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

Examination of bone metabolism in systemic sclerosis and inflammatory rheumatic diseases

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The Examination takes place at Building B, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 19th April 2023, 9 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, 19th April 2023, 10:30 am

1 Introduction

Inflammatory rheumatological diseases and systemic autoimmune diseases are well recognized risk factors for low BMD resulting in a greater risk of fragility fractures. The prevalence of osteoporosis (OP) in these pathologies is constantly increasing as rheumatological patients age and have better life prospects. In addition to immobility as a result of inflammation and active disease, other metabolic factors are also involved in bone loss.Corticosteroid (CS) therapy, which is often used during treatment, may also contribute to the development of osteoporosis.

1.1 Osteoporosis

OP is associated with an increased risk of bone fractures due to the microarchitectural deterioration of bone tissue and a marked gradual loss of structural integrity. It has been called as "silent epidemic" as it is a growing problem and many patients are asymptomatic. Bone loss is a result of an imbalance between bone-destroying osteoclasts and bone-forming osteoblasts. Bone is characterized by continuous remodeling to adapt the structure to variate mechanical needs, to repair micro-injuries, and to prevent the accumulation of aged bone stock. In addition to hormonal changes, the remodeling process is influenced by many other factors, in which the main regulators are the RANKL (receptor activator of nuclear factor-kappa β ligand) / OPG (osteoprotegerin) and Wnt (Wingless) pathways. RANKL is a cytokine belonging to the tumor necrosis factor (TNF) family, which regulates the activity of osteoclasts with its natural "trap receptor", OPG. RANKL is essential for osteoclast maturation and development. In contrast, the Wnt/β-catenin system regulates osteoblast differentiation by activating the transcription of osteoblast-specific genes. The Wnt/β-catenin signaling pathway not only enhances bone formation, but also inhibits bone resorption by blocking osteoblast apoptosis and increasing OPG expression. Dickkopf-related protein 1 (DKK1) and sclerostin (SOST) are natural

inhibitors of the Wnt signaling pathway, influencing the differentiation and function of osteoblasts. DKK1 increases the expression of macrophage colony-stimulating factor (GM-CSF) and RANKL, enhances RANKL-RANK interaction and decreases OPG expression. In addition, elevated levels of DKK1 and sclerostin correlate with the activity of osteoclasts, and there is a direct interaction between DKK1 and sclerostin, in animal models inhibition of DKK1 also reduces sclerostin production.

1.2 Inflammation and bone

During synovial inflammation, many proinflammatory cytokines are released, such as TNF-α, IL-1, IL-6, IL-17, which on the one hand, directly regulate the differentiation of osteoclasts and bone resorption and, on the other hand, indirectly increase the expression of RANKL on mesenchymal cells. A low OPG/RANKL ratio has been shown to correlate with radiological progression of joint destruction in RA. It is also known that TNF-α reduces bone formation by inducing DKK1 and sclerostin production. Based on the results in clinical trials, TNF-α inhibitors hamper bone destruction and reduce radiological progression in RA. In addition to local effects on bone, this treatment also affects generalized bone loss in patients treated with RA and ankylosing spondylitis (AS). In case of AS there is much controversy about the effect of TNF-α inhibitors on bone formation, probably due to the fact that SOST exerts different effects in inflammatory and non-inflammatory environments.

1.3 The role of vitamin D

The role of vitamin D in bone homeostasis has been known for a long time, but in recent years its immunomodulatory role in autoimmune and inflammatory rheumatic diseases has also been published. Receptor of vitamin D is found on immune cells (T- and B-lymphocytes, antigen-presenting cells), and these immune cells are also capable of synthesizing active vitamin D, that can regulate both the natural and the adaptive immune response in this specific

immunological environment. It inhibits B-cell proliferation, differentiation and immunoglobulin secretion. It also inhibits T-cell proliferation, shifting the balance towards the Th2 phenotype. By influencing the maturation of T-cells, it reduces the formation of Th17 cells, induces the formation of regulatory T (Treg) cells, thus reducing the level of proinflammatory cytokines (IL-17, 21) and increasing the level of anti-inflammatory cytokines (e.g. IL-10). It inhibits the differentiation and maturation of dendritic cells (DC), and by preserving immature DCs, it plays an important role in maintaining immune tolerance. In addition, its antifibrotic effect has been proven in animal experiments.

