





Soluble Vascular Biomarkers in Rheumatoid Arthritis and Ankylosing Spondylitis: Effects of 1-year Antitumor Necrosis Factor- α Therapy

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ABSTRACT. Objective. Rheumatoid arthritis (RA) and ankylosing spondylitis (AS) have been associated with cardiovascular disease. The treatment of arthritis by tumor necrosis factor- α (TNF- α) inhibitors may decrease the serum concentrations of vascular biomarkers. We determined circulating levels of oxidized low-density lipoprotein (oxLDL)/ β_2 glycoprotein I (β_2 -GPI) complexes, antibodies to 60 kDa heat shock protein (anti-Hsp60), soluble urokinase plasminogen activator receptor (suPAR), and B-type natriuretic peptide (BNP) fragment in sera of RA and AS patients undergoing anti-TNF treatment.

Methods. Fifty-three patients with RA/AS were treated with etanercept or certolizumab pegol for 1 year. Circulating oxLDL/ β_2 -GPI complex (AtherOx), anti-Hsp60 IgG, and BNP8-29 fragment levels were assessed by ELISA. suPAR levels were determined by suPARnostic Quick Triage test. Flow-mediated vasodilation (FMD), carotid intima-media thickness (CIMT), and arterial pulse wave velocity (PWV) were determined by ultrasound.

Results. One-year anti-TNF treatment significantly decreased oxLDL/ β_2 -GPI levels, as well as suPAR levels in patients with critically high suPAR levels at baseline. In RA, BNP levels were higher in seropositive vs seronegative patients. Serum levels of these vascular biomarkers variably correlated with lipids, anticitrullinated protein antibodies, rheumatoid factor, and C-reactive protein. CIMT positively correlated with BNP, and PWV with suPAR and anti-Hsp60, whereas FMD inversely associated with anti-Hsp60. In repeated measures ANOVA analysis, disease activity supported the effects of anti-TNF treatment on 12-month changes in oxLDL/ β_2 -GPI. CIMT supported the effects of therapy on changes in anti-Hsp60 and suPAR.

Conclusion. These biomarkers may be involved in the pathogenesis of atherosclerosis underlying RA/AS. TNF inhibition variably affects the serum levels of oxLDL/ β_2 -GPI, suPAR, and BNP.

Key Indexing Terms: angiogenesis, anti-TNF therapy, atherosclerosis, biomarkers, rheumatoid arthritis

Rheumatoid arthritis (RA) and ankylosing spondylitis (AS) have been associated with inflammatory atherosclerosis, increased cardiovascular (CV) morbidity, and mortality.^{1,2,3,4,5} Early endothelial dysfunction and activation may precede

these events already in the preclinical phase of arthritides.^{1,2,3,5} Soluble vascular biomarkers, such as von Willebrand factor antigen⁶ or circulating endothelial cell adhesion molecules,⁷ are released from the endothelial surface. Numerous

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proteins may serve as biomarkers of inflammatory atherosclerosis.⁸ The treatment of arthritis by tumor necrosis factor- α (TNF- α) inhibitors may decrease the serum concentrations of these biomarkers.^{6,7}

Oxidized low-density lipoprotein (oxLDL) and β_2 glycoprotein I (β_2 -GPI) antigens, as well as antibodies to these antigens, have been implicated in atherosclerosis associated with autoimmune diseases^{9,10,11,12,13} and atherosclerosis in general.^{10,13,14,15} Unlike native LDL, oxLDL binds to β_2 -GPI to form complexes, which are readily detectable in the blood.^{9,16,17} Macrophages uptake these complexes by scavenger receptors, and this mechanism is relevant for the development of foam cells within the atherosclerotic lesions.^{16,17} High circulating oxLDL/ β_2 -GPI levels have been associated with disease severity and prognosis in acute coronary syndrome.¹⁸ Serum oxLDL/ β_2 -GPI complex levels may also reflect vascular damage in systemic lupus erythematosus (SLE) and other autoimmune diseases, such as antiphospholipid syndrome and systemic sclerosis (SSc).^{9,17,19} In 1 study, there was a slight increase in oxLDL/ β_2 -GPI levels in RA.¹⁹ However, neither the role of these complexes in AS nor the effects of anti-TNF therapy on serum oxLDL/ β_2 -GPI complex levels have been evaluated.

Heat shock proteins (Hsp), including the human Hsp60 and the mycobacterial Hsp65, have been implicated in the pathogenesis of atherosclerosis¹⁰ and inflammation.²⁰ Anti-Hsp antibodies have been detected in patients with CV disease (CVD)^{21,22} and autoinflammatory diseases including RA and AS.^{11,22,23,24,25} These antibodies were associated with early atherosclerotic changes.^{11,22}

The urokinase plasminogen activator (uPA) receptor (uPAR; CD87) is expressed mainly on immune cells, smooth muscle cells, and endothelial cells, favoring extracellular matrix degradation, cell adhesion, and cell proliferation, and regulating cell migration.^{26,27,28} Signaling of uPA/uPAR results in the release of numerous inflammatory mediators and synovial fibroblast activation.²⁷ We detected uPAR in the RA synovium.²⁶ suPAR is the soluble form of the cell membrane-bound protein uPAR released by GPI-anchor cleavage from the cell surface.^{28,29} Elevated serum levels of suPAR may reflect increased activation of immune system, as well as vascular pathology in inflammatory diseases.^{29,30,31,32} In RA, suPAR levels have been associated with disease activity.^{30,32}

B-type natriuretic peptide (BNP) and its N-terminal pro hormone BNP (NT-proBNP) exert a key role in CV homeostasis with biologic actions, including natriuresis, diuresis, vasorelaxation, and inhibition of renin and aldosterone secretion.³³ BNP is synthesized as a prohormone in cardiomyocytes. Upon release into the circulation, proBNP is cleaved into BNP and NT-proBNP fragments in equimolar proportions.³³ A high concentration of BNP in the bloodstream is indicative of heart failure and also predicts prognosis after acute coronary syndrome.³³ BNP is also a good biomarker in an emergency setting.³³ Increased BNP production has been associated with cardiac manifestations and may serve as a prognostic factor in various autoinflammatory diseases, such as SSc.^{34,35} In RA and AS, NT-proBNP levels were assessed and anti-TNF therapy decreased NT-proBNP in these diseases.^{36,37} BNP fragments have not yet been assessed in RA and AS.

