SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Effects of targeted therapy on arthritis comorbidities and their biomarkers

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Members of the Examination Committee:	Zsófia Kardos, MD, PhD
	Zsuzsanna Baloghné Bereczky, MD, PhD

The Examination takes place at Building B, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 30th April 2024, 11:00 am.

Head of the **Defense Committee**:	Árpád Illés, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 30th April 2024, 13:00 pm.

1. INTRODUCTION

1.1. Risk of autoimmune atherosclerosis

Rheumatoid arthritis (RA) and ankylosing spondylitis (SPA) are chronic, progressive inflammatory rheumatological diseases that cause severe structural and functional damage and can lead to a deterioration in quality of life if not properly treated and cared for. Compared to the general population, rheumatological diseases are associated with a higher risk of developing a number of comorbidities, of which cardiovascular diseases are responsible for 30-50% of deaths. The effector mechanisms of these diseases, including the production of proinflammatory cytokines (IL-1, IL-6, TNFα) in the locally affected joints, the accumulation of reactive oxygen species (ox-LDL), the altered production of nitric oxide (NO) and the presence of certain autoantibodies (ACPA, RF), can activate endothelial cells both directly and indirectly, which triggers endothelial dysfunction, an early proatherogenic process. Tumour necrosis factor-alpha (TNF-α) is a pleiotropic cytokine that promotes inflammation and plays an important role in the development of RA and SPA. TNF-alpha also has atherogenic effects such as reducing the expression of nitric oxide synthase, activating the nuclear factor-kappa B (NF-kB) signalling pathway and increasing the accumulation of reactive oxygen species, which directly impair endothelial function. Anti-TNF therapy may have a beneficial effect on cardiovascular risk, not only by reducing inflammation, but also by inhibiting the deleterious effect of TNFα on the process of atherosclerosis. In our first study, we investigated the effect of 12 months of anti-TNF on vascular biomarkers involved in the development of autoimmune-mediated vascular inflammation and oxidative stress and whose elevated concentrations have been detected in various arthritic diseases.

β2-glycoprotein I (β2-GPI) is a plasma protein that can bind directly to oxidized LDL, forming a stable and pathogenic *oxLDL/B2GPI complex.* In the presence of the anti-β2GPI antibody IgG, oxLDL uptake by macrophages and surface expression of the scavenger receptors CD36 and FcγRI are accelerated. In addition, the *oxLDL/β2GPI/anti-β2GPI complex* promotes the endothelial inflammatory response through the TLR4-mediated NF-κB signalling pathway and enhances the migratory

capacity of vascular smooth muscle cells. The presence of the complex is associated with a 3.5-fold increased risk of coronary artery disease severity and adverse outcome.

Heat shock protein 60 (HSP60) is constitutively expressed at low levels under normal physiological conditions and plays an important role in the biosynthesis of certain proteins. Its expression can be significantly increased in response to various stress effects. In addition, HSP60 is able to restore tissue homeostasis, fine-tuning inflammation through the activity of TLR4 and TLR2. At low concentrations it helps to maintain immune balance. Certain risk factors, such as free radicals and oxLDL, directly stimulate arterial wall cells, resulting in high expression of HSP60. HSP60, when released into the intracellular space, binds to TLR4/CD4 receptors, induces the expression of adhesion molecules, stimulates VSMC migration and proliferation and leads to the production of pro-inflammatory cytokines through monocyte activation. At the same time, macrophages present antigens to T and B cells, which produce autoreactive cells and autoantibodies against HSP, contributing to endothelial damage and the vascular wall inflammatory response.

The *urokinase plasminogen activator receptor (uPAR)* is a highly glycosylated polypeptide that binds to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. In terms of its function, it is capable of regulating the plasminogen activation system and facilitates cell-ECM interactions and the initiation of intracellular signalling cascades that induce cell adhesion, cell migration, differentiation, proliferation. The enzymes responsible for cleavage of uPAR are various proteases and phospholipases which, depending on the site of cleavage, generate three different forms of suPAR. Different fragments of suPAR influence the direction of signaling induced by suPAR and participate in the process of migration and adhesion of inflammatory cells in different ways, and by binding uPA, acting as a competitive inhibitor of uPAR, they are able to inhibit proteolysis mediated by uPAR and uPA. It has been shown that suPAR is a strong predictor of atherosclerosis and endothelial dysfunction and is significantly associated with the risk of coronary artery disease and ischemic stroke.

The *B-type natriuretic peptide (BNP) is* produced primarily by ventricular myocytes during ventricular myocardial failure due to volume expansion or pressure overload. Increasing its levels improves myocardial relaxation. In addition, BNP is able

to regulate the production of some key inflammatory molecules, such as the production of reactive oxygen species (ROS and RNS), and contributes to the increase in leukotriene B4 (LTB4) and prostaglandin E2 (PGE2). The resulting PGE2 effectively inhibits the release of IL-12, TNF-α by dendritic cells and is able to enhance the production of IL-10 macrophages. The action of BNP thus exerts a dual proinflammatory effect through the regulation of ROS, RNS, NO2, LTB4 production, while supporting the anti-inflammatory effect by increasing the levels of PGE2 and IL-10.

1.2 The process of angiogenesis and its relationship with chronic inflammation

The specific conditions of atherosclerosis include oxidative stress, inflammation and intimal thickening, which induce a hypoxic state in the arteries, thereby triggering increased blood vessel formation (angiogenesis) within the inflamed tissue. The emergence of angiogenic factors increases the endothelial surface area, leading to thickening of the vessel wall and migration of inflammatory cells, thus promoting plaque progression. Among the factors involved in angiogenesis, *vascular endothelial growth factor (VEGF)* is a key regulator of inflammatory angiogenesis, directly stimulating angiogenesis. *Angiopoietin-1 (Ang-1)* binds to the Tie2 receptor, inhibits endothelial apoptosis, induces new blood vessel sprouting, reduces vascular permeability. *Angiopoietin-2 (Ang-2)* acts as an antagonist of angiopoietin-1 by binding to a common receptor, inhibits vessel growth and destabilizes the endothelium. *Platelet-derived growth factor BB (PDGF-BB)* also stimulates angiogenesis, promotes smooth muscle cell proliferation and extracellular matrix (ECM) synthesis. Thrombospondin-1 *(TSP-1)* is a potent endogenous inhibitor of angiogenesis. Angiogenesis and chronic inflammation may be triggered by the same molecular events and are thus closely related. High levels of TNFα are not only involved in the development of endothelial dysfunction, but also in the process of angiogenesis, as they have a direct effect on endothelial cell migration, proliferation and the formation of new blood vessels.

