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BRIEF REPORT

Lack of Replication of an Association Between Anti–Citrullinated Fibrinogen and Subclinical Atherosclerosis in Patients With Rheumatoid Arthritis

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Objective. **Results of a recent study suggested that the excess cardiovascular (CV) risk observed in patients with rheumatoid arthritis (RA) could be partially explained by the presence of immune complexes of antibodies against citrullinated proteins that locally promote and perpetuate inflammation and progression of atherosclerotic plaques. The present study was undertaken to attempt to replicate one of the observations supporting this hypothesis, i.e., association between anti–citrullinated fibrinogen (anti–Cit-fibrinogen) positivity and subclinical atherosclerosis.**

Methods. **Three surrogate markers of atherosclerosis were assessed in 124 RA patients with no previous history of CV events: carotid intima-media thickness (CIMT) assessed by carotid ultrasonography, carotid plaques assessed by carotid ultrasonography, and Coronary Artery Calcification Score (CACS) determined by multidetector computed tomography (CT) scanning. We analyzed the relationship of these 3 subclinical atherosclerosis markers to the presence and levels of autoantibodies, including anti–Cit-fibrinogen, anti–cyclic citrullinated peptide 2 (anti–CCP-2), and rheumatoid factor (RF).**

Results. **Carotid plaques and CIMT >0.90 mm were present in 69.4% and 15.3%, of the patients, respectively, and the CACS was moderate or high in 21.0%. None of these surrogate markers of atherosclerosis showed a significant association with positivity for or the level of anti–Cit-fibrinogen antibodies (either against the whole protein [present in 33.9% of the patients] or against an immunodominant peptide [present in 23.4%]), anti–CCP-2 (present in 60.7%), or RF (present in 58.1%) in this series of patients with RA.**

Conclusion. **Our results do not support the notion that there is a relationship between anti–Cit-fibrinogen antibodies and subclinical atherosclerosis in RA, thus calling into question the claim that these antibodies have a role in the increased risk of CV disease observed in patients with RA.**

Patients with rheumatoid arthritis (RA) have an increased risk of cardiovascular (CV) disease that cannot be explained solely by the presence of classic CV risk factors (1,2). Recently, it was proposed that in addition to the CV risk associated with chronic inflammation in these patients (1,2), reactivity with citrullinated proteins in the atherosclerotic plaque was a possible factor in the excess risk (3). This hypothesis was based on the association of CV events with seropositive RA, the demonstration of citrullinated fibrinogen (Cit-fibrinogen) and other citrullinated proteins in atherosclerotic plaques, and the positive correlation between anti–Cit-fibrinogen antibody titers and aortic calcification scores, which were used as surrogate markers of atherosclerotic burden and CV risk (3). Nonetheless, more research on this issue is needed to confirm these findings. In the present study, we aimed to replicate the potential association between titers of anti–Cit-fibrinogen and subclinical atherosclerosis in patients with RA.

PATIENTS AND METHODS

Patients, atherosclerosis, and CV risk assessment. One hundred twenty-four patients were included in the present study; clinical information on most of the patients (104 of the

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124) has been reported previously (4). Briefly, the study population consisted of consecutive patients who were diagnosed as having RA and had no history of CV events (ischemic heart disease, cerebrovascular accident, peripheral artery disease, or heart failure). All patients provided written informed consent to participate, and the study was approved by the local ethics committee of Cantabria.