In this study, we aimed to assess the effects of 1-year anti-TNF therapy on vascular biomarkers described above. We also wished to associate these biomarkers with markers of disease activity, autoantibodies, lipids, and vascular imaging results. Moreover, we wished to determine the predictors of these variables at baseline, as well as the determinants on 1-year change in these variables. This study may improve our understanding of vascular biomarkers underlying inflammatory atherosclerosis.

METHODS

Patients. Fifty-three patients with inflammatory arthritis (36 RA and 17 AS) selected for the initiation of anti-TNF therapy were enrolled in the study. Patient characteristics are seen in Table 1. The cohort included 34 women and 19 men with mean age of 52.0 ± 12.1 (range: 24–83) years. Mean disease duration was 8.5 ± 7.9 (range 1–44) years, while mean age at diagnosis was 43.5 ± 12.1 (range: 23–62) years. Exclusion criteria included unstable hypertension (blood pressure > 140/90 mmHg), congestive heart failure, diabetes mellitus, current inflammatory disease other than RA or AS, infectious disease, or renal failure (serum creatinine ≥ 117 mmol/L). None of the patients received aspirin, clopidogrel, heparin or warfarin, or vasoactive drugs at the time of inclusion. Patients with active disease were recruited prior to initiating a biologic therapy. All patients started on an anti-TNF therapy at baseline and received the same biologic treatment at 1 year. Among the 36 patients with RA, 20 received etanercept (ETN) 50 mg/week subcutaneous (SC), and 16 received certolizumab pegol (CZP; 400 mg at 0, 2, and 4 weeks, and thereafter 200 mg twice weekly SC). Altogether, 18 patients with RA were treated with ETN, and 13 with CZP in combination with methotrexate (MTX). The other patients received monotherapy. All 17 patients with AS received 50 mg/week ETN monotherapy SC. RA patients did not take disease-modifying antirheumatic drugs (DMARDs) other than MTX. Altogether, 12 RA and 2 AS patients currently took low-dose (6 mg/day) methylprednisolone. Disease activity was determined by Disease Activity Score in 28 joints (DAS28) and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) in RA and AS, respectively.

The study was approved by the Hungarian Scientific Research Council Ethical Committee (approval no. 14804-2/2011/EKU). Written informed consent was obtained from each patient, and assessments were carried out according to the Declaration of Helsinki.

Laboratory measurements. After overnight fasting, blood samples were taken from the patients for total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG). Lipids were determined using routine laboratory methods.

Serum high sensitivity C-reactive protein (hsCRP; normal: ≤ 5 mg/L) and IgM rheumatoid factor (RF; normal: ≤ 50 IU/mL) were measured by quantitative nephelometry (Cobas Mira Plus, Roche), using CRP and RF reagents (both Dialab). Anticitrullinated protein/peptide antibodies (ACPA/anti-CCP) were detected in serum samples using a second-generation Immunoscan-RA CCP2 ELISA test (Euro Diagnostica; normal: ≤ 25 IU/mL).

Circulating oxLDL/ β_2 -GPI complexes (U/mL) were assessed by an AtherOx ELISA system (Corgenix) according to the manufacturer's instructions.

Antihuman Hsp60 IgG levels were measured by an in-house ELISA as described previously.^{21,38} In brief, plates were coated with $0.1 \mu\text{g/well}$ recombinant human Hsp60 (SPP-740; StressGen). After washing and blocking (phosphate-buffered saline [PBS], 0.5% gelatin), the wells were incubated with $100 \mu\text{L}$ of serum samples diluted to 1:500 (PBS, 0.5% gelatin, 0.05% Tween 20). Bound anti-Hsp60 antibodies were detected by antihuman IgG peroxidase-labeled antibodies and *o*-Phenylenediamine (both Sigma). Optical density (OD) values were assessed, and concentrations were expressed in AU/mL.

Table 1. Patient characteristics.

	RA	AS	Total
N	36	17	53
Sex, F:M	31:5	3:14	34:19
Age, yrs, mean \pm SD (range)	55.9 \pm 9.8 (35–83)	43.6 \pm 12.4 (24–72)	52.0 \pm 12.1 (24–83)
Disease duration, yrs mean \pm SD (range)	9.1 \pm 8.3 (1–44)	7.2 \pm 7.0 (1–26)	8.5 \pm 7.9 (1–44)
Age at diagnosis, yrs	47.0 \pm 8.7 (28–62)	36.4 \pm 11.6 (23–50)	43.5 \pm 12.1 (23–62)
Smoking, current	7	7	14
Positive CV history	8	1	9
RF positivity, n (%)	26 (72)	–	–
ACPA positivity, n (%)	21 (58)	–	–
DAS28 (baseline), mean \pm SD	5.00 \pm 0.86	–	–
BASDAI (baseline), mean \pm SD	–	5.79 \pm 1.19	–
Treatment (ETN, CZP)	20 ETN, 16 CZP	17 ETN	37 ETN, 16 CZP

ACPA: anticitrullinated protein antibody; AS: ankylosing spondylitis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; CZP: certolizumab pegol; CV: cardiovascular; DAS28: Disease Activity Score in 28 joints; ETN: etanercept; RA: rheumatoid arthritis; RF: rheumatoid factor.

Circulating suPAR levels were assessed by suPARnostic Quick Triage test (ViroGates A/S) that gives fully quantitative results within the range of 2–16 ng/mL. Results were read by the suPARnostic Quick Test Reader (ViroGates). Serum levels were expressed in ng/mL. According to the manufacturer, suPAR levels in the normal population are in the range of 2–3 ng/mL. Serum levels < 4, 4–5.5, 5.5–9, and > 9 ng/mL are considered as low, observe, high, and critical, respectively.