1.3. Relationships between vascular and bone status in patients with arthritis

People with rheumatic diseases are at higher risk of developing a number of comorbidities, with cardiovascular and osteoporotic diseases being the most common and having the greatest impact on mortality. In addition to traditional risk factors,

ongoing chronic inflammation may be another prominent factor in the outcome of both diseases. The increased expression of adhesion molecules associated with inflammatory processes, the abundance of cells secreting proinflammatory cytokines, the process of neovascularisation and the release of collagen-degrading enzymes may all contribute to bone and cartilage erosion of joints and the instability of atherosclerotic plaques. These associations suggest that common pathological mechanisms may link the development of these diseases in arthritis. Current TNFα-inhibitor therapies may prevent systemic bone loss associated with the disease and reduce the risk of cardiovascular disease. The process of calcification may also be regulated by factors known to play a role in bone regeneration.

The serum *type I procollagen N-terminal propeptide (P1NP)* biomarker and *osteocalcin (OC) are* proteins synthesized by osteoblasts. They are traditional markers for assessing the rate of bone formation in osteoporosis. OC directly affects the process of VSMC calcification driven by Wnt.

Elevated levels of *type I collagen C-terminal telopeptide (CTX-I*) reflect increased osteoclast activity and local bone erosion. CTX levels may predict carotid artery wall thickening in the elderly population. *cathepsin K (CATHK)* is a cysteine protease responsible for osteoclast-mediated bone resorption and fibroblast-mediated cartilage destruction in unstable atherosclerotic plaques, which may accelerate the process of atherosclerosis and cause plaque instability.

The *receptor activator nuclear factor kappa B ligand (RANKL)* and its receptor RANK are essential for the formation and activation of osteoclasts. *Osteoprotegerin (OPG)* acts as a soluble decoy receptor for RANKL, protecting bone from excessive bone resorption by binding to RANKL. The RANKL/OPG ratio is a crucial determinant of bone resorption, and its ratio is important for bone mass and skeletal integrity. RANKL promotes the pathological differentiation of vascular smooth muscle cells (VSMCs) into osteoblastic phenotype cells through the expression of bone morphogenetic protein (BMP). *Dickkopf-1 (DKK-1)* is a natural inhibitor of the Wnt signalling pathway, thereby blocking bone formation and differentiation. It promotes bone resorption by enhancing the expression of macrophage colony stimulating factor, which is involved in osteoclast activity. DKK-1 may contribute to the progression of atherosclerotic lesions by promoting endothelial activation, leukocyte migration and

inflammation. Its concentration was significantly correlated with the presence of carotid plaques and coronary calcification. *Sclerostin (SOST)* is a glycoprotein with a role in the inhibition of osteoclast-mediated osteoblast bone formation and stimulation of RANKL secretion, which generates enhanced bone resorption. SOST may be an independent predictor of arterial stiffness in healthy adults and has a positive correlation with carotid artery calcification in non-dialyzed renal patients and carotid-femoral pulse wave velocity (PWV) in postmenopausal osteoporosis. Parathyroid *hormone (PTH)* produced by the parathyroid gland is a master regulator of calcium homeostasis, activates bone layer cells, reduces osteoblast apoptosis and stimulates osteoblast activity by enhancing growth factors. Several clinical studies have shown a correlation between serum PTH and the number of narrowed coronary arteries, hypertension, carotid stiffness. *Vitamin 25-OH-D3 (vitamin D3)* is a steroid hormone, plays an important role in bone and calcium metabolism. Vitamin D deficiency is associated with reduced mineral density, increased bone turnover and increased fractures. It also has an immunomodulatory effect, which inhibits the production of several pro-inflammatory cytokines, stimulates Th2 lymphocytes, regulates anagen-presenting cells, reduces B cell proliferation and differentiation. Vitamin D also has vasoprotective effects, reduces inflammatory processes, improves endothelial dysfunction and inhibits vascular smooth muscle cell proliferation.

2. OBJECTIVES

First study

In patients with RA and SPA, we investigated vascular biomarkers (oxLDL/β2GPI complex, aHsp60, suPAR, BNP fragments) that may be involved in the development of autoimmune-mediated vascular inflammation and oxidative stress.

- We determined the effect of anti-TNF therapy on serum levels of biomarkers 6 and 12 months after the start of treatment
- Correlations were sought between vascular biomarkers and disease activity (DAS28/BASDAI), markers of systemic inflammation (CRP, We), lipid parameters (TC, TG, HDL-C, LDL-C), autoantibodies (RF, ACPA) and vascular physiological ultrasound (IMT, FMD, PWV)

Second study

In the second study, we used data from two previous studies by our team. The data were based on patients with the same vascular and bone status. Our aim was to determine the relationship between bone and vascular markers and to compare pre- and post-treatment data to determine the relationship between said statuses by examining the effect of anti-TNF therapy. The statistical analyses we used were:

- Cross-sectional analysis to analyse the magnitude and direction of the linear relationship between the values.
- Univariable and multivariable regression analyses were used to assess the causal relationships between each value.
- Generalized linear model (GLM) with repeated measures analysis of variance (RM-ANOVA) - we examined the effect of two independent factors on the continuous dependent variable.
- Repeated measures multifactorial analysis of variance (MANOVA) was used to examine the effect of one or more independent variables on two or more dependent variables.

3. PATIENTS AND METHODS

3.1. The patient population studied

The patients included in the study were recruited from the specialised clinics of the University of Debrecen Clinical Centre, Rheumatology Clinic. The inclusion criteria were the diagnosis of RA patients according to ACR (American College of Rheumatology) and SPA patients according to ASAS (Assessment of Spondyloarthritis International Society) criteria. Inclusion was conditional on ineffectiveness or side effects of previous DMARD or biological therapy that warranted a change in therapy, and persistent arthritis as evidenced by the disease activity index value above DAS28>5,2 in the case of RA patients and value above BASDAI>4 in the case of SPA patients.

