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Triple positivity for autoantibodies in patients with rheumatoid arthritis is associated with a severe course of the disease but not with bone turnover markers

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Abstract

Introduction: Rheumatoid arthritis (RA) is a prevalent autoimmune disorder characterized by chronic joint inflammation and progressive bone erosion. Traditional autoantibodies, such as anti-citrullinated peptide antibodies (ACPAs) and rheumatoid factor (RF), are established markers associated with disease severity. Recent studies have identified anti-carbamylated protein (anti-CarP) antibodies as potential indicators of disease progression. Additionally, bone turnover markers and specific single nucleotide polymorphisms (SNPs) may influence RA pathogenesis. This study aimed to evaluate the correlation between autoantibody profiles, disease activity, bone turnover markers, and selected SNPs in a cohort of Polish RA patients.

Material and methods: A total of 138 RA patients from the Department of Rheumatology, Medical University of Lodz, were enrolled. Disease activity was assessed using the Disease Activity Score in 28 joints by C-reactive protein (DAS28-CRP). Serum levels of RF, ACPAs, anti-CarP antibodies, and bone turnover markers (sclerostin, periostin, and Dickkopf-1) were measured using immunoassays. Genotyping for SNPs in PADI4 (rs2240340), STAT4 (rs7574865), and PTPN22 (rs2476601) genes was performed. Patients were categorized into two groups: those positive for anti-CarP antibodies, RF, and ACPA (triple-positive, $n = 27$) and those with other antibody combinations ($n = 111$).

Results: Demographic characteristics, including age (mean approx. 61 years), gender distribution (approx. 75% female), treatment rates (approx. 75%), and glucocorticosteroid use (approx. 40%), were comparable between groups. The triple-positive group exhibited higher disease activity, with a greater number of painful joints (mean 10.07 vs. 7.72; $p = 0.017$), higher Visual Analogue Scale (VAS) scores for pain (mean 6.26 vs. 5.06; $p = 0.018$), elevated DAS28-CRP scores (mean 4.75 vs. 4.07; $p = 0.037$), and increased erythrocyte sedimentation rate (ESR) (mean 32.92 mm/h vs. 22.82 mm/h; $p = 0.019$). Serologically, the triple-positive group had significantly higher levels of anti-CarP (mean 29.19 ng/ml vs. 16.29 ng/ml; $p < 0.0001$) and ACPAs (mean 395.45 vs. 368.70; $p < 0.0001$), but lower RF levels (mean 164.01 vs. 453.40; $p = 0.004$). Bone turnover markers showed no significant differences between groups, though the difference in sclerostin levels approached statistical significance ($p = 0.085$), suggesting a possible association of higher bone formation inhibition with triple-positive status. No significant associations were found between the autoantibody profiles and the selected SNPs.

Conclusions: The presence of anti-CarP antibodies, RF, and ACPA is associated with increased disease activity in RA patients. However, these autoantibody profiles do not significantly correlate with bone turnover markers or the selected genetic polymorphisms in this Polish cohort. Further research is warranted to elucidate the complex interactions between autoantibodies, bone metabolism, and genetic factors in RA.

Key words: rheumatoid arthritis, anti-citrullinated peptide antibodies, anti-carbamylated protein antibodies, bone turnover markers.

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Introduction

Rheumatoid arthritis (RA), the most common autoimmune inflammatory disease of the joints, is one of the best-described and understood collagen tissue disorders [1]. However, gaps still exist in our understanding of its pathogenesis and clinical course. Identifying biomarkers that help predict disease severity and endotypes is crucial for both patients and clinicians. One of the most well-described prognostic factors is the presence of autoantibodies, mainly anti-citrullinated peptide antibodies (ACPAs) and rheumatoid factor (RF) [2, 3]. The presence of these classical autoantibodies has been linked to 1) faster bone destruction and erosion formation [4], 2) a worse response to treatment [5, 6], 3) potentially worse prognosis, and 4) more pronounced inflammation [6]. However, not all studies confirm these findings [7].