BNP8-29 fragment levels were assessed by commercially available ELISA kit (Biomedica). The fragment consisted of the 8-29 C-terminal part of proBNP. Measurements were performed according to the manufacturer's instructions.

All laboratory assessments were performed at baseline, as well as 6 and 12 months after treatment initiation.

Assessment of vascular physiology by ultrasound. Brachial artery flow-mediated vasodilation (FMD) was assessed as described previously.^{23,39} In brief, ultrasound (US) examination was performed on the right arm by a single trained sonographer using a 10-MHz linear array transducer (US system: HP Sonos 5500), which after 30 minutes rested in a temperature-controlled room (basal value for FMD). A B-mode longitudinal section of the brachial artery was obtained above the antecubital fossa. In order to assess FMD, reactive hyperemia was induced by release of a pneumatic cuff around the forearm inflated to suprasystolic pressure for 4.5 minutes. After deflation, the maximal flow velocity and the arterial diameter were 90 seconds long, continuously recorded. Flow velocities, the baseline diameter, as well as FMD, were electrocardiographically gated and detected offline. FMD values were expressed as a percent change from baseline (resting) value.

The carotid intima-media thickness (CIMT) measurements were carried out as described before.^{23,39} Briefly, a duplex US system (HP Sonos 5500, 10 MHz linear array transducer) was used to assess the common carotid arteries by a single observer. Longitudinal high-resolution B-mode US scans were employed over both right and left common carotid arteries and were R-synchronized and recorded. The offline measurements were performed 1 cm proximal to the carotid bulb in the far wall. CIMT was defined as the distance between the first and second echogenic lines from the lumen, taking the average of 10 measurements on both sides. CIMT values were expressed in mm.

With respect to arterial stiffness, pulse-wave velocity (PWV) was calculated automatically by a TensioClinic arteriograph system (TensioMed Ltd.) as the quotient of the distance between the jugular fossa and symphysis, as described before.^{39,40} If an artery is elastic, PWV is low. With decreased arterial elasticity, PWV rises. The arteriograph assesses this variable from the oscillometric data obtained from the 35 mmHg suprasystolic pressure of the

brachial artery. In order to obtain reproducible results, the patient had to rest in a supine position for at least 10 minutes before the assessment in a quiet room. PWV is expressed in m/s.

Statistical analysis. Statistical analysis was performed using SPSS version 22.0 (IBM Corp.) software. Data are expressed as mean \pm SD for continuous variables, and percentages for categorical variables. Continuous variables were evaluated by paired 2-tailed *t* test and Wilcoxon test. Nominal variables were compared between groups using the chi-square or Fisher exact test, as appropriate. Correlations were determined by Pearson analysis. Univariable and multivariable regression analyses using the stepwise method were applied to investigate independent associations between angiogenic biomarkers (dependent variables) and other clinical, laboratory, and imaging variables (independent variables). The β standardized linear coefficients showing linear correlations between 2 variables were determined. The B (95% CI) regression coefficient indicated independent associations between dependent and independent variables during changes. Repeated measures (RM)-ANOVA was performed in order to determine the additional effects of multiple variables on changes of vascular imaging markers between baseline and 12 months. The dependent variables were anti-oxLDL/ β_2 -GPI, anti-Hsp60, suPAR, and BNP. Partial η^2 is given as indicator of effect size, with values of 0.01 suggesting small, 0.06 medium, and 0.14 large, effects. In all analyses, *P* values < 0.05 were considered significant.

RESULTS

Effects of TNF inhibition on vascular biomarkers. First, TNF- α inhibition significantly decreased disease activity in RA and AS patients. In the RA cohort (*n* = 36), ETN and CZP treatment resulted in significant decreases in DAS28 after 6 months (3.13 \pm 0.84, *P* < 0.001) and 12 months of treatment (3.02 \pm 0.96, *P* < 0.001) compared to baseline (5.00 \pm 0.86; data not shown). In AS (*n* = 17), BASDAI significantly decreased from 5.79 \pm 1.19 at baseline to 2.00 \pm 1.03 (*P* < 0.001) and 1.86 \pm 1.04 (*P* < 0.001) at 6 and 12 months, respectively (data not shown).

In the mixed cohort of 53 arthritis patients, the circulating levels of oxLDL/ β_2 -GPI significantly decreased after 12 months of anti-TNF therapy (0.20 \pm 0.11 U/mL) compared to baseline (0.24 \pm 0.10 U/mL, *P* = 0.014; Figure 1).

Anti-Hsp60 antibody levels did not change after 6 months (158.6 \pm 138.6 AU/mL) and 12 months (167.3 \pm 143.3 AU/mL) compared to baseline (170.3 \pm 140.4 AU/mL; Figure 1).