Exclusion criteria included the presence of untreated hypertension (>140/90 mm Hg), congestive heart failure, diabetes mellitus, inflammatory disease other than RA and SPA, infection, and kidney disease. None of the patients were receiving aspirin, clopidogrel, heparin, warfarin or vasoactive drug therapy. Patients did not suffer from primary osteoporosis before diagnosis of RA or SPA. Patients who had previously received vitamin D or calcium were no longer receiving any additional drug supplementation at least 3 months before starting therapy. Patients with hypertension who had stabilised disease at least 6 months before the start of the study were on appropriate medication with no change in anti-TNF therapy.

A total of 53 patients were included in the study, 34 women and 19 men, with a mean age of 52.0±12.1 (range: 24-83) years, a mean age at diagnosis of 43.5±12.1 (range: 23-62) years, and a mean duration of disease of 8.5±7.9 (range: 1-44) years. At baseline, RA and SPA patients had a mean DAS28 of 5.00±0.86 and a mean BASDAI of 5.79±1.19.

Among the 36 RA patients, 20 patients received etanercept (ETN) therapy (50mg/week) and

16 patients received certolizumab pegol (CZP) therapy (400 mg in weeks 0, 2 and 4 then 200 mg every 2 weeks). Among them, 18 RA patients on ETN therapy, 13 on CZP therapy in combination with methotrexate, and the remaining patients on

monotherapy received biological therapy. 17 SPA patients received ETN monotherapy at 50mg/week. 12 RA and 2 SPA patients received additional low-dose methylprednisol treatment (<6mg/day). In all cases, treatment was started after the washout period of the previous therapy and after the required screening tests. Examinations were performed before starting therapy and at 6 and 12 months after treatment. In accordance with the Declaration of Helsinki, patients gave informed consent to participate in the study by signing a consent form after being duly informed orally and in writing. The study was approved by the Hungarian Scientific Research Council Ethical Committee (approval number 14804-2/2011/EKU).

3.2. Laboratory tests

At each visit, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) values were recorded as part of routine laboratory tests during fasting blood sampling. High-sensitivity CRP (hsCRP; normal≤5mg/l) and IgM-type rheumatoid factor (RF; normal ≤50 IU/ml) levels were determined by quantitative nephelometry (Cobas Mira Plus-Roche) using CRP and RF reagents (Dialab), and serum levels of anticyclic citrullinated peptide antibody (ACPA/aCCP) were determined using a second-generation RA-immunosensitive CCP2 ELISA test (Euro Diagnostica; normal≤25 IU/ml).

3.3 Vascular pathophysiology ultrasound examination

Measurement of *branchial flow-mediated dilation (FMD) and* bilateral *arteria carotis communis intima-media thickness (IMT) was* determined using a 10 MHz linear transducer (ultrasound system: HP Sonos 5500) according to the device's instructions for use, based on international measurement methods we measured it. The FMD was expressed as a percentage of the deviation from the initial (resting) state, while the IMT values were expressed in mm.

The TensioClinic arteriograph system (Tensiomed Ltd, Budapest, Hungary) was used to measure the *pulse wave velocity (PWV).* the PWV is calculated and expressed in m/s.

3.4 Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM) software. Nominal variables between groups were evaluated using Pearson's chi-square test or Fisher's test, and the percentage distribution of variables was given. Continuous variables were compared using a two-sample t-test and Wilcoxon's test, and the values were expressed as mean±SD. Correlations were determined by Pearson's or Spearman's correlation analysis.

Univariable and multivariable regression analyses were used to model the causal relationship between the dependent and independent variables. The probability level is considered significant from $p< 0.05$. In the multivariable regression analysis, we used the stepwise method to examine which independent variables have a real effect on the dependent variable

In a general linear model (GLM) repeated measures analysis of variance (RM-ANOVA), the effect of two independent factors on the continuous dependent variable was examined. A value of $p<0.05$ is considered significant. The partial eta-squared (η^2) is weak at 0.01, medium at 0.06 and strong at 0.14.

3.5. First study

Soluble vascular biomarker level measurements

During the clinical visits, a vacuum blood collection tube (red, native, gel-free BD#367815) was taken during fasting blood sampling between 8-10 am. After coagulation for one hour at room temperature, it was centrifuged at 1200g for 20 minutes. The resulting supernatant was pipetted into Eppendorf tubes and stored at -70C° until use.

3.5.1. Detection of oxLDL/β2GPI antigen complex

The oxLDL/β2GPI complex of each sample was quantified using the Corgenix AtherOx® ELISA test kit (Corgenix, Broomfield, CO, USA) according to the manufacturer's protocol. The resulting colour intensity was determined using a spectrophotometer (ELISA-reader) at 450 nm.

3.5.2 Determination of anti-human Hsp60 immunoglobulin G (IgG) levels

IgG-type antibodies to HSP-60 family proteins were determined by ELISA. First, ELISA microtiter plates were coated with 0.1 μg/well of recombinant human HSP60 [recombinant human hsp60 (StressGen, SPP-740, Victoria, Canada)]. Binding of anti-HSP60 antibodies was determined using γ-chain specific anti-human IgG peroxidase labelled antibodies (Sigma, St. Louis, MO, USA) and o-phenylenediamine (Sigma). Optical densitometry was measured at 490nm (reference 620nm). The values obtained were then converted to the AU/ml value for this standard.

3.5.3. Measurement of soluble uPAR levels

To measure the suPAR level, the suPARnostic® Quick Triage test (Virogates A/s, Birkerød, Denmark), based on the principle of a lateral flow immunoassay, was used. The results were determined using the suPARnostic® Quick Test Reader (Virogates) which quantifies the results in the detection range 2-15 ng/ml suPAR.

3.5.4. Determination of BNP fragment level

The enzyme-linked immunosorbent assay (ELISA) method was used for the determination using the BNP fragment ELISA test kit (Biomedica, Vienna) following the manufacturer's protocol. The values of the samples on the microtiter plate were determined by ELISA reader at 450 nm (reference 630 nm).

3.6. Second study

In the second study, we analysed published results from previous studies of the same patient group using new criteria.