In recent years, several novel autoantibodies have been described and linked to the clinical course of the disease [8, 9]. Among them, anti-carbamylated protein (anti-CarP) antibodies seem promising [9, 10], as they may be linked to disease pathogenesis and exposure to environmental inhalants. Carbamylation refers to the post-translational modification of proteins by the addition of cyanate, resulting in the formation of carbamylated proteins [11]. In RA, this process may contribute to the development and progression of the disease [12]. Proteins such as fibrinogen [13], vimentin [14], and α-enolase [15] are frequently carbamylated in RA. Anti-carbamylated protein antibodies are generated in response to carbamylated peptides. These antibodies contribute to the autoimmune response seen in RA, leading to inflammation and joint damage [12]. Higher levels of anti-CarP antibodies are often associated with increased disease severity in RA patients. Therefore, anti-CarP antibodies may serve as prognostic markers of RA progression [10, 16].

Rheumatoid arthritis is characterized by chronic synovitis leading to enhanced production of pro-inflammatory cytokines and further activation of osteoclasts [1]. Activation of osteoclasts occurs as a result of pro-inflammatory cytokines, which lead to a decrease in the ratio of osteoprotegerin to receptor activator of nuclear factor κB ligand (OPG/RANKL) [17]. In the progression of RA, markers of bone remodeling have been reported to be involved in both bone formation and bone resorption [18]. This phenomenon results in local periarticular osteoporosis and bone erosions, as well as generalized osteoporosis [18]. It has been proven that the degree of bone destruction depends on the activity of joint inflammation; higher inflammation parameters lead to faster progression of bone changes [17, 18].

The etiology of autoimmune diseases, including RA, involves specific interactions between environmental factors and genetic predisposition. The influence of genes from the human leukocyte antigen (HLA) and non-HLA single nucleotide polymorphisms (SNPs) has been demonstrated [19]. Single nucleotide polymorphisms in genes other than HLA are likely involved in RA pathogenesis. Large genome-wide association studies (GWAS) have identified risk factors for RA, with several SNPs in genes including protein tyrosine phosphatase non-receptor type 22 (*PTPN22*, rs2476601), peptidylarginine deiminase type 4 (*PADI4*, rs2240340), tumor necrosis factor associated factor 1 (*TRAF1*, rs3761847), signal transducer and activator of transcription 4 (*STAT4*, rs7574865), and cluster of differentiation 40 (*CD40*, rs4810485) [19–21]. The products of these genes are involved in RA pathogenesis, providing a reasonable explanation for the role of these SNPs in RA. Studies have confirmed that polymorphisms involving the *PADI4* gene, whose protein is a major factor in citrullination, are present in the Polish RA population [22]. The same was found for the *STAT4* gene, which is involved in inflammation of the synovial joint [23].

The aim of this study was to assess the correlation of the autoantibody profile with disease activity, bone turnover markers, and the occurrence of selected SNPs in a population of Polish patients with RA.

Material and methods

Patients

The study included patients from the Department of Rheumatology, Medical University of Lodz (Poland) who fulfilled the diagnostic criteria of EULAR/ACR (European Alliance of Associations for Rheumatology/American College of Rheumatology). The study cohort comprised 138 patients diagnosed with RA, with a mean age of 61.29 ±13.29 years. The majority were female (n = 125), with 13 male participants. Regarding treatment, 108 patients were receiving disease-modifying antirheumatic drugs (DMARDs), including methotrexate (n = 79), leflunomide (n = 19), sulfasalazine (n = 9), and tocilizumab (n = 1). Additionally, 61 patients were on glucocorticosteroids (GCs), specifically prednisone. The mean Visual Analogue Scale (VAS) score for pain was 5.27 ±2.44 mm. Disease activity, assessed by the Disease Activity Score 28 with C-reactive protein (DAS28-CRP score – DAS < 1.7 was defined as remission, DAS > 1.7 and < 2.6 was defined as low disease activity, and DAS28 above 5.1 was defined as high disease activity), averaged 4.27 ±1.36, with 34 patients (21.66%) exhibiting high disease activity (DAS28-CRP > 5.1). The mean age at symptom onset was 48.99 ±14.20 years, and the average disease duration was approximately 12.39 ±9.88 months. Inflammatory markers included a mean C-reactive protein (CRP) level

of 13.61 ±18.09 mg/dl and an erythrocyte sedimentation rate (ESR) of 25.56 ±21.24 mm/h.