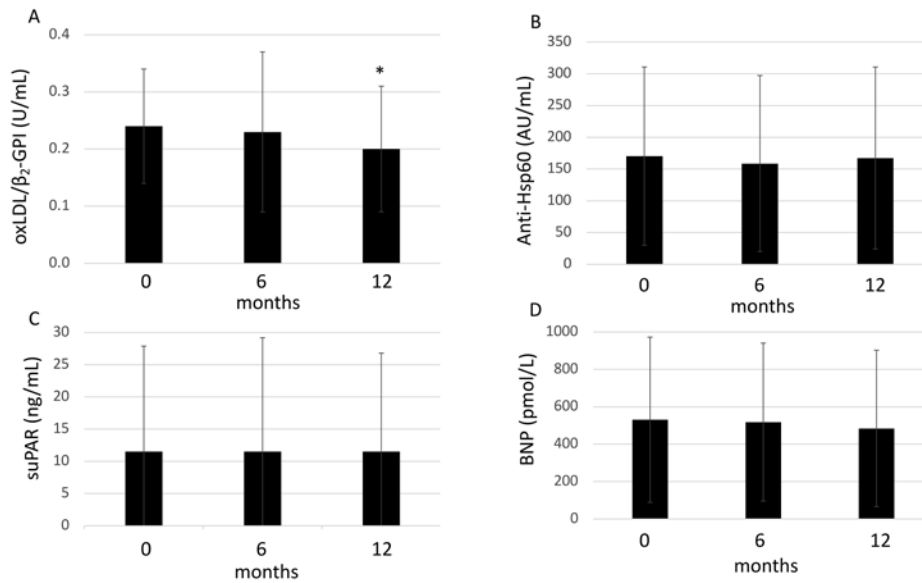


Figure 1. Effects of 1-year anti-TNF therapy on (A) oxLDL/ β_2 -GPI complexes, (B) anti-Hsp60, (C) suPAR, and (D) BNP fragments in RA and AS. Asterisk indicates significant change ($P < 0.05$). anti-Hsp60: antibodies to 60 kDa heat shock protein; AS: ankylosing spondylitis; β_2 -GPI: β_2 glycoprotein I; BNP: B-type natriuretic peptide; oxLDL: oxidized low-density lipoprotein; RA: rheumatoid arthritis; suPAR: soluble urokinase plasminogen activator receptor.

suPAR levels did not change significantly after 6 months (11.3 ± 17.7 ng/mL) and 12 months (10.3 ± 15.3 ng/mL) vs baseline (11.5 ± 16.4 ng/mL; Figure 1). Among the patients, 21.2% had low, 36.4% observe, 9.1% high, and 33.3% critical suPAR levels, according to the classification described above (data not shown). When these 4 serum level categories were considered, suPAR concentrations exerted significant decrease in RA patients with critical suPAR levels (> 9 ng/mL; $P = 0.04$; Figure 2).

BNP fragment levels did not change significantly after 6 months (518.2 ± 422.4 pmol/L) and 12 months ($484.1 \pm$

418.2 pmol/L) vs baseline (530.8 ± 441.8 pmol/L; Figure 1). However, serum BNP levels at baseline and after 12 months were significantly increased in ACPA-positive vs -negative RA patients (baseline: 670.6 ± 323.0 vs 138.0 ± 436.4 pmol/L, $P = 0.030$; and 12 months: 652.9 ± 283.2 vs 456.5 ± 423.1 pmol/L, $P = 0.021$), as well as in RF-positive vs -negative RA patients (baseline: 680.6 ± 381.6 vs 292.9 ± 198.3 pmol/L, $P = 0.007$, and 12 months: 668.9 ± 346.5 vs 312.2 ± 207.0 pmol/L, $P = 0.001$; Figure 3).

Correlations of vascular markers with other variables. First, the

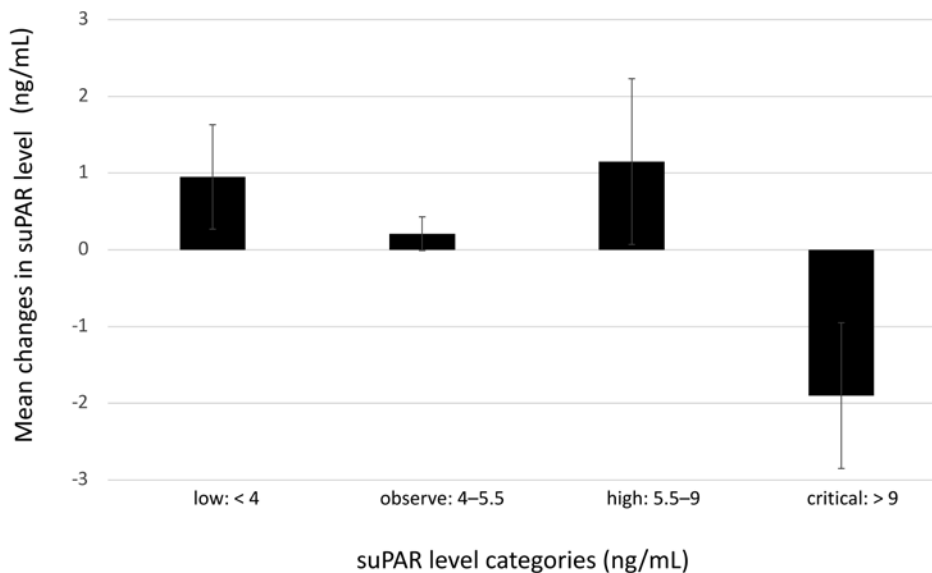


Figure 2. Effects of 1-year anti-TNF therapy on suPAR levels according to serum concentration quartiles. Asterisk indicates significant change ($P < 0.05$). suPAR: soluble urokinase plasminogen activator receptor.

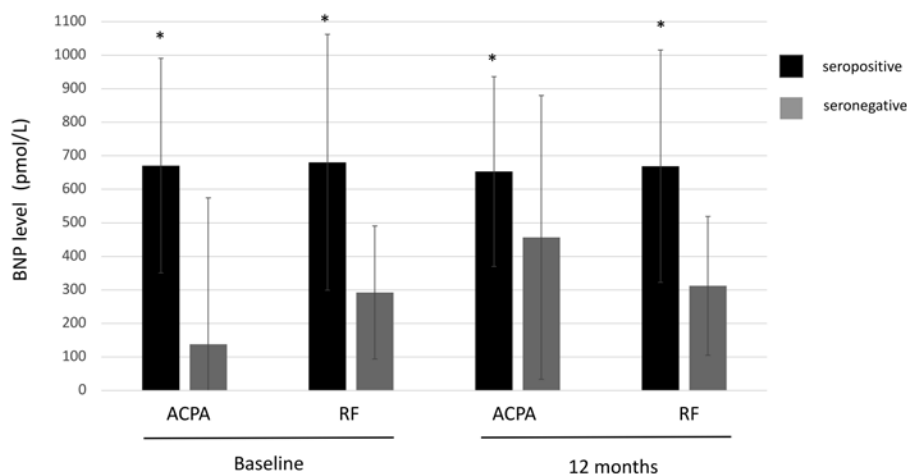


Figure 3. Effects of 1-year anti-TNF therapy on BNP levels according to ACPA and RF seropositivity vs seronegativity. Asterisk indicates significant change ($P < 0.05$). ACPA: anticitrullinated protein antibodies; BNP: B-type natriuretic peptide. RF: rheumatoid factor.