3.6.1. Bone density measurement by DXA and qCT

Te DXA and QCT assessments carried out in the very same cohort were performed and published previously. Dual-energy X-ray absorptiometry (DXA) was used to measure the mineral density (BMD) of the lumbar spine (L1-L4) and femur. Measurements were performed on patients using a LUNAR Prodigy (GE-Lunar Corp.,

Madison, WI, USA) densitometer by the same two experienced technicians at 3 different time points during the study. The results were expressed as g/cm^2 calculated as the T-score value relative to the mean mineral content of the bone of a young person of the same sex. Based on the obtained value, osteoporosis was defined at a T-score ≤ 2.5 SD according to the WHO established criteria. The mineral density of the volumetric bone was determined in the total area and in the cortical and trabecular regions using peripheral quantitative computed tomography (pQCT) (Stratec XCT-2000. Stratec Medizintechnik Gmb H, Pforzheim, Germany), by examining the ultradistal region of the dominant forearm of the patients.

Data analysis was performed by XCT 6.00 software (Stratec). The radius and threshold density of the measuring mask was 269 mg/mm3. BMD values were expressed as mg/mm .[3] The radius of the measuring mask was set to 269 mg/mm3 during the measurements.

3.6.2. Biomarkers of bone metabolism

During each visit, the following biomarkers were determined from samples taken during morning fasting blood sampling using the ELISA method: serum calcium (Ca; Roche Diagnostics; normal, 2.1-2.6 mmol/l); phosphate (P; Roche Diagnostics; normal, 0.8-1.45 mmol/l); parathyroid hormone (PTH; Roche Diagnostics; normal, 1.6-6.9 pmol/l); vitamin 25-OH-D3 (DiaSorin; normal, ≥ 75 nmol/l); osteocalcin (OC; Roche Diagnostics; normal, < 41 μg/l), I.-type I procollagen N-terminal propeptide (P1NP; Roche Diagnostics; normal, < 75 μg/l), type Itype I collagen C-terminal telopeptide (CTX-1; Roche Diagnostics; normal, < 0.57 μg/l), osteoprotegerin (OPG; Biomedica; median, 2.7 pmol/l), sclerostin (SOST; Biomedica; median, 24.14 pmol/l), Dickkopf-1 protein (DKK-1; Biomedica; median, 36 pmol/l), soluble receptor activator of nuclear factor kappa B ligand (sRANKL; Biomedica; median, 0.14 pmol/l), and catepsin-K (cathK; Biomedica; median, 8.7 pmol/l). These values have been published in a previous study by our group.

3.6.3. Cardiovascular biomarkers

In a previous reported study by our group, the following vascular serum biomarkers were determined by ELISA before, 6 and 12 months after the start of anti-

TNF treatment: vascular endothelial growth factor (VEGF; V-Plex, Meso Scale Diagnostics; pg/ml), platelet-derived growth factor (PDGF-BB; DuoSet ELISA, R&D Systems; pg/ml), angiopoietin-1 (Ang-1; DuoSet ELISA, R&D Systems; pg/ml), angiopoietin-2 (Ang-2; QuantiKine ELISA, R&D Systems; pg/ml) and thrombospondin-1 (TSP-1; DuoSet ELISA, R&D Systems; ng/ml).

3.6.4. Statistical analysis

In repeated measures multifactorial analysis of variance (MANOVA), the dependent variables were the correlated bone and vascular biomarkers. As in RM-ANOVA, the F-ratio (F) and significance value (p), indicate the effect of the independent variables on the dependent variable. A value of $p<0.05$ is considered significant. The partial eta-square (η^2) is weak at 0.01, medium at 0.06 and strong at 0.14.

4. RESULTS

4.1. First study

The 53 patients included in the study (36 RA, 17 SPA) received 12 months of etanercept (ETN)/certolizumab pegol (CZP) therapy. Assessments were performed before initiation of therapy and at months 6 and 12 post-treatment. TNFα inhibitor therapy significantly reduced disease activity in both patient groups. In RA patients (n=36), ETN and CZP therapy resulted in a significant reduction in activity compared to baseline DAS28 (5.00±0.86) at month 6 (3.13±0.84; $p<0.001$) and month 12 (3.02±0.96; $p<0.001$). The baseline BASDAI (5.79±1.19) of SPA patients (n=17) also significantly decreased after 6 months (2.00±1.03; $p<0.001$) and 12 months (1.86±1.04; $p<0.001$) of treatment.

4.1.1. Relationships between vascular biomarkers and other parameters

The values of vascular biomarkers measured at 2 time points (months 0,12) were compared with the values of lipid parameters (TC, TG, LDL-C, HDL-C), autoantibodies (ACPA, RF), inflammatory parameters (CRP, We), imaging parameters (FMD, IMT, PWV) and the patients' medical history at the time of inclusion by Pearson correlation analysis. Based on these lipid parameters, for values before treatment initiation, the oxLDL/β2GPI complex showed a strong correlation with total cholesterol (TC-0) ($R=0.532$; $p<0.001$) and LDL cholesterol (LDL-C-0) ($R=0.648$; $p<0.001$). The levels of aHSP60, suPAR and BNP measured before the initiation of therapy also showed a strong correlation with initial triglyceride (TG-0) ($R=0.462$, $p=0.040$; $R=0.382$, $p=0.028$; $R=0.303$, $p=0.041$). In the analysis of RA specific autoantibodies, we find a correlation in two cases. Firstly, the initial value of suPAR correlated with the initial values of ACPA ($R=0.613$; $p<0.001$) and RF ($R=0.413$; $p=0.024$), and the initial level of BNP showed significance between the initial levels of ACPA ($R=0.591$; $p<0.001$) and RF ($R=0.479$; $p=0.004$). Furthermore, BNP levels after 12 months correlated with patients' age at diagnoses ($R=0.330$; $p=0.023$) the initial CRP ($R=0.372$; $p=0.010$) and 12-month CRP levels ($R=0.356$; $p=0.014$). Among the imaging parameters, the initial aHSP60 value correlated with the values of FMD ($R=-0.380$, $p=0.022$) values after12 months and initial PWV ($R=0.564$, $p=0.040$). The initial suPAR and suPAR at month 12 were significantly

correlated with PWV-12 (R=0.390, p=0.045; R=0.393, p=0.042), while the initial BNP level was significantly correlated with IMT (R=0.375, p=0.016) before starting therapy. Finally, we also correlated the individual vascular biomarkers. As a result, a positive correlation was found in one case, between initial BNP levels (BNP-0) and initial suPAR levels (suPAR-0) (R=0.427, p=0.013). None of the vascular biomarkers correlated with disease activity (DAS28, BASDAI) and erythrocyte sedimentation rate (We).