Sample collection

Nine milliliters of peripheral blood were collected from patients for serum sample isolation. For DNA isolation, 9 ml of peripheral blood was collected in K3-EDTA tubes. Samples were cryopreserved at –80°C until the experiments were performed.

Analysis of rheumatoid factor and anti-citrullinated protein antibodies

Rheumatoid factor and ACPAs were measured in a commercial diagnostic laboratory using immunoturbidimetric assay for RF (IU/ml; DxC systems Beckman Coulter) and ELISA for ACPA (RU/ml).

Analysis of bone turnover markers

Protein bone turnover markers were measured in serum samples of RA patients using commercially available enzyme-linked immunosorbent assays (ELISA). For the quantitative measurement of sclerostin (SOST), Dickkopf-1 (DKK-1) and periostin we used the Human SOST ELISA Kit (ab221836-1, ABCAM), Dickkopf-1 measurement was performed using the Human DKK1 ELISA Kit (Dickkopf-1) (ab100501-1, ABCAM), and evaluation of periostin level in serum was performed using the Periostin Human ELISA Kit (EHPOSTN, Thermo Fisher Scientific). For each assay the dilution of the samples had to be optimized. For periostin analysis, the samples were diluted 1 : 3 and 1 : 19 in the case of high concentration (serum : diluent); DKK1 1 : 1; SOST 1 : 1. Assay procedures were followed according to the manuals provided by the manufacturer. Optical density was measured using a plate reader at 450 nm (Multiskan FC Thermo Scientific). Concentrations were automatically interpolated from the standard curve. The results were calculated by multiplying by the dilution factor to determine the final concentrations in serum samples.

Analysis of anti-carbamylated peptide antibodies

For qualitative detection of immunoglobulin G (IgG) antibodies to anti-CarP in patients and the control group we used the Human Anti-Carbamylated Protein Antibody ELISA Kit (BG-HUM09021 NOVATEIN) according to the manufacturer's protocol. Values were obtained using formulas present in the protocol. Optical density was measured using a plate reader (Multiskan FC Thermo Scientific) at 450 nm. Values above the 95th percentile of healthy donors (> 16.31 ng/ml; $n = 28$) were classified as positive [24].

Genotyping

Genomic DNA (gDNA) was isolated using the GeneMatrix Blood DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's protocol. Genotypes were determined by TaqMan SNP Genotyping Assay as described previously [22].

Statistical analysis

The statistical analysis was performed using the Statistica 13.3 software package (TIBCO Software Inc.). The normality of data distribution for all continuous variables was assessed with the Shapiro-Wilk test. For variables with a normal distribution and equal variances, Student's *t*-test was applied. In cases where these assumptions were not met, the Mann-Whitney *U* test was used. To analyze dichotomous variables and individual genotypes of the studied genes, the χ^2 test and Fisher's exact test were utilized. Statistical significance was set at $p \leq 0.05$.

Bioethical standards

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Bioethics Committee of the Medical University of Lodz (Poland) (no. RNN/07/18/KE, approved date: 16 January 2018).