4 vascular biomarkers were correlated with each other. We found only 1 significant correlation: baseline BNP (BNP-0) and suPAR-0 showed positive association with each other ($P = 0.013$; Supplementary Table 1, available from the authors on request).

When these biomarkers were correlated with other markers, oxLDL/ β_2 -GPI complex, suPAR, BNP, and anti-Hsp60 levels positively correlated with some lipids at baseline ($P < 0.05$; Table 2, Supplementary Table 1, available from the authors on request). suPAR-0 levels also positively correlated with ACPA-0 ($P < 0.001$). BNP-0 strongly correlated with RF-0 ($P = 0.004$) and ACPA-0 ($P < 0.001$). Moreover, BNP-12 correlated with age at disease onset ($P = 0.023$), CRP-0 ($P = 0.010$), and CRP-12 ($P = 0.014$; Supplementary Table 1). None of the vascular biomarkers correlated with disease activity as determined by DAS28 or BASDAI (data not shown).

Results of the univariable and multivariable regression analyses are indicated in Table 2. In the univariable analysis, the predictor of suPAR-0 was TG-0 ($P < 0.05$), while that of suPAR-12 was CRP-12 ($P < 0.05$). BNP-0 was determined by TG-0 ($P < 0.05$), while BNP-12 was determined by age at disease onset, CRP-0, and CRP-12 ($P < 0.05$). The multivariable analysis confirmed the significant association of BNP-12 and CRP-0

only ($P = 0.028$). Thus, most correlations found in univariable analysis, except for 1, was lost during the multivariable analysis.

When vascular biomarkers were correlated with imaging markers of vascular physiology, BNP-0 showed positive association with CIMT-0 ($P = 0.016$). Anti-Hsp-60-0 inversely correlated with FMD-12 ($P = 0.022$) and positively with PWV-0 ($P = 0.040$). Both suPAR-0 ($P = 0.045$) and suPAR-12 ($P = 0.042$) were positively associated with PWV-12 (Supplementary Table 1, available from the authors on request). Finally, generalized linear model RM-ANOVA was performed to assess combined determinants of vascular biomarker changes over the 12-month period. The change of oxLDL/ β_2 -GPI complex levels between baseline and 12 months was determined by the anti-TNF treatment together with higher baseline disease activity (DAS28/BASDAI-0, $P = 0.014$). In addition, TNF inhibition and higher CIMT-0 determined anti-Hsp-60 ($P = 0.015$) and suPAR changes over the 1-year period ($P = 0.041$; Table 3).

DISCUSSION

In this study, we determined changes in various vascular biomarkers upon 1-year anti-TNF therapy. We also found associations of circulating levels of these biomarkers with CRP, lipids, and vascular imaging markers. In the same cohort, we previously

Table 2. Univariable and multivariable regression analysis of vascular biomarkers.

Dependent Variable	Independent Variable	β	P	Univariable		β	P	Multivariable	
				B	95% CI			B	95% CI
suPAR-0	TG-0	0.382	0.028	8.314	0.948–15.680			NS	
suPAR-12	CRP-12	0.525	0.002	0.991	0.402–1.580			NS	
BNP-0	TG-0	0.303	0.041	185.559	8.242–362.788			NS	
BNP-12	Age at onset	0.330	0.023	14.161	2.007–26.316			NS	
	CRP-0	0.372	0.010	8.995	2.263–15.728	0.316	0.028	7.460	0.843–14.077
	CRP-12	0.356	0.014	19.019	4.020–34.018			NS	

-0: baseline; -12: 12 months; BNP: B-type natriuretic peptide; CRP: C-reactive protein; NS: not significant; suPAR: soluble urokinase plasminogen activator receptor; TG: triglyceride.

Table 3. Significant results of the generalized linear model repeated measures ANOVA test determining the effects of treatment and other independent variables on vascular biomarkers as dependent variables.

Dependent Variable	Effect	F	P	Partial η^2
oxLDL/ β_2 -GPI 0–12	Treatment * DAS28/ BASDAI-0	6.617	0.014	0.155
aHsp60 0–12	Treatment * CIMT-0	6.533	0.015	0.161
suPAR 0–12	Treatment * CIMT-0	4.294	0.041	0.177

DAS28: Disease Activity Score in 28 joints; aHsp60: antiheat shock protein 60 kDa; CIMT: carotid intima-media thickness; oxLDL/ β_2 -GPI: oxidized low-density lipoprotein/ β_2 glycoprotein I complex; suPAR: soluble urokinase plasminogen activator receptor.

published the effects of anti-TNF therapy on vascular pathophysiology using imaging techniques.⁴¹ Anti-TNF therapy significantly decreased serum levels of oxLDL/ β_2 -GPI complexes. We and others suggested the involvement of both oxLDL and β_2 -GPI, as well as anti-oxLDL and anti- β_2 -GPI, in inflammatory atherosclerosis and CVD.^{9,11,12,14,15} Serum oxLDL/ β_2 -GPI levels have been associated with acute coronary syndrome¹⁸ as well as vascular damage in various autoimmune rheumatic diseases.^{9,16,17,19,42} To the best of our knowledge, this is the first study to evaluate oxLDL/ β_2 -GPI complexes in AS and to assess the effects of anti-TNF biologics on these complexes. In addition, we found correlations between baseline levels of oxLDL/ β_2 -GPI complexes and lipids. RA and AS disease activity in combination with 1-year anti-TNF treatment determined 12-month changes in oxLDL/ β_2 -GPI, as shown by RM-ANOVA. The clinical relevance of oxLDL/ β_2 -GPI levels needs to be validated.