In the univariable regression analysis, the initial value of suPAR (suPAR-0) was correlated with the initial value of triglyceride (TG-0), while the value of suPAR at month 12 (suPAR-12) was correlated with the value of CRP at month 12 (CRP-12). The initial value of BNP (BNP-0) was correlated with the initial value of triglyceride (TG-0), while the value of BNP at month 12 was correlated wih age at onset, initial CRP (CRP-0) and CRP at month 12 (CRP-12) ($p<0.05$). No significant effect of independent variables was detected for oxLDL/β2GPI complex and HSP60.

Multivariable regression analysis revealed significance in only one case, between the 12-month BNP value (BNP-12) and the initial CRP value (CRP-0) (p=0.028).

In the general linear model repeated measures analysis of variance (GLM RM-ANOVA) significance ($p<0.05$) was tested for the effect of two independent factors simultaneously on the continuous dependent variable. The change of oxLDL/β2GPI 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). And the change in aHSP60 (aHSP60 0-12) and suPAR (suPAR 0-12) levels during one year of treatment was determined by the treatment and IMT initial value (IMT-0). The partial root mean square (partial $\eta^{2)}$) was above 0.14 in all three cases, demonstrating a strong effect between the variables.

4.1.2. Effect of TNFα inhibitors on vascular biomarkers

In the combined patient group (RA+SPA), the baseline oxLDL/β2GPI circulating level (0.24±0.10 U/ml) was significantly reduced after 12 months of anti-TNF therapy (0.20±0.11 U/ml; p=0.014). Anti-HSP60 antibody levels were unchanged at 6 months (158.6±138.6 AU/ml) and 12 months (167.3±143.3 AU/ml) after treatment compared to baseline (170.3±140.4 AU/ml). The initial level of suPAR

(11.5±16.4 ng/ml) did not change significantly at 6 months (11.3±17.7 ng/ml) and 12 months (10.3±15.3 ng/ml) after treatment, nor did the initial value of BNP fragments (530.8±441.8 pmol/l) show significant changes at 6 months (518.2±422.4 pmol/l) and 12 months (484.1±418.2 pmol/l) after treatment.

In further analysis of each biomarker, for the suPAR level, patients were divided into 4 different groups according to the reference ranges for the serum samples set by the manufacturer. Based on these, for initial suPAR levels, 21.1% of patients were classified as low (<4 ng/ml) (11 patients), 36.4% as adequate (4-5.5 ng/ml) (20 patients), 9.1% as high (5.5-9 ng/ml) (5 patients), and 33.3% as critical (>9 ng/ml) (17 patients). Looking at the categories individually, RA patients with an initial suPAR of critical showed a significant decrease over the one-year treatment (>9 ng/ml) (p=0.04).

The serum BNP level, in the patient group as a whole, did not change significantly after one year of anti-TNF treatment. However, when comparing seropositivity, we found the following correlations in both initial and post-treatment values. In ACPA positive patients, compared to ACPA negative patients, a significantly increased BNP level was observed (initial value: 670.6±323.0 versus 138.0±436.4 pmol/l; p=0.030 and the 12th month: 652.9±283.2 versus 456.5±423.1 pmol/l; p=0.021) for both time points. The same correlation was also demonstrated in RF positive patients, where the BNP level was significantly higher compared to RF negative patients (initial value: 680.6±381.6 versus 292.9±198.3 pmol/l; p=0.007 and the month 12: 668.9±346.5 vs. 312.2±207.0 pmol/l; p= 0.001) at baseline and after one year of treatment.

4.2. Second study

The vascular and bone metabolism, imaging and laboratory parameters of the same patient group included in the first study, have already been published as a result of previous work by our group. In the present study, we reanalysed these data from a new perspective, focusing on the assessment of the relationships between bone and vascular status.

4.2.1. Relationships between vascular, bone metabolism biomarkers and imaging parameters

When comparing the results of vascular and bone imaging parameters, the following correlations were found. The value of IMT was variably and inversely correlated with total and trabecular BMD as determined by QCT measurements at different time points ($p<0.05$). Similarly, PWV measured at 12 months inversely correlated with DXA femoral neck BMD, as well as with QCT total BMD.

For vascular imaging and bone laboratory biomarkers, FMD and osteoprotegerin (OPG) showed a positive correlation at different time points. For IMT, a positive correlation was found with sclerostin (SOST), while an inverse correlation was found for osteocalcin (OC), type I procollagen N-terminal polypeptide (P1NP) and vitamin D3 (VITD3) ($p<0.05$).

Vascular laboratory biomarkers were associated with the following bone imaging and laboratory parameters. Platelet-derived growth factor BB (PDGFBB) was positively correlated with DXA femoral neck BMD at different time points. Angiopoietin 1 (Ang1), angiopoietin 2 (Ang2) and PDGFBB showed positive correlation with dickkopf-1 protein (DKK-1). In addition, Ang2 showed positive correlation with OPG levels and inversely correlation with receptor activator nuclear factor kappa ligand (RANKL) levels. Thrombospondin-1 (TSP1) correlated with OPG and SOST levels, while BNP correlated with C-terminal telopeptide (CTX) and OC.

Univariable and multivariable regression analyses were used to model the causal relationship between vascular and bone status. These showed a positive correlation between baseline FMD and baseline OPG ($p=0.026$), a positive correlation between IMT and SOST at month 12 ($p=0.014$), and an inverse correlation between IMT at month 6 and baseline OC ($p=0.032$). For PWV, a determinant independent variable was confirmed at all three time points. The initial PWV was correlated with the initial VITD3 ($p=0.042$), the PWV measured at month 6 was correlated with parathyroid hormone (PTH6) measured at the same time point ($p=0.047$) while the PWV measured at month 12 was correlated with the initial cathepsin (CATHK-0) ($p=0.013$). In addition, initial QCT trabecular BMD was inversely associated with initial ($p=0.023$) and 6-

month IMT (p=0.002), while QCT total BMD was also inversely associated with IMT at month 12 (p=0.011).