The Systematic Coronary Risk Evaluation (SCORE) and the European League Against Rheumatism (EULAR) modified SCORE (mSCORE) (1), which assess the 10-year risk of fatal CV events in members of the general population and patients with RA, respectively, were calculated based on age, sex, smoking history, systolic blood pressure, and total cholesterol level. All patients underwent computed tomography (CT) imaging of coronary arteries using a 32-slice multidetector CT scanner (Lightspeed, Pro 32; GE Healthcare) to determine the Coronary Artery Calcification Score (CACS) (5). The CACS is the sum of the calcium scores (measured as Agatston scores of all calcifications) in the left main coronary artery, left anterior descending artery, left circumflex coronary artery, right coronary artery, and posterior descending artery. Patients were stratified into 4 groups: CACS 0 (normal), CACS 1–100 (low-to-moderate CV risk), CACS 101–400 (moderate-to-high CV risk), and CACS >400 (high CV risk). They were also assessed by carotid ultrasonography using a Mylab 70 scanner equipped with a 7–12−MHz linear transducer (Esaote). This assessment included measurement of carotid intima-media thickness (CIMT) in the common carotid artery (Quality Intima Media Thickness in real time; Esaote), and detection of focal plaques in the extracranial carotid tree (6). As observed in the general population, CIMT >0.90 and the presence of plaques have been found to be predictors of CV events in patients with RA (7).

Autoantibody determinations. Titers of IgG anti–cyclic citrullinated protein 2 (anti–CCP-2) were determined using an EDIA anti–CCP-2 enzyme-linked immunosorbent assay (ELISA) kit (Euro-Diagnostica). IgM rheumatoid factor (RF) was measured by rate nephelometry (IMMAGE Immunochemistry System; Beckman Coulter). Antibodies against Cit-fibrinogen were measured, after subtraction of reactivity against native fibrinogen, using an in-house ELISA with the citrullinated whole protein obtained commercially (Cayman Chemical). The ELISA protocol was as previously described (8). Titers of the most prevalent peptide-specific anti–Cit-fibrinogen antibodies in RA patients were also determined by ELISA, as previously described (9). The antigen for this assay was the biotinylated and citrullinated amino acids 36–52 peptide of the fibrinogen β-chain (citFibβ 36-52−NEEGFFSA[cit]GHRPLDKK; Schafer-N). A standard curve made with serial dilutions from a single lot of pooled positive sera was used to transform optical density in arbitrary units by 4-parameter logistic regression curve. The cutoff for positivity was set at the 95th percentile level in 44 healthy donors (1.7 for anti–Cit-fibrinogen, and 2.1 for anti–citFibβ 36-52).

Statistical analysis. Statistica 7.0 software (StatSoft) was used for all statistical calculations. Spearman's rank order correlation test was used for analysis of the relationships between variables. Three atherosclerosis outcomes were assessed: presence of carotid plaques, CIMT, and CACS. As the CACS was 0 in many patients, we considered this parameter either as an ordinal variable with the 4 levels mentioned above or as a log-transformed (ln[CACS + 1]) standardized variable, as previously described (10). Generalized linear models were fitted to these 2 CACS variables as well as to CIMT, and logistic regression analysis of carotid plaque presence was performed. In addition, quantile regression for the conditional median was applied to the untransformed CACS values using the Quantreg R package (11), because Sokolove et al used this analysis (3). These analyses were performed with and without adjustment for the variables addressed by Sokolove et al (anti-CCP titer, age, time since disease onset, systolic blood pressure, body mass index, smoking, and low-density lipoprotein:high-density lipoprotein cholesterol ratio) or for the variables that showed association with subclinical atherosclerosis in our patients (age, sex, systolic blood pressure, time since disease onset, and erythrocyte sedimentation rate). Finally, contingency tables according to antibody/atherosclerosis status were analyzed by chi-square test. Post hoc power of the