Elevated serum levels of suPAR have been detected in various rheumatic diseases, such as RA and SSc.^{29,30,31,43} Moreover, according to other studies, suPAR levels may reflect disease activity in RA,^{30,32} which was not confirmed in our cohort. In our cohort, baseline suPAR levels correlated with ACPA and TG levels. Again, very few studies evaluated the effects of biologics on suPAR production. We observed that 1-year TNF inhibition did not change suPAR levels significantly. In another study, similarly to ours, infliximab treatment did not modify suPAR levels in pediatric inflammatory bowel disease patients.⁴⁴ Interestingly, although TNF inhibition did not affect suPAR levels in the full cohort, it significantly reduced suPAR production in patients with arthritis who had critical suPAR levels at baseline. In addition, CRP-12 may be a predictor of suPAR-12, suggesting that if, in spite of 1-year biologic therapy, CRP still remains higher, suPAR would also stay high. In other words, suPAR may be a marker of sustained inflammation despite anti-TNF therapy in arthritis. Finally, in another cohort, suPAR was a good predictor of adalimumab responsiveness in RA.⁴⁵ We did not find any association between suPAR and clinical response to anti-TNF therapy in our cohort. Baseline suPAR correlated with BNP, and anti-TNF treatment together with baseline CIMT determined 12-month changes in suPAR. The clinical relevance of suPAR levels needs to be validated.

Increased production of BNP and NT-proBNP have been associated with CVD and heart failure.^{36,46} NT-proBNP may also play a role in CV comorbidities of SSc, RA, and other

rheumatic diseases.^{34,35,37} Other investigators reported that TNF inhibition reduced NT-proBNP levels in RA and AS.^{36,37} However, the BNP fragment has not yet been previously studied in these diseases. In our present study, anti-TNF therapy did not change BNP levels significantly in the full cohort. However, circulating BNP levels were significantly elevated in seropositive RA patients compared to seronegative ones. Moreover, TG-0 was a predictor of BNP-0, whereas age, CRP-0, and CRP-12 were determinants of BNP-12. Thus, BNP may be associated with systemic inflammation and some lipids. Moreover, the association between CRP-0 and BNP-12 suggests that the extent of inflammation at baseline may predict how BNP levels would change after 1-year TNF inhibition.

Finally, anti-Hsp60 antibodies have been detected in patients with CVD^{21,22,38} as well as in rheumatic diseases including RA and AS.^{11,22,23,24,25} In our cohort, anti-Hsp60 levels at baseline correlated with TG. Biologics did not change anti-Hsp60 levels over time. However, TNF inhibition combined with baseline CIMT determined the 12-month changes in anti-Hsp60 as shown by RM-ANOVA. Again, we have not found any other studies where the effects of biologics on anti-Hsp antibodies were evaluated. The clinical relevance of anti-Hsp60 levels needs to be validated.

There have certainly been other studies that analyzed vascular biomarkers in arthritides. For example, angiotensin-2 levels correlated with CVD in RA.⁴⁷ Anti-TNF therapy reduced resistin levels in RA.⁴⁸ In patients with AS, osteoprotegerin correlated with disease activity and vascular damage.⁴⁹ Finally, we found that the release of angiogenic vascular biomarkers also changed upon TNF inhibition in RA and AS patients.⁵⁰

When comparing the 4 analyzed vascular markers, 1-year anti-TNF therapy generally decreased oxLDL/ β_2 -GPI levels and suppressed suPAR in patients with critical levels at baseline. BNP levels were higher in ACPA- and RF-seropositive patients. Multiple correlations were found between any of these 4 markers and CRP, lipids, ACPA, and RF. According to the regression analysis, CRP and TG may be important predictors of BNP and suPAR at baseline or after 12 months of treatment. Among imaging markers of vascular pathophysiology, CIMT correlated with BNP and PWV with suPAR and anti-Hsp60, whereas FMD correlated inversely with anti-Hsp60, suggesting that various biomarkers may be associated with different pathophysiological alterations in the vasculature. Baseline disease activity or CIMT

may significantly influence the effects of anti-TNF treatment on changes of oxLDL/ β_2 -GPI, anti-Hsp60, and suPAR overtime.

The strength of this study is its complexity. Here we compared 4 vascular biomarkers with disease activity and multiple laboratory variables, as well as imaging of vascular physiology in RA and AS. Limitations may include the relatively low number of RA and AS patients. The majority of these surrogate markers, except BNP, have not been clinically validated, so it is difficult to interpret their everyday practical relevance. This study does not have a control group and power was not calculated, both of which may also be limitations.

In conclusion, among the 4 vascular markers assessed in this study, oxLDL/ β_2 -GPI was affected by anti-TNF therapy. Moreover, suPAR levels changed in patients with the highest levels at baseline. These markers are variably associated with vascular pathophysiology, CRP, ACPA, RF, and lipids. oxLDL/ β_2 -GPI complexes, anti-Hsp60 antibodies, suPAR, and BNP may be involved in inflammatory and vascular mechanisms underlying RA and AS. These proteins may also serve as potential biomarkers in inflammatory atherosclerosis and CVD.