Results

In this study, 2 patient groups were compared: 1 positive for anti-CarP, RF, and ACPA antibodies ($n = 27$), and another with other combinations of these antibodies ($n = 111$). The 2 groups had similar demographics, including age (mean approx. 61 years), gender distribution (approx. 75% female), treatment rates (approx. 75%), and GCs use (approx. 40%), with no significant differences. However, the triple-positive group exhibited higher disease activity, evidenced by a greater number of painful joints (mean 10.07 vs. 7.72; $p = 0.0170$), higher VAS scores for pain (mean 6.26 vs. 5.06; $p = 0.0176$), elevated Disease Activity Score (DAS) with CRP (mean 4.75 vs. 4.07; $p = 0.0372$), and increased ESR (mean 32.92 mm/h vs. 22.82 mm/h; $p = 0.0193$). Serologically, the triple-positive group had significantly higher levels of anti-CarP (mean 29.19 ng/ml vs. 16.29 ng/ml; $p < 0.0001$) and ACPAs (mean 395.45 vs. 368.70; $p < 0.0001$), but lower RF levels (mean 164.01 vs. 453.40; $p = 0.0042$).

Markers of bone turnover

The triple-positive group had a mean SOST level of 1,132.26 ±404.97 pg/ml, while the other combination

Table I. Characteristics of triple-positive vs. non-triple-positive RA patients

Factor	Triple positive (anti-CarP+, ACPA+, RF+) (n = 27)	Not triple positive (n = 111)	p
Age [years]	61.037 ±11.044	61.712 ±13.407	0.5583
Female sex (%)	20 (74.07)	88 (79.28)	0.5565
Treatment with DMARDs (%)	20 (74.07)	88 (79.28)	0.5565
GCs (%)	11 (40.74)	50 (45.45)	0.6588
Number of painful joints	10.074 ±5.784	7.721 ±6.672	0.0170
Number of swollen joints	4.481 ±3.251	3.955 ±4.832	0.1324
VAS (0–10)	6.259 ±2.263	5.064 ±2.428	0.0176
DAS28 > 5.1	8 (29.63)	26 (24.76)	0.6059
Age at first symptoms [years]	47.667 ±12.162	49.910 ±14.684	0.3585
Duration of disease [years]	13.370 ±8.806	11.802 ±9.939	0.2577
Anti-CarP > 16.31 [ng/ml]	29.187 ±13.400	16.293 ±11.529	0.0000
Periostin [ng/ml]	43.936 ±40.645	57.705 ±111.037	0.9179
Sclerostin [pg/ml]	1,132.261 ±404.971	987.416 ±393.723	0.0850
Dickkopf-1 [pg/ml]	6,206.162 ±4,306.751	5,208.027 ±2,705.094	0.6768
Anti-CarP+	27 (100)	26 (26.80)	< 0.0001
Anti-CCP+	27 (100)	55 (55.00)	< 0.0001
RF+	27 (100)	63 (58.88)	< 0.0001
RF [IU/ml]	164.013 ±245.629	453.404 ±3,445.928	0.0042
Anti-CCP [RU/ml]	395.452 ±701.715	368.696 ±2,587.527	0.0000
DAS28-CRP	4.748 ±0.855	4.071±1.414	0.0372
CRP [mg/dl]	12.219 ±12.662	13.686 ±19.691	0.4386
ESR [mm/h]	32.923 ±25.349	22.824 ±20.413	0.0193

Anti-CarP – anti-carbamylated peptide, anti-CCP – anti-cyclic citrullinated protein, CRP – C-reactive protein, DAS28 – Disease Activity Score in 28 joints, DAS28-CRP – Disease Activity Score in 28 joints by C-reactive protein, DMARDs – disease-modifying antirheumatic drugs, ESR – erythrocyte sedimentation rate, GCs – glucocorticosteroids, RF – rheumatoid factor, VAS – Visual Analogue Scale.

group had a mean level of 987.42 ±393.72 pg/ml. This difference was not statistically significant (p = 0.0850), but suggests that triple-positivity is potentially associated with higher inhibition of bone formation. The mean periostin level was 43.94 ±40.65 ng/ml in the triple-positive group and 57.71 ±111.04 ng/ml in the other combination group. The difference was not statistically significant (p = 0.9179). Also, mean DKK-1 levels did not differ significantly and were 6,206.16 ±4,306.75 pg/ml in the triple-positive group and 5,208.03 ±2,705.09 pg/ml in the other combination group (Table I).