Several clinical studies have shown low vitamin D concentrations in patients with RA, systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), and non-differentiated collagenosis (NDC). Scleroderma cohort studies also reported low vitamin D levels among patients.

1.4 Systemic sclerosis

SSc is a rare, clinically heterogeneous connective tissue disease characterized by fibrosis of the skin and internal organs.

Three main factors play a role in its pathogenesis: microvascular damage, fibrosis of the skin and internal organs with increased collagen deposition, and pathological functioning of the immune system, characterized by T-cell activation and autoantibody production.

The manifestations of the disease are heterogeneous. The disease usually begins with Raynaud's syndrome. Clinically - based on the extent of the skin symptoms, internal organ involvement and the autoantibodies - we can distinguish two main subgroups. In patients belonging to the limited cutaneous (lcSSc) group, Raynaud's syndrome typically precedes the appearance of other skin and internal organ symptoms by years, and the association with anticentromere antibodies (ACA) is more common. In patients suffering from the diffuse cutaneous form (dcSSc), the characteristic symptoms of the disease develop soon after the first

episode of the Raynaud's syndrome, which are more severe and progressive than in the previous group. Regarding autoantibodies, the anti-topoisomerase I antibody (anti-Scl-70) and the anti-RNA polymerase III autoantibody appear in this group more frequently.

The main symptoms are gastrointestinal (GI), cardiac, pulmonary and renal manifestations, of which interstitial lung disease (ILD) and pulmonary arterial hypertension (PAH) are the most common causes of the mortality of the disease. At the same time, we also have to payattention to musculoskeletal symptoms and OP during the course of the disease, which might significantly affect the patients' quality of life. Based on literature data, the prevalence of OP can be estimated between 3 and 51% among patients with scleroderma. In addition to classic OP risk factors, SSc-specific factors also play a role in the development of osteoporosis, such as chronic inflammation, early menopause as a complication of cyclophosphamide therapy, which has been widely used in recent decades, immobilization due to flexion contractures, soft tissue calcification, skin alteration, renal failure, gastrointestinal malabsorption. In addition, hypothyroidism, often associated with the disease, may also affect bone mass.

1.5 Rheumatoid arthritis

RA is a chronic, autoimmune disease characterized by symmetrical synovial inflammation of the small joints. The prevalence of RA can be estimated at 0.5-1% in the population, and it is characterized by a female predominance. Due to the inflammatory nature of the disease, without treatment - through damage to the joints - it leads to severe functional impairment, reduced quality of life and life expectancy. The disease is not limited to the joints, since in almost 40% of patients we have to reckon with extra-articular symptoms, e.g. with the appearance of rheumatoid nodules, vasculitis, scleritis, pulmonary fibrosis, pericarditis. RA is often accompanied by comorbidities, cardiovascular abnormalities, early atherosclerosis, malignant tumors, infections, and often OP can be observed in addition to inflammation of the joints.

The exact etiology of RA is unknown, but both environmental and genetic factors play a role in its development. The disease is associated with synovial inflammation, hyperplasia, production of autoantibodies (rheumatoid factor [RF] and anti-citrullinated protein antibody [ACPA]), cartilage and bone destruction, and systemic symptoms. In the pathogenesis of the disease, the role of TNF-α, IL-1 and IL-6 cytokines can be emphasized, which exert their effect on inflammatory cells and osteoclasts and cause synovitis and erosive changes.

Local bone loss (periarticular osteopenia) accompanied by reduction of bone trabeculae and bone marrow edema is a characteristic symptom of early RA. It develops as a consequence of the effect of inflammatory cytokines (IL-6, TNF-α) in the area of the joints and can be detected more often in ACPA-positive patients. In addition to localized bone loss, generalized OP is a frequent extra-articular manifestation of the disease, especially in case of long-standing and poorly controlled disease. The prevalence of OP in RA can be as high as 30%, and its incidence can be twice as high compared to the average population. In addition, patients with RA may develop an OP fracture even with higher BMD values compared to the non-RA population.

Some risk factors of OP and RA overlap, such as female gender and smoking. Systemic inflammation associated with activity, the local bone-destroying effect of inflammatory cells, corticosteroid therapy and reduced physical activity can be considered as additional risk factors in RA. At the molecular level, among the inflammatory cytokines, TNF-α is known to induce bone loss through the stimulation of RANKL and directly affect the function of osteoclasts. The inflammatory cytokines IL-1, IL-6 and IL-17 have similar bone effects, which also play a role in the pathogenesis of the disease.

