (15.3% vs 4.0%). This is interesting because both groups are healthy individuals, and FDR only stands apart by having genetic relationship with patients with RA, which renders it a sub-group with a higher risk of developing RA.[11,38] The integration of these findings with the presence of anti-CarP, anti-Ca-Fib2 and anti-Ca-Fib3 antibodies in the FDR group may confirm the contention that anti-CCP antibodies are not the only autoantibodies that precede and generate a risk of developing RA. In addition, in this group of individuals, some subjects already have symptoms that involve inflammation and pain in the joints, together with the presence of anti-CarP antibodies. This may indicate that autoantibodies contribute to the breakdown of tolerance at the joint level, generating further autoimmune processes.

Genetic predisposition is associated with the breakdown of tolerance. Considering the high frequency of the HLA-DRB1 SE allele observed in this FDR risk group and the hypothesis that the primary anchor pockets of HLA-DRB1 SE selectively binds arthritogenic peptides for the presentation to CD4 + helper T cells, several works have focused on the binding of proteins containing citrulline,[38-40] thus leading to studies of other PTMs. This may reflect the importance of research aimed at additional potential targets, to understand the pathogenesis of RA, especially in the pre-clinical phases.

Kolfenbach describes genetic and environmental factors in American individuals, reporting that HLA-DRB1 SE alleles are present in 55% of the FDR population.[28] Similarly, Sparks reported a frequency of 54.9% for HLA-DR4 SE alleles in parents, children or siblings of patients with RA.[29] These previous data are similar to those reported here, as we recorded a frequency greater than 50% in the FDR group. These findings support the notion that this sub-group of individuals are genetically susceptible to having some characteristics and risk factors that can lead to RA development. In contrast, Radstake reported a higher frequency of -DRB1 SE alleles in 79% of Dutch FDR cases, possibly because of the ethnic characteristics of the population.[41]

Regarding the presence of anti-FCS-Carp antibodies with HLA-DRB1 alleles in FDR, we found an association between these antibodies and the presence of the HLA-DRB1 SE *1402 allele and of anti-Ca-Fib2 antibodies, as well as an association with the presence of the HLA-DRB1 non-SE *1501 and *0901 alleles. These data compared with those reported by the Koppejan study, which reported on anti-FCS-Carp antibodies, showed that they were more frequent in SE-positive subjects (OR = 3.1; $P = .03$). However, this association between SE positivity and anti-CarP positivity is not supported in multivariate models.[17] Therefore, future studies using a greater number of FDR samples would allow the confirmation of this association.

The presence of PD must be analyzed as an environmental event associated with RA.[12] Both are chronic and inflammatory conditions which produce bone damage.[41,42] In the study reported by Unriza et al, the authors found that 79% of the FDR individuals had a diagnosis of periodontitis compared with 56% in the control group.[5] In our study, we observed a similar trend of a higher frequency of PD in FDR; there was a significantly higher involvement of periodontal clinical variables in this group compared with the controls, which reflects the importance of the link between periodontal inflammation and the pre-clinical phase of the disease in these individuals, as reported previously in early RA.[4]

Although the presence of *P gingivalis* is not a marker for PD, infection with this bacterium is involved in the development of potential RA triggers.[42,43] The discovery and characterization of the peptidylarginine deiminase enzyme (PPAD) expressed by this pathogen, has shown a close relationship with citrullination and further development of RA-related autoantibodies. This enzyme is able to citrullinate both bacterial and mammalian proteins, leading to the hypothesis that describes the citrullination of PPAD-mediated proteins at inflamed periodontal sites as a trigger of several cascades of

events that culminate in the production of anti-citrullinated protein antibodies.[44,45] *P gingivalis* was present in 62.1% of FDR compared with 42.7% of the controls, similar to the previous study of Bello et al[4]

Although most carbamylation in tissues is believed to take place during inflammation when myeloperoxidase (MPO) is released from neutrophil, PD (both gingivitis and periodontitis) is a classic inflammatory condition inextricably linked with neutrophil recruitment and function suggesting that carbamylation occurring in inflamed periodontal tissues is high.[46-48] However, no association was found in the FDR group with anti-Carp antibodies and the presence of antibodies against *P gingivalis* or the presence of the bacteria in this study.

All the joint inflammatory signs and the differences in rheumatic indices shown in this study indicate that follow-up studies could be carried out on these subjects with genetic risk in the development of RA in the future.

5 | CONCLUSION

The presence of anti-FCS-Carp, anti-Ca-Fib3 and anti-Ca-Fib2 antibodies may have an important prognostic value because of the relationship with joint inflammatory manifestations. These data may suggest a role for these antibodies as early biomarkers of the development of RA, probably including additional mechanisms related with other non-SE alleles, especially in high-risk individuals. Finally, the anti-peptide antibodies proposed in the present study may represent a simpler way to identify antibodies directed to a specific target.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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