On the other hand, when we analysed the determinants of bone status according to vascular parameters, both univariable and multivariable analyses indicated that PDGFBB was positively associated with DXA femoral BMD (p=0.036), while IMT was inversely correlated with QCT total BMD (p<0.001).

In the general linear model repeated measures analysis of variance (GLM RM-ANOVA), only IMT as the dependent variable yielded results. In this regard, the anti-TNF therapy has a combined effect with initial osteocalcin for the change in IMT levels at 12 months (IMT 0-6-12, p=0.045) and between months 6 and 12 (IMT 6-12, p=0.038). It also has an effect with P1NP with respect to the change in the IMT in the total duration (0-6-12, p=0.036) and between 0 to 6 months (IMT 0-6, p=0.024), and with vitamin D3 level for the change of IMT between 6 and 12 months (IMT 6-12, p=0.041). All three of these cases showed an inverse relationship.

The MANOVA method was used to evaluate the determinants of two correlated vascular and bone marker covariates. Our results showed that baseline disease activity (DAS28/BASDAI) had a significant effect on the correlations between IMT and QCT trabecular BMD at month 12 (p=0.028), PWV and QCT trabecular BMD at month 12 (p=0.042) and an inverse correlation between Ang2 and RANKL at month 6. The positive correlation between OC and BNP at month 12 was significantly affected by initial CRP (p=0.031). We also assessed the effects of ani-TNF treatment-related changes of disease activity between baseline and 12 months on various biomarkers. We found that DAS28 (0-12) or BASDAI (0-12) between baseline and 12 months had a significant effect on the positive correlations between IMT and sclerosin (p=0.021), as well as PWV and catepsin K (p=0.021).

4.2.2 Effect of TNF inhibitors on the correlation of vascular and bone biomarkers

Finally, we investigated the effect of anti-TNF therapy on inflammatory vascular and bone interactions. To this end, we compared the patterns of correlations between vascular and bone biomarkers measured at baseline and after 12 months. In univariable and multivariable analyses, associations between osteoprotegerin and FMD, vitamin D3 and PWV or PDGFBB and DXA femoral BMD were observed at baseline. These

correlations were not detectable after 12 months of treatment. In contrast, after one year of treatment, compared to baseline values, the following correlations were found. Significant correlations were found between osteocalcin, CTX, P1NP and IMT and between cathepsin K (CATHK) and PWV at 12 months, and for imaging parameters, between IMT or PWV and total BMD of QCT after one year of treatment.

In many cases, we find correlations where some baseline vascular biomarker was able to determine bone status after one year of therapy, and the same was true backwards. In the MANOVA analysis, changes in disease activity or disease activity and baseline CRP values determined more correlations between vascular and bone biomarkers in patients treated after 12 months.

5. DISCUSSION

The aim of **our first study** was to investigate vascular biomarkers that are associated with inflammatory processes and play a role in endothelial dysfunction and plaque formation, where elevated levels of these biomarkers are confirmed in some arthritic diseases. Another aim was to investigate the effect of anti-TNF therapy on serum levels of biomarkers 6 and 12 months after the start of treatment. In addition, we looked for correlations between markers of systemic inflammation, disease activity indices, lipid parameters, autoantibodies, and vascular imaging scores in RA and SPA patients. Our group has previously published results showing changes in IMT, FMD, and PWV ultrasound scan values in response to TNFα-inhibitor treatment. Elevated levels of the oxLDL/β2GPI complex have been shown in antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and rheumatoid arthritis (RA). In the present study, we investigated the changes in oxLDL/β2GPI complex levels after one year of anti-TNF therapy in RA and SPA patients. Our results showed that 12 months of treatment significantly reduced serum levels of the complex in the whole patient group. This is the first study to investigate the change in oxLDL/β2GPI complex in response to anti-TNF therapy in RA and SPA patients. However, several studies have demonstrated that the complex is not only higher in certain rheumatic diseases, but that elevated levels are associated with an increased risk of developing acute coronary syndromes and other vascular lesions. The association examined in the present study suggests that a significant reduction in the biomarker is likely to be associated with a reduction in the risk of certain cardiovascular complications. To investigate this further, we plan to conduct a longer-term follow-up. In addition, we found correlations between the initial values of the complex and the initial values of some lipids (TC, LDL). Although no correlations were found between inflammatory parameters, disease activity and imaging values, the results of statistical analysis (RM-ANOVA) suggest that the 12-month change in oxLDL/β2GPI complex after 1 year of treatment is determined by the initial values of disease activity (DAS28/BASDAI). Consequently, the change in oxLDL/β2GPI complex with anti-TNF therapy over a 1-year time course can be predicted from the initial disease activity value of each patient.

Elevated levels of anti-HSP (aHSP) antibodies in autoimmune diseases have been demonstrated in several studies. Higher levels of antibodies are a predictor of worse outcome in atherosclerosis, but in RA and SPA patients no association with disease activity or progression has been demonstrated to date. In the present study, we investigated the effect of one year of anti-TNF inhibitor treatment on anti-HSP60 levels. To our knowledge, there have been no previous studies that have investigated the change with biological therapy in RA or SPA patients. Although our present results suggest that biological therapy did not significantly change aHSP60 levels after 12 months of treatment, the change in initial anti-HSP60 (aHSP60) levels over one year of treatment was able to determine the effect of treatment and initial IMT. No correlation was found between initial aHSP60 levels and initial disease activity index values (DAS28/BASDAI), initial CRP or We. These results are consistent with previous studies that aHSP60 antibody levels do not reflect inflammatory status in autoimmune rheumatology patients. However, initial levels correlated with initial PWV, FMD and triglyceride levels which reflect endothelial cell abnormalities.