Table 1. Clinical characteristics of the 124 patients with RA*

Women	72.6
Age, median (IQR) years	59 (54–66)
Age at diagnosis, median (IQR) years	48 (42–57)
Disease duration, median (IQR) years	9 (5–15)
RF	58.1
RF titer, mean ± SD†	335 ± 508
Anti–CCP-2‡	60.7
Anti–CCP-2 titer, mean ± SD†	1,060 ± 931
Seropositive RA (RF or anti-CCP)	71.5
Erosive arthritis	46.8
Extraarticular manifestations	12.1
No. of previous DMARDs, median (IQR)	2 (1–3)
Biologic treatment	57.3
Glucocorticoid treatment	78.2
Smoking	51.6
ESR, median (IQR) mm/hour	12 (6–20)
CRP, median (IQR) mg/liter	2 (0.8–6.8)
Total cholesterol, median (IQR) mg/dl	218 (184–242)
LDL:HDL cholesterol, ratio median (IQR)	2.1 (1.5–2.7)
BMI, median (IQR) kg/m^2	27 (24–31)
Systolic blood pressure, median (IQR) mm Hg	132 (121–150)
SCORE, median (IQR)	1.5 (1.0–3.0)
mSCORE, median (IQR)	1.75 (1.0–3.0)
CIMT >0.90 mm	15.3
CIMT, median (IQR) mm	0.74 (0.63–0.83)
Carotid plaques	69.4
Unilateral	25.8
Bilateral	43.5
CACS, median (IQR)	8.5 (0–85.5)
CACS level	
Normal (0)	40.3
Low-to-moderate (1–100)	38.7
Moderate-to-high (101–400)	12.9
High (>400)	8.1
Anti−Cit-fibrinogen	33.9
Anti−Cit-fibrinogen titer, mean ± SD†	2.3 ± 6.9
Anti−citFibβ 36-52	23.4
Anti−citFibβ 36-52 titer, mean ± SD†	13.5 ± 124.9

* Except where indicated otherwise, values are the percent of patients. RA = rheumatoid arthritis; IQR = interquartile range; RF = rheumatoid factor; anti–CCP-2 = anti–cyclic citrullinated peptide 2; DMARDs = disease-modifying antirheumatic drugs; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; LDL = low-density lipoprotein; HDL = high-density lipoprotein; BMI = body mass index; SCORE = Systematic Coronary Risk Evaluation; mSCORE = European League Against Rheumatism modified SCORE; CIMT = carotid intima-media thickness; CACS = Coronary Artery Calcification Score; anti–Cit-fibrinogen = anti–citrullinated fibrinogen.
† Among patients with positive titers.
‡ Data missing on 2 patients.

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Table 2. Analysis of the relationship of anti–Cit-fibrinogen and anti–citFibβ 36-52 antibody titers to subclinical atherosclerosis assessed according to 3 different outcomes in patients with RA*

	Coefficient†			
	Anti−Cit-fibrinogen		Anti−citFibβ 36-52	
	Without adjustment	Adjusted	Without adjustment	Adjusted
CACS quantile regression	4.4	13.3	−2.6	8.1
zCACS GLM	0.06	0.06	−0.07	−0.05
CACS GLM	0.06	0.04	−0.08	−0.07
CIMT GLM	−0.02	−0.03	−0.11	−0.09
Carotid plaque LR	0.0	−0.03	−0.71	−0.78

* Covariates in the adjusted analyses were anti-CCP titer, age, duration of disease, systolic blood pressure, BMI, smoking, and LDL:HDL cholesterol. zCACS = log-transformed (ln[CACS + 1]) standardized CACS (see Table 1 for other definitions).
† Coefficients were standardized coefficients or β for CACS quantile regression, and unstandardized coefficients or B for the generalized linear model (GLM) and the logistic regression (LR). None of the unadjusted or adjusted coefficients were statistically significant.

study was evaluated with G*Power, using as a reference the previously reported results and a linear regression model (12).

RESULTS

Clinical characteristics of the patients. The study included 124 RA patients with no history of CV events. Clinical characteristics are shown in Table 1. Presence of subclinical atherosclerosis was evaluated by carotid ultrasonography and coronary multidetector CT scanning. Most patients (81.4%) showed at least one sign: 69.4% had carotid plaques, 21.0% had moderate or high risk based on the CACS, and 15.3% had CIMT >0.90 mm. The 3 atherosclerosis markers are predictors of CV disease (5–7,10). Their increased frequencies in relation to frequencies in the general population (carotid plaques in 18.1–46.7% [13,14], CACS >400 in 7.4–9.6% [15,16], and median CIMT 0.70–0.71 mm [13,17]) have already been evidenced in previous studies (4,6). In contrast to the high frequency of these atherosclerosis signs, CV risk based on sex, age, cholesterol levels, systolic blood pressure, and smoking status was low or moderate for most patients. This was reflected in a median SCORE of 1.5% (estimated risk of CV death in 10 years), and only 13 patients exhibited a ≥5% risk of fatal events in 10 years according to classic risk factors.