Lack of correlations with single nucleotide polymorphisms

The distribution of genotypes for the SNPs rs2240340, rs3761847, rs7574865, rs4810485, and rs2476601 shows no statistically significant differences between the triple-positive group and the other combination group (Table II).

Discussion

Our study showed that patients positive for triple autoantibodies – anti-CarP, RF, and ACPA – exhibited higher disease activity, as indicated by increased DAS28-CRP scores, VAS pain scores, and ESR levels. This suggests that the presence of multiple autoantibodies may correlate with more severe disease manifestations. These results are in line with previous publications showing that presence of various autoantibodies, especially in high titers, is closely correlated with worse prognosis and prompt disease progression [6, 25–28].

High levels of RF are known predictor of severe RA [6, 29, 30]. Patients with both IgM RF and IgG ACPA, commonly referred to as "double-positive" patients, tend to experience more significant bone destruction, including more frequent and larger bone erosions. Furthermore, the disease activity is elevated in these double-positive patients, with higher pro-inflammatory cytokine levels compared to patients without RF [31, 32]. Anti-carbamylated

Table II. Presence of SNPs in a cohort of RA patients with different antibody profiles

	Triple-positive patients (anti-CarP, RF and ACPA) (*n* = 24)	Not triple positive (*n* = 85)	*p*
rs2240340			
C/T	12 (50.00)	43 (50.59)	0.8656
T/T	5 (22.83)	14 (16.47)	
C/C	7 (29.17)	28 (32.94)	
rs3761847			
A/A	6 (25.00)	26 (30.59)	0.8152
G/G	5 (20.83)	14 (16.47)	
A/G	13 (54.17)	45 (52.94)	
rs7574865			
G/T	8 (33.33)	34 (40.00)	0.6401
G/G	13 (54.17)	45 (52.94)	
T/T	3 (12.50)	6 (7.06)	
rs4810485			
G/G	15 (62.50)	57 (67.06)	0.6370
G/T	9 (37.50)	26 (30.59)	
T/T	0	2 (2.35)	
rs2476601			
G/A	9 (37.50)	24 (28.24)	0.5736
G/G	13 (54.17)	56 (65.88)	
A/A	2 (8.33)	5 (5.88)	

anti-CarP – anti-carbamylated peptide antibodies, ACPA – anti-citrullinated peptide antibodies, RF – rheumatoid factor.

peptide antibodies are another biomarker linked to RA, often detectable years before the onset of symptoms, and the titers of anti-CarP antibodies tend to rise gradually prior to symptom manifestation, marking them as potential early indicators of RA development in individuals with joint pain [10, 33]. They are referred to as predictors of RA onset in people with joint pain [10, 11]. These antibodies are associated with accelerated progression of bone lesions, particularly in patients who do not test positive for ACPA [34]. Moreover, research of Kolarz et al. [35] suggested that anti-CarP, but not anti-PAD4, may serve as a valuable biomarker for identifying ACPA/RF-negative RA cases. This makes anti-CarP particularly relevant for diagnosing and assessing RA in these ACPA/RF-negative cases.

The presence of all 3 antibodies – RF, ACPA, and anti-CarP – can be used to identify individuals at risk of developing RA [28, 36]. Identifying patients with multiple autoantibodies is therefore essential for tailoring treatment strategies, as these individuals are likely to experience more aggressive disease progression.

In our study, we hypothesized that patients with triple autoantibody positivity would exhibit significantly elevated markers of bone turnover and regulation. However, this hypothesis was not confirmed. Although the triple-positive group showed higher average sclerostin levels, this was not statistically significant. This difference may nonetheless suggest a potential association of increased bone formation inhibition with triple autoantibody positivity. Given sclerostin's role as an inhibitor of the Wnt/β-catenin signaling pathway, which affects bone metabolism, this possibility could have important implications for bone health in RA patients with multiple autoantibodies.