REFERENCES

1. Agca R, Heslinga SC, Rollefstad S, Heslinga M, McInnes IB, Peters MJL, et al. EULAR recommendations for cardiovascular disease risk management in patients with rheumatoid arthritis and other forms of inflammatory joint disorders: 2015/2016 update. *Ann Rheum Dis* 2017;76:17-28.
2. Kerekes G, Szekanez Z, Dér H, Sandor Z, Lakos G, Muszbek L, et al. Endothelial dysfunction and atherosclerosis in rheumatoid arthritis: a multiparametric analysis using imaging techniques and laboratory markers of inflammation and autoimmunity. *J Rheumatol* 2008;35:398-406.
3. Bodnár N, Kerekes G, Seres I, Paragh G, Kappelmayer J, Némethné ZG, et al. Assessment of subclinical vascular disease associated with ankylosing spondylitis. *J Rheumatol* 2011;38:723-9.
4. Nurmohamed MT, Heslinga M, Kitis GD. Cardiovascular comorbidity in rheumatic diseases. *Nat Rev Rheumatol* 2015;11:693-704.
5. López-Mejias R, Castañeda S, González-Juanatey C, Corrales A, Ferraz-Amaro I, Genre F, et al. Cardiovascular risk assessment in patients with rheumatoid arthritis: the relevance of clinical, genetic and serological markers. *Autoimmun Rev* 2016;15:1013-30.
6. Kerekes G, Soltész P, Szucs G, Szamosi S, Dér H, Szabó Z, et al. Effects of adalimumab treatment on vascular disease associated with early rheumatoid arthritis. *Isr Med Assoc J* 2011;13:147-52.
7. Gonzalez-Gay MA, Garcia-Unzueta MT, De Matias JM, Gonzalez-Juanatey C, Garcia-Porrúa C, Sanchez-Andrade A, et al. Influence of anti-TNF-alpha infliximab therapy on adhesion molecules associated with atherogenesis in patients with rheumatoid arthritis. *Clin Exp Rheumatol* 2006;24:373-9.
8. Csernok E, Hellmich B. Usefulness of vasculitis biomarkers in the era of the personalized medicine. *Autoimmun Rev* 2020;19:102514.
9. Bassi N, Ghirardello A, Iaccarino L, Zampieri S, Rampudda ME, Atzeni F, et al. OxLDL/beta2GPI-anti-oxLDL/beta2GPI complex and atherosclerosis in SLE patients. *Autoimmun Rev* 2007;7:52-8.
10. Shoenfeld Y, Harats D, George J. Heat shock protein 60/65, beta 2-glycoprotein I and oxidized LDL as players in murine atherosclerosis. *J Autoimmun* 2000;15:199-202.
11. Sherer Y, Cerinic MM, Bartoli F, Blagojevic J, Conforti ML, Gilburd B, et al. Early atherosclerosis and autoantibodies to heat-shock proteins and oxidized LDL in systemic sclerosis. *Ann N Y Acad Sci* 2007;1108:259-67.
12. George J, Blank M, Hohnik M, Bar-Meir E, Koike T, Matsuura E, et al. Oxidized low-density lipoprotein (ox-LDL) but not LDL aggravates the manifestations of experimental antiphospholipid syndrome (APS). *Clin Exp Immunol* 1997;108:227-33.
13. Suciú CF, Prete M, Ruscitti P, Favoino E, Giacomelli R, Perosa F. Oxidized low density lipoproteins: the bridge between atherosclerosis and autoimmunity. Possible implications in accelerated atherosclerosis and for immune intervention in autoimmune rheumatic disorders. *Autoimmun Rev* 2018;17:366-75.
14. Laczik R, Szodoray P, Veres K, Szomják E, Csípo I, Sipka S Jr, et al. Assessment of IgG antibodies to oxidized LDL in patients with acute coronary syndrome. *Lupus* 2011;20:730-8.
15. Veres K, Lakos G, Kerényi A, Szekanez Z, Szegedi G, Shoenfeld Y, et al. Antiphospholipid antibodies in acute coronary syndrome. *Lupus* 2004;13:423-7.
16. Matsuura E, Kobayashia K, Koikeb T, Shoenfeld Y, Khamashta MA, Hughes GR. Atherogenic autoantigen: oxidized LDL complexes with beta2-glycoprotein I. *Immunobiology* 2003;207:17-22.
17. Matsuura E, Kobayashi K, Inoue K, Lopez LR, Shoenfeld Y. Oxidized ldl/beta2-glycoprotein I complexes: New aspects in atherosclerosis. *Lupus* 2005;14:736-41.
18. Greco TP, Conti-Kelly AM, Anthony JR, Greco T Jr, Doyle R, Boisen M, et al. Oxidized-LDL/beta2-glycoprotein I complexes are associated with disease severity and increased risk for adverse outcomes in patients with acute coronary syndromes. *Am J Clin Pathol* 2010;133:737-43.
19. Lopez LR, Kobayashi K, Matsunami Y, Matsuura E. Immunogenic oxidized low-density lipoprotein/beta2-glycoprotein I complexes in the diagnostic management of atherosclerosis. *Clin Rev Allergy Immunol* 2009;37:12-9.
20. Karlsson-Parra A, Söderström K, Ferm M, Ivanyi J, Kiessling R, Klareskog L. Presence of human 65 kD heat shock protein (Hsp) in inflamed joints and subcutaneous nodules of RA patients. *Scand J Immunol* 1990;31:283-8.
21. Prohászka Z, Duba J, Horváth L, Császár A, Karádi I, Szébeni A, et al. Comparative study on antibodies to human and bacterial 60 kDa heat shock proteins in a large cohort of patients with coronary heart disease and healthy subjects. *Eur J Clin Invest* 2001;31:285-92.
22. Sherer Y, Gerli R, Bocci EB, Gilburd B, Vaudo G, Bistoni O, et al. Heat-shock protein 65 autoantibodies are differently associated with early atherosclerosis in rheumatoid arthritis and in healthy subjects. *Ann N Y Acad Sci* 2007;1108:408-13.
23. Bodnár N, Szekanez Z, Prohászka Z, Kemény-Beke A, Némethné-Gyurcsik Z, Gulyás K, et al. Anti-mutated citrullinated vimentin (anti-MCV) and anti-65 kDa heat shock protein (anti-Hsp65): new biomarkers in ankylosing spondylitis. *Joint Bone Spine* 2012;79:63-6.
24. Mantej J, Polasik K, Piotrowska E, Tukaj S. Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. *Cell Stress Chaperones* 2019;24:283-7.
25. Horváth L, Czirják L, Fekete B, Jakab L, Prohászka Z, Cervenak L, et al. Levels of antibodies against cIq and 60 kDa family of heat shock proteins in the sera of patients with various autoimmune diseases. *Immunol Lett* 2001;75:103-9.
26. Szekanez Z, Haines GK, Koch AE. Differential expression of the urokinase receptor (CD87) in arthritic and normal synovial tissues. *J Clin Pathol* 1997;50:314-9.
27. Dinesh P, Rasool M. uPA/uPAR signaling in rheumatoid arthritis: shedding light on its mechanism of action. *Pharmacol Res* 2018;134:31-9.
28. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. *Nat Rev Mol Cell Biol* 2010;11:23-36.
29. Legány N, Toldi G, Distler JHW, Beyer C, Szalay B, Kovács L, et al. Increased plasma soluble urokinase plasminogen activator receptor