The EULAR task force suggested using a modified SCORE to correct for the excess CV risk in patients with RA (1). The mSCORE incorporates a 1.5× factor if patients have 2 of the 3 following characteristics: disease duration >10 years, seropositive RA, or extraarticular manifestations (1) (present in 41.9%, 71.5%, and 12.1% of our patients, respectively). EULAR mSCORE values were slightly increased when compared with those obtained applying the SCORE. However, the number of patients with high/very high CV risk was still small (17 patients had an mSCORE of ≥5%). It is important to note that although these parameters measure different aspects of CV risk, all are related. This was shown by clear positive correlations between the 3 atherosclerosis surrogate markers and between these markers and the SCORE values (all correlations $P < 0.003$), and by the significant enrichment of the 3 markers in the upper tertile of mSCORE values (data not shown).

Besides the conventional RA autoantibodies RF and anti–CCP-2 we assessed autoantibodies against the whole molecule of Cit-fibrinogen and against a prevalent immunodominant peptide, citFibβ 36-52 (Table 1). The presence of the different autoantibodies was correlated. Most (75.9%) of the patients with antibodies against Cit-Fibβ 36-52 (n = 29 [23.4% of the total group]) were also positive for antibodies in the assay with the whole protein. Similarly, most patients with anti–Cit-fibrinogen antibodies (n = 42 [33.9% of the total group]) were also positive for anti–CCP-2 (85.4%) and, to a lower extent, for RF (76.2%).

Lack of association of anti–Cit-fibrinogen antibodies with subclinical atherosclerosis. Taking advantage of the results with regard to the 3 surrogate markers of subclinical atherosclerosis (CIMT, carotid plaques, and CACS), we explored the association of each of them with the autoantibodies. As shown in Tables 2 and 3, no significant associations were identified. Of particular interest were the results obtained for titers of anti–Cit-fibrinogen (Table 2), because this was the antibody that was reported to be associated with subclinical atherosclerosis and because Cit-fibrinogen was reported to be present in atherosclerotic plaques (3). In these analyses, CACS was assessed using multiple approaches, because there is no consensus on the best way to analyze this type of variable with a large number of RA patients having a score of 0. However, the results derived from the analysis of all these models were negative (Table 2). In addition, no significant association was observed when patients were assessed as a whole group (Tables 2 and 3) or according

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Table 3. Percent of patients with subclinical atherosclerosis outcomes according to RA autoantibody status*

	Anti−Cit-fibrinogen		Anti−citFibβ 36-52		Anti-CCP		RF		Seropositive RA†	
	+	−	+	−	+	−	+	−	+	−
CACS										
Normal	42.9	39.0	27.6	44.2	36.5	47.9	36.1	46.2	35.3	52.8
Low-to-moderate	38.1	39.0	58.6	32.6	43.4	29.2	41.2	34.6	39.8	36.1
Moderate-to-high	19.0	9.7	10.3	13.7	12.2	14.6	15.3	9.6	17.0	2.8
High	0.0	12.2	3.5	9.5	8.1	8.3	6.9	9.6	8.0	8.3
CIMT >0.9 mm	21.4	12.2	17.2	14.7	13.5	18.8	18.1	11.5	15.9	13.8
Carotid plaques	71.4	68.3	79.3	66.3	68.9	68.8	75.0	61.5	71.6	63.9

* There were no statistically significant differences between the patient groups by RA autoantibody status, for any of the subclinical atherosclerosis outcomes assessed. See Table 1 for definitions.
† RF or anti-CCP.

to sex, mSCORE (≥5, or tertiles), RA seropositivity status (RF or anti–CCP-2), combined positivity for anti–Cit-fibrinogen and RF, combined positivity for RF and anti–CCP-2, or combined atherosclerosis surrogate markers (Table 3 and data not shown). Similarly, results were negative regardless of adjustment for relevant covariates (Table 2).