Sclerostin, a regulator of bone metabolism, is implicated in the pathogenesis of RA and has been found to be present at higher circulating levels in RA patients compared to healthy individuals [37, 38]. However, consistent with previous studies, our results did not show a correlation between serum sclerostin levels and disease activity, bone erosions, or other bone biomarkers in RA patients [39]. The correlations with disease activity are not consistently observed in studied cohorts of patients with RA [37].

Additionally, we did not find any correlation between disease activity and other bone turnover markers such as periostin and DKK-1. Dickkopf-1, an endogenous inhibitor of the canonical Wnt pathway, is implicated in RA

pathogenesis. Recent research involving 1,305 RA patients found significantly higher levels of DKK-1 in RA patients compared to controls [40]. Levels of DKK-1 can be linked to structural damage and activity of the disease [41, 42]. However, the findings regarding the correlation between ACPAs and DKK-1 are contradictory [42, 43].

Periostin, a matricellular protein involved in tissue development and repair, plays a key role in processes such as cell adhesion, migration, and tissue remodeling, particularly within the extracellular matrix [44]. In RA patients, periostin levels positively correlate with disease activity as assessed by the DAS28 index and with RF and CRP levels [45]. Elevated periostin concentrations may indicate higher disease activity and an increased risk of bone fractures in RA patients [46].

The lack of significant associations between the studied SNPs linked with RA in the Polish population (rs2240340, rs7574865, and rs2476601) and autoantibody profiles suggests that these genetic variants may not influence the presence of multiple autoantibodies in RA patients [22]. Our findings align with the meta-analysis conducted by Elshazli et al. [47], which demonstrated that polymorphisms in *PTPN22* (rs2476601) and *STAT4* (rs7574865) are associated with increased susceptibility to RA across various ethnic groups, independently of RF and ACPAs status.

In studies on RA, anti-CarP antibodies were found to be associated with specific genetic variants within the HLA region. Research suggests that HLA-B*08 carrying Asp-9 as the MHC locus is a significant marker for RA patients who test positive for anti-CarP antibodies but negative for ACPAs [48]. Additionally, this antibody profile – positivity for anti-CarP antibodies and negativity for ACPA – has been linked to the *HLA-DR3* variant [49–51]. In contrast, Jiang et al. [52] reported no association between anti-CarP antibodies and most genetic or environmental factors, including smoking, PTPN22 polymorphisms, or general *HLA-DRB1* alleles, except for a specific association between anti-CarP-FCS and the *HLA-DRB1*03* allele. Changes in the levels of RF, ACPA, and anti-CarP antibodies are influenced by the use of DMARDs but do not significantly impact DAS scores [53]. Furthermore, the presence of anti-CarP and ACPA independently affects the progression of RA [54].

Study limitations

The cross-sectional design of the study limits the ability to infer causal relationships between autoantibody profiles and disease activity or bone turnover. Longitudinal studies are necessary to assess how these factors interact over time. Additionally, investigating other genetic polymorphisms and environmental factors could yield a more comprehensive understanding of their roles in RA pathogenesis and progression. Future plans include expanding the study group for long-term evaluation in a larger cohort of RA patients, with particular emphasis on studies related to SNPs.

Conclusions

Patients positive for anti-CarP, RF, and ACPA antibodies exhibit higher disease activity, as demonstrated by an increased number of painful joints, elevated VAS scores, higher DAS28-CRP scores, and elevated ESR levels. However, no significant association was found between the studied SNPs and disease severity or elevated autoantibody levels. Additionally, bone turnover marker levels did not correlate with the presence of the studied SNPs, disease severity, or the presence of RF, ACPA, and anti-CarP antibodies.

Disclosures

Conflict of interest: The authors declare no conflict of interest.

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Ethics approval: The study was approved by the Institutional Bioethics Committee of the Medical University of Lodz (Lodz, Poland) (no. RNN/07/18/KE, approved date: 16 January 2018).

Data availability: The data that support the findings of this study are available on request from the corresponding author (J.M.).

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