- levels in systemic sclerosis: possible association with microvascular abnormalities and extent of fibrosis. *Clin Chem Lab Med* 2015;53:1799-805.
30. Toldi G, Bekó G, Kádár G, Mácsai E, Kovács L, Vászrhelyi B, et al. Soluble urokinase plasminogen activator receptor (suPAR) in the assessment of inflammatory activity of rheumatoid arthritis patients in remission. *Clin Chem Lab Med* 2013;51:327-32.
 31. Vasarhelyi B, Toldi G, Balog A. The clinical value of soluble urokinase plasminogen activator receptor (suPAR) levels in autoimmune connective tissue disorders. *EJIFCC* 2016;27:122-9.
 32. Slot O, Brünner N, Locht H, Oxholm P, Stephens RW. Soluble urokinase plasminogen activator receptor in plasma of patients with inflammatory rheumatic disorders: increased concentrations in rheumatoid arthritis. *Ann Rheum Dis* 1999;58:488-92.
 33. Weber M, Hamm C. Role of B-type natriuretic peptide (BNP) and NT-proBNP in clinical routine. *Heart* 2006;92:843-9.
 34. Bosello S, De Luca G, Berardi G, Canestrari G, de Waure C, Gabrielli FA, et al. Cardiac troponin T and NT-proBNP as diagnostic and prognostic biomarkers of primary cardiac involvement and disease severity in systemic sclerosis: a prospective study. *Eur J Intern Med* 2019;60:46-53.
 35. Allanore Y, Komocsi A, Vettori S, Hachulla E, Hunzelmann N, Distler J, et al. N-terminal pro-brain natriuretic peptide is a strong predictor of mortality in systemic sclerosis. *Int J Cardiol* 2016;223:385-9.
 36. Heslinga SC, Van Sijl AM, De Boer K, Van Halm VP, Nurmohamed MT. Tumor necrosis factor blocking therapy and congestive heart failure in patients with inflammatory rheumatic disorders: a systematic review. *Curr Med Chem* 2015;22:1892-902.
 37. Peters MJL, Welsh P, McInnes IB, Wolbink G, Dijkmans BA, Sartar N, et al. Tumor necrosis factor α blockade reduces circulating N-terminal pro-brain natriuretic peptide levels in patients with active rheumatoid arthritis: results from a prospective cohort study. *Ann Rheum Dis* 2010;69:1281-5.
 38. Prohaszka Z, Duba J, Lakos G, Kiss E, Varga L, Jánoskúti L, et al. Antibodies against human heat-shock protein (hsp) 60 and mycobacterial hsp65 differ in their antigen specificity and complement-activating ability. *Int Immunol* 1999;11:1363-70.
 39. Soltész P, Dér H, Kerekes G, Szodoray P, Szücs G, Dankó K, et al. A comparative study of arterial stiffness, flow-mediated vasodilation of the brachial artery, and the thickness of the carotid artery intima-media in patients with systemic autoimmune diseases. *Clin Rheumatol* 2009;28:655-62.
 40. Baulmann J, Schillings U, Rickert S, Uen S, Düsing R, Illyes M, et al. A new oscillometric method for assessment of arterial stiffness: comparison with tonometric and piezo-electronic methods. *J Hypertens* 2008;26:523-8.
 41. Végh E, Kerekes G, Pusztai A, Hamar A, Szamosi S, Váncsa A, et al. Effects of 1-year anti-TNF- α therapy on vascular function in rheumatoid arthritis and ankylosing spondylitis. *Rheumatol Int* 2020;40:427-36.
 42. Bassi N, Zampieri S, Ghirardello A, Tonon M, Zen M, Beggio S, et al. OxLDL/beta2GPI complex and anti-oxLDL/beta2GPI in SLE: prevalence and correlates. *Autoimmunity* 2009;42:289-91.
 43. Toldi G, Szalay B, Bekó G, Kovács L, Vászrhelyi B, Balog A. Plasma soluble urokinase plasminogen activator receptor (suPAR) levels in ankylosing spondylitis. *Joint Bone Spine* 2013;80:96-8.
 44. Kolho KL, Valtonen E, Rintamaki H, Savilahti E. Soluble urokinase plasminogen activator receptor suPAR as a marker for inflammation in pediatric inflammatory bowel disease. *Scand J Gastroenterol* 2012;47:951-5.
 45. Koga T, Okada A, Kawashiri S, Kita J, Suzuki T, Nakashima Y, et al. Soluble urokinase plasminogen activator receptor as a useful biomarker to predict the response to adalimumab in patients with rheumatoid arthritis in a Japanese population. *Clin Exp Rheumatol* 2011;29:811-5.
 46. Carella DM. Brain Natriuretic Peptide: it's not about the brain or just another smart polypeptide--it's about the heart. *Neonatal Netw* 2015;34:355-9.
 47. López-Mejías R, Corrales A, Genre F, Hernández JL, Ochoa R, Blanco R, et al. Angiotensin-2 serum levels correlate with severity, early onset and cardiovascular disease in patients with rheumatoid arthritis. *Clin Exp Rheumatol* 2013;31:761-6.
 48. Gonzalez-Gay MA, Garcia-Unzueta MT, Gonzalez-Juanatey C, Miranda-Filloo JA, Vazquez-Rodriguez TR, De Matias JM, et al. Anti-TNF-alpha therapy modulates resistin in patients with rheumatoid arthritis. *Clin Exp Rheumatol* 2008;26:311-6.
 49. Genre F, López-Mejías R, Miranda-Filloo JA, Ubilla B, Carnero-López B, Palmou-Fontana N, et al. Osteoprotegerin correlates with disease activity and endothelial activation in non-diabetic ankylosing spondylitis patients undergoing TNF-alpha antagonist therapy. *Clin Exp Rheumatol* 2014;32:640-6.
 50. Balogh E, Végh E, Kerekes G, Pusztai A, Hamar A, Hodosi K, et al. Effects of one-year anti-TNF- α therapy on biomarkers of angiogenesis and functional vascular parameters in arthritides. *Rheumatol Orthop Med* 2019;4:1-8.