Recent years/decades have brought significant progress in the treatment of the disease, many new therapeutic targets have appeared among the therapeutic options, causing a dramatic improvement in the outcome of the disease. Among the biological disease-modifying agents (bDMARDs), the most important medications are targeting TNF-α, IL-6 and the IL-1 receptor,

as well as inhibition of T-cell costimulation, and CD20 antigen surface expression of B cells are also listed as important therapeutic targets. In the last decade, targeted low-molecular-weight synthetic disease-modifying therapies (tsDMARD), which exert their effect through the inhibition of Janus kinase (JAK), have entered the therapeutic palette. With these tsDMARDs, we can effectively reduce disease activity, which is accompanied by stopping bone loss. Based on literature data, TNF-α inhibitors stop bone destruction and reduce radiological progression in RA.

1.6 Ankylosing spondylitis

AS (axial spondyloarthritis based on the new nomenclature) is an entity that belongs to the group of seronegative spondyloarthritides. These members share common characteristics: the presence of HLA-B27 positivity, peripheral, asymmetric oligoarthritis, sacroiliitis, enthesitis, dactylitis and uveitis. AS is a chronic inflammatory disease that primarily affects the axial skeletal system and can cause fusion of the vertebrae and stiffness of the spine in the long term. During the disease course, two opposing bone remodeling processes take place at the same time in the spine: on the one hand, a pathological new bone formation can be seen in the cortical zone of the vertebrae and along the ligaments, and an increased trabecular bone loss in the center of the vertebrae leading to osteoporosis. Low bone mineral content (BMD) is a common and significantly underestimated comorbidity among patients, its prevalence can be over 50% based on literature data. Based on clinical studies, patients have a higher risk of vertebral fractures, but the results are contradictory in case of non-vertebral fractures. The peak of increased fracture risk occurs an average of two and a half years after the established diagnosis. The prevalence of vertebral fractures varies widely in different studies, reaching a prevalence of up to 30% in some studies.

In addition to inflammation and reduced mobility, the characteristic OP risk factors for the disease are balance and coordination problems due to spinal deformation and reduced vision, a higher risk of falls, a longer duration of the disease and the value of the wall-occiput distance.

The standard examination procedure that are used to measure bone density in this group may show erroneously high values due to syndesmophyte formation in the lumbar spine, so as an alternative measurement tool, lateral lumbar dual-energy X-ray photon absorptiometry (DXA) and peripheral quantitative computer tomography (pQCT) can be a good alternative when examining AS patients.

In terms of treatment, agents that inhibit the two most important cytokine pathways involved in the disease, anti-TNF-α and anti-IL23/17 inhibitors, and JAK inhibitors, the latter that provide broader cytokine inhibition, can be used effectively during the therapy of patients.

2 Objectives

Based on the previous literature data, our objective was to investigate the effects of inflammatory rheumatological diseases and autoimmune diseases, as well as TNF-α inhibitors, on the skeletal system.

In the first study, we studied the bone metabolism, vitamin D status, and fracture risk of patients with systemic sclerosis. In the second study, we investigated the effect of a one-year TNF-α inhibitor treatment on the skeletal system among our RA and AS patients.

2.1 A complex study of BMD, fracture risk, vitamin D status and bone metabolism in patients with systemic sclerosis

Our objectives were the following:

• To assess the patients' vitamin D supply, markers of bone metabolism and the occurrence of osteoporosis in our SSc patients.

• To examine the bone quality of SSc patients using pQCT.

• Examination of the correlations found between the territorial and volumetric BMD values, the fracture risk calculated on the basis of FRAX, the levels of vitamin D and bone metabolism markers.

2.2 Assessing the effects of one year of TNF-α inhibitor treatment on BMD and bone biomarkers among our RA and SPA patients

Our objectives were the following:

• Determination of the short- and long-term effects of TNF-α inhibitor treatment on bone density and bone biomarkers.

• Determination of the effect of TNF-α inhibitory treatment on serum OPG, RANKL, DKK1 and sclerostin levels.

• Examination of biomarkers of bone remodeling and correlations between disease activity and BMD values before and after TNF-α inhibitor treatment in RA and AS patients.