The activation of the immune system and the development of an inflammatory response leads to increased levels of soluble urokinase activator receptor (suPAR), which has been described as a valuable indicator in a number of inflammatory rheumatological diseases. In the present study, we compared initial suPAR levels with initial disease activity index values (DAS28/BASDAI) and initial CRP or We values and found no correlation between suPAR levels and the degree of initial inflammation. In patients with seropositive rheumatoid arthritis (RA), a correlation between the suPAR value and the RF and ACPA values was detectable before the initiation of therapy, suggesting a role for suPAR as a prognostic biomarker. Few studies have investigated the effect of ani-TNF therapies on suPAR. In our case, anti-TNF therapy did not reduce suPAR levels at 12 months of treatment when the entire patient population was studied. However, after categorising patients according to their suPAR level, we observed a significant reduction in patients with critically high baseline suPAR levels. Correlation and regression analysis showed that there was a correlation between suPAR and CRP values measured at 12 months, suggesting that in cases where CRP remained high despite one year of therapy, suPAR values also remained high. SuPAR is receiving increasing attention as a promising new biomarker for

cardiovascular disease, with elevated levels associated with the risk of coronary heart disease and ischaemic stroke. Our study found a correlation between initial BNP and initial suPAR levels, an important biomarker of congestive heart failure. Additionally, we found that the initial carotid intima-media thickness (cIMT) was able to determine the 12-month change in suPAR with treatment effect.

B-type natriuretic peptide (BNP) and N-terminal pro B-type natriuretic peptide (NT-proBNP) are prognostic biomarkers that are routinely used in the diagnosis of heart failure, coronary artery disease and aortic valve stenosis. Higher NT-proBNP levels have been detected in some autoimmune diseases. In case of RA disease, higher BNP levels have been observed in active patients than in moderately active or well-controlled patients. Some studies have shown that TNF inhibitors were effective in reducing NT-proBNP levels in RA and SPA patients, while others have not observed significant changes in response to the TNF therapy. In our own study, we found that anti-TNF therapy did not show a significant change in BNP levels for the whole group. Our results do not support that these targeted therapies are protective against heart failure. However, we did find higher BNP levels in seropositive patients compared to seronegative patients. The relationship between NT-proBNP and CRP has been studied in a number of cases. In the present study, correlation and regression analysis showed an association between initial BNP level and initial and 12th month CRP levels. The results of multiple regression analysis suggest that the degree of initial inflammation may predict how BNP levels will change after one year of treatment. Several studies have investigated the relationship between natriuretic peptides and atherosclerosis. In the present study, we find significant associations between the patients' age, the initial BNP levels, the triglyceride levels of the lipid parameters and the IMT of the vascular imaging parameters. These results raise the possibility that the relationship between BNP and acute phase markers may be direct through the association between atherosclerosis and inflammation.

In the **second study,** we used data from two previous studies of our group, which included vascular and bone status results from the same group of patients at the same time point, analysed according to new criteria. Our aim was to determine the associations between bone and vascular markers and to compare pre- and post-treatment

data to determine the relationship between bone and vascular status by examining the effect of anti-TNF therapy.

The higher incidence of cardiovascular and bone disease in arthritis is associated with chronic systemic inflammatory processes. Based on these observations, several studies have investigated the impact of targeted therapies in RA and SPA on specific vascular and bone biomarkers. However, very few follow-up studies have examined their combined changes in these markers in the context of a follow-up study. In RA and SPA, elevated levels of pro-inflammatory molecules such as CRP and pro-inflammatory cytokines (IL-6, IL-1, TNFα) play a key role in the pathogenesis of cardiovascular and osteoporotic diseases. TNFα is able to affect bone structure and remodelling, and has a negative effect on bone health by stimulating osteoclast formation. In the pathology of atherosclerosis, elevated levels of TNFα contribute to abnormal endothelial activation, vascular smooth muscle cell migration and proliferation, and cholesterol accumulation in the vasculature, leading to the growth of atherosclerotic plaques. Based on these findings, targeted therapies for RA and SPA, such as the TNFα inhibitor etanercept and certolizumab pegol, may have a beneficial effect on the development of these comorbidities by reducing chronic inflammation.

In our statistical analysis, the most relevant significant correlations, which showed an inverse correlation with each other, were between PWV and femoral neck BMD parameters, and between IMT and QCT measured at different time points. These results suggest that persistent systemic inflammation affects both bone and blood vessels, and that one year of therapy may result in correlated changes not only in rapidly changing inflammatory markers but also in structural parameters. The positive correlation between FMD and osteoprotegerin (OPG) was also confirmed by our correlation and regression analysis results. In addition, PWV and IMT showed correlations with several bone markers. Additional correlations were found between angiogenic and bone biomarkers, for example, between platelet-derived growth factor BB (PDGF-BB) and femoral neck BMD.

Regression analysis was used to examine the causal relationships between each imaging and laboratory marker. The relationship correlation between baseline FMD and OPG values revealed by correlation analysis was also confirmed by our regression

analysis. Osteoprotegerin, which is known to play an important role in inhibiting osteoclastogenesis in bone by binding to RANKL, was also associated with the degree of vascular calcification High levels of osteoprotegerin may predict a higher vascular risk in RA and SPA.

A positive correlation was found between sclerostin (SOST) and IMT values at month 12. Recent studies have shown that under arteriosclerotic conditions, vascular smooth muscle cells may undergo a phenotypic transition towards osteoblasts that are capable of expressing certain bone markers such as sclerostin. Based on the results of our MANOVA analysis, the change in the one-year level of disease activity was able to influence the relationship between IMT and sclerostin at 12 months. This suggests that inflammation may play a key role in the correlation between the two markers.

We investigated the relationship between markers of angiogenesis and changes in bone tissue. We found a positive correlation between initial PDGF-BB levels and DXA femoral neck BMD at 12 months. PDGF-BB plays an important role in promoting angiogenesis during atherosclerosis and is also known to be a regulatory factor in bone tissue repair and regeneration.

Our regression analysis revealed additional correlations between bone and vascular imaging parameters. We found that qCT trabecular BMD at baseline and total BMD at 12 months were inversely correlated with baseline and 12-month IMT, respectively. In addition, 12-month IMT was inversely correlated with qCT total BMD at 12 months. These results provide further evidence that there is a strong association between inflammatory bone loss and the development of atherosclerosis.

Among the vascular imaging parameters, PWV is correlated with artery narrowing by plaque. In our study, the PWV measured at month 12 determined the initial cathepsin K (CATHK) value. Cathepsin K is highly expressed in unstable plaques and its production is closely associated with inflammatory processes within the vascular wall. This relationship was confirmed by our MANOVA analysis, in which the one-year change in disease activity indices (DAS28/BASDAI) values influenced the relationship between PWV and CATHK measured at month 12.