DISCUSSION

In the present study, we found that none of 3 well-validated surrogate markers of subclinical atherosclerosis and CV disease (CIMT, carotid plaques, and CACS) (5–7,10) were associated with the presence or titer of anti–Cit-fibrinogen antibodies. In addition, no association was found with other RA autoantibodies, including the citFibβ 36-52 peptide and anti–CCP-2, or using a wide array of statistical tests and covariate adjustments. Therefore, the lack of replication of the previously described association between anti–Cit-fibrinogen antibodies and subclinical atherosclerosis (3) was a consistent result in our series of patients. Our data thus do not support the notion that anti–citrullinated protein antibody immune complexes are involved in the progression and local inflammation of atherosclerotic plaques, as was previously postulated to be a factor contributing to the increased CV risk in patients with RA (3).

Our study was sufficiently powered (1–β >0.90) to detect the difference that was previously reported, because the sample sizes in this study and the study by Sokolove et al (3) were similar (124 and 134, respectively), but most notably because the effect size observed in the earlier study was very large (a change of 383.6 calcification score units per each SD change in anti–Cit-fibrinogen titer). However, with a study that includes more patients than currently available, it would be possible to determine whether a small effect is present. In addition, there were differences between the studies that could explain the contradictory results. For example, Sokolove and colleagues analyzed these anti–Cit-fibrinogen antibodies in relation to thoracic aortic calcifications detected by electron beam CT, and no other markers of atherosclerosis. Thoracic aortic calcium scores were not available for our patients, and we cannot exclude the possibility that the lack of replication might be due to the absence of this type of information. However, the CACS and thoracic aortic calcifications have been shown to correlate well with one another, and the CACS is a better predictor of CV risk than aortic calcification, as demonstrated in large studies (18,19). In addition, the power of CIMT and carotid plaques, which have not been directly compared with aortic calcifications, for CV risk assessment is similar to that of the CACS, and complement the information provided by this technique (10,20).

There were also differences between the studies in terms of ethnicity and nationality of the patients, which could possibly affect risk of CV events (patients in the US [with ethnicity not indicated] in the study by Sokolove et al, versus European Caucasians in ours). However, CV disease risk is considered to be relatively low in both countries (http://www.who.int/cardiovascular_diseases/en/cvd_atlas_13_coronaryHD.pdf?ua=1). Other differences between the studies include some clinical and demographic characteristics of the patients, e.g., male/female distribution and percent RF seropositivity, but they are unlikely to contribute to the lack of replication since we performed stratified and adjusted analyses. Additionally, there were differences in the method of measuring levels of anti–Cit-fibrinogen antibodies (bead-based multiplex assay versus ELISA) and possibly the fibrinogen antigen.

The hypothesis linking the excess risk of CV events in patients with RA with anti–citrullinated protein antibodies through immune complexes in the atherosclerosis plaque includes other elements that we did not address in the present study (of particular relevance, the presence of

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citrullinated proteins in the atherosclerotic plaque [3], and the increased CV risk in seropositive patients [1]). However, neither of these two elements exhibits a clear relationship. Citrullinated proteins were also demonstrated in plaque of subjects without RA (3), possibly reflecting ongoing inflammation unrelated to autoimmunity. In addition, the increased CV risk in seropositive patients could be due to the more severe disease and increased systemic inflammation in this subgroup of patients (2), and it is not specific to positivity for anti–citrullinated protein antibodies (1). These limitations were acknowledged by Sokolove et al. Therefore, the correlation between anti–Cit-fibrinogen titers and subclinical atherosclerosis was an important element in the hypothesis, and lack of replication of this association in the current study consequently casts doubt concerning this hypothesis.

In summary, we did not find any association between anti–Cit-fibrinogen antibodies and subclinical atherosclerosis manifested either in the CACS or as CIMT or carotid plaques. These results raise questions regarding the hypothesis that these autoantibodies form immune complexes in atherosclerotic plaques, promoting and perpetuating inflammation and contributing to increased CV risk in patients with RA.

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AUTHOR CONTRIBUTIONS

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

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