Vitamin D3 regulates a wide range of physiological and pathological processes and plays an important role through its vascular protective effect from the early

endothelial activation stage to plaque formation. Low levels are associated with an increased risk of coronary calcification. Our study confirmed a significant inverse causal relationship between initial PWV and vitamin D3.

In RM-ANOVA analysis, we found that one year of biological treatment could predict changes in IMT over the course of therapy, which was achieved by combining baseline levels of different bone biomarkers (OC, P1NP, VITD3). The correlation was always inverse. Low levels of OC, P1NP and VITD3 are known to predict decreased bone turnover and increased fracture incidence. These observations provide further evidence that reduced bone mineral density is associated with increased incidence of atherosclerotic vascular abnormalities.

The MANOVA analysis revealed that disease activity or systemic inflammation (CRP) and their anti-TNF inhibitor associated changes between baseline and after one year of treatment may influence the relationship between certain bone and vascular biomarkers. Our results show that initial DAS28/BASDAI values inversely influenced the associations between IMT, PWV measured at 12 months and QCT trabecular BMD for some bone and vascular imaging parameters. It also inversely influenced the relationship between angiopoietin 2, which is involved in the process of angiogenesis, and bone marker RANKL at 6 months. Changes in disease activity indices at 1 year influenced the relationship between IMT and sclerostin and PWV and cathepsin K measured at 12 months as described previously. Initial levels of CRP influenced the association between osteocalcin, a marker of bone formation, and BNP, a prognostic marker of cardiovascular disease, at month 12. Based on these associations, one year of therapy may have a significant impact on vascular and bone interactions.

Our results showed that in for RA and SPA patients treated with TNF inhibitors, we found a number of bone markers were able to predict the vascular status after 12 months of treatment, and vice versa. In addition, systemic inflammation and arthritic disease activity were also able to influence the relationship between vascular and bone markers. Based on some of the effects described in the RA-ANOVA and MANOVA analyses, the initial high inflammatory status may produce a number of relationships between vascular and bone markers. These can be more frequently observed at baseline than after one year of treatment. One explanation for this may be that after one year of

therapy, the level of the basic inflammatory process decreases, which is associated with a change in the activity of the molecules and cells involved in the inflammation, which affects the effect of these inflammatory agents on the vascular and skeletal system as well.

In addition to their many strengths, both tests have some limitations when testing RA and SPA patients. Due to the small number of patients, in most cases a mixed cohort group had to be used for the evaluations, which meant that no comparison between RA and SPA patient groups was possible. Similarly, we were not able to compare the efficacy of etanercept and certolizumab pegol biologic therapies due to the low number of patients. The study also lacked a control group, which caused additional difficulties in interpreting the results. In addition, most of the markers tested were not clinically validated, which made it difficult to assess the relevance of the results to everyday practice.

6. SUMMARY

Rheumatological diseases are associated with a higher risk of developing a number of co-morbidities. Current TNFα inhibitor therapies may help prevent systemic bone loss associated with the disease and reduce the risk of developing cardiovascular disease.

In the **first study,** we investigated the effect of one year of etanercept/certolizumab pegol therapies for RA and SPA diseases on soluble vascular biomarkers (oxLDL/β2GPI complex, suPAR, aHSP60, BNP fragments), whose elevated levels are associated with the development of inflammation-related atherosclerosis.This is the first study to investigate the long-term changes of these biomarkers in response to anti-TNF therapy in these diseases.

Our results are summarised below:

1. A significant reduction in oxLDL/β2GPI complex levels was observed in the whole group of RA and SPA patients after one year of anti-TNF therapy. In addition, a similar effect was observed in patients with critically high suPAR levels. The resulting reduction is likely to lead to a reduction in the risk of certain cardiovascular complications.
2. RF and ACPA seropositivity were associated with higher suPAR and BNP levels, which may predict a worse prognosis.
3. We confirmed the association of certain biomarkers with pathophysiological changes in the vasculature. According to these results, all 4 biomarkers showed correlation with one of the baseline lipid parameters, and vascular ultrasound examination showed correlation with several of the biomarkers we investigated.
4. Based on the results of the regression analysis, CRP and triglyceride may be important predictors of BNP and suPAR levels 12 months before or 12 months after treatment.
5. Based on the results of analysis of variance, disease activity or IMT may significantly influence the effects of anti-TNF treatment on changes in oxLDL/β2GPI complex, aHsp60 and suPAR.

In the **second study,** we used the results of two previous studies of our working group. Both of them evaluated the vascular[196,197] and bone biomarkers[198,199] in a one-year anti-TNF therapy of RA and SPA patients. This is the first study to simultaneously assess vascular and bone status in patients with arthritis treated with biological agents. Several vascular and bone imaging and laboratory parameters were evaluated to better understand possible interactions.

Our results are summarised below:

1. In a correlation analysis of the relationship between bone and vascular parameters, we observed significant inverse correlations between IMT and qCT parameters and PWV and femoral neck BMD at different time points. Crucially, we observed correlations not only between inflammatory biomarkers but also between structural markers.
2. We showed a positive correlation between FMD and OPG. Our results show that the association between OPG and FMD persists before and after anti-TNF therapy, further demonstrating the role of OPG as a biomarker in determining vascular risk and prognosis.
3. IMT and PWV were associated with a number of bone markers. Among the vascular biomarkers, PDGF-BB showed a positive correlation with femoral neck BMD, and several angiogenic markers were associated with several bone biomarkers.
4. Our regression analysis found a number of bone markers that were able to predict vascular status after 12 months of treatment, and the reverse was also true. In addition, we found a correlation between femoral BMD assessed by DXA and PDGF-BB, as well as between some BMD values assessed by qCT and IMT.
5. Based on the results of our RM-ANOVA analysis, the one-year biological treatment combined with baseline levels of different bone biomarkers (OC, P1NP, VITD3) was able to predict changes in IMT during therapy. The analysis confirmed that initial reduced bone density may be associated with an increased incidence of vascular abnormalities.

6. The MANOVA analysis revealed that disease activity (DAS28/BASDAI) or systemic inflammation (CRP) and their changes associated with anti-TNF inhibitor between baseline and after one year of treatment may influence the relationship between certain bone and vascular biomarkers. This suggests that arthritis increases both cardiovascular and osteoporotic disease early in the course of the disease.

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List of publications related to the dissertation

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