3 Materials and methods

3.1 Patients and controls

3.1.1 Systemic sclerosis

We included randomly selected 44 SSc patients (36 women, 8 men) undergoing regular follow-ups at the outpatient clinic of the Division of Rheumatology, University of Debrecen between March 2016 and December 2017. They had a mean age of 64.1±9.0 years (range: 41-82 years) and a mean disease duration of 18.0±5.9 years. We selected these patients from the 252 SSc patients followed in our clinic (198 women and 54 men; mean age: 61.2±7.8 years; mean disease duration: 16.8±5.2 years). The study was approved by the local ethics committee and all patients signed a written informed consent. All patients fulfilled the 2013 ACR/EULAR classification criteria for SSc. The control group consisted of 33 age-matched healthy volunteers from the hospital staff. Patients were classified as dcSSc and lcSSc. Patients with diseases that may alter bone metabolism, such as those with endocrine disorders, chronic renal failure, liver disease, malignant hematopoetic diseases or bone tumors, were excluded.

3.1.2 Rheumatoid arthritis and ankylosing spondylitis

Fifty-three patients with inflammatory arthritis (36 RA and 17 AS) were enrolled in the study. 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. Patients with active disease were recruited prior to initiating a biological therapy. Inclusion criteria included definitive diagnosis of RA or AS; high disease activity (DAS28>5.1, BASDAI>4 after at least 3 months of combined conventional DMARD therapy); clinical indication of biological therapy. Exclusion criteria included acute/recent infection, standard contraindications to anti-TNF therapy, chronic renal or liver failure. None of the patients had known primary osteoporosis prior to the diagnosis

of RA or AS. None of the patients received replacement vitamin D therapy at the time of inclusion. At baseline RA patients had a mean DAS28 of 5.00±0.86, while AS patients exerted mean BASDAI of 5.79±1.19. All patients were biologic-naïve, started on an anti-TNF therapy at baseline and received the same biological treatment at one year. Clinical assessments were performed at baseline, and after 3, 6 and 12 months of therapy. Among the 36 RA patients, 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 28 RA patients were administered TNF inhibitor in combination with methotrexate (MTX). These patients had been on MTX prior to the initiation of biologics and MTX dose was not changed. All 17 AS patients received ETN 50 mg/week SC in monotherapy. Although most RA patients and some AS patients may have received corticosteroids prior to the study, none of the patients were on corticosteroids for at least 3 months prior to and during the study period.

3.2 Clinical assessment

3.2.1 Systemic sclerosis

We recorded the following clinical parameters: age, disease duration, organ involvement, menopausal status and all relevant clinical risk factors (previous OP fractures, parental hip fracture history, BMI, alcohol use, smoking and CS therapy) included in the FRAX by clinical charts and also by using a questionnaire. Vascular disease was defined by the presence of Raynaud's syndrome and digital ulcers. With respect to digital ulcers, we counted lesions, that were greater than 3 mm in diameter as a consequence of epithelium loss. Pulmonary manifestations were diagnosed by chest X-ray and/or high resolution CT (HRCT) scan, and functional abnormalities were determined by pulmonary function test (PFT) including diffusing capacity for carbon monoxide (DLCO). Cardiac involvement was evaluated by echocardiography, while in cases of reasonable suspicion of pulmonary arterial hypertension

(PAH), right heart catheterisation (RHC) was performed. Gastrointestinal manifestation was defined by the presence of dysphagia, gastroesophageal reflux disease (GERD) and malabsorption syndrome. The diagnosis of the latter was made based on the symptoms (weight loss, low BMI, chronic diarrhea, anemia, lower iron level), the swallowing x-ray and the glucose hydrogen breath test.

FRAX was assessed for all patients and controls using the online FRAX tool provided by the Centre for Metabolic Bone Diseases at Sheffield University (http://www.shef.ac.uk/FRAX/tool.jsp) (6) applying the Hungarian population reference.

3.2.2 Rheumatoid arthritis and ankylosing spondylitis

Disease activity of RA and AS was calculated as DAS28-ESR (3 variables) and BASDAI, respectively.

3.3 Immunolaboratory analyses and biomarkers of bone metabolism

3.3.1 Systemic sclerosis

The following serological tests were performed to detect autoantibodies: antinuclear antibodies (ANA) were determined by indirect immunfluorescence on Hep2-cells (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany). Anticentromere (ACA; Viro-Immun Labor-Diagnostika GmbH) and anti-Scl70 antibodies (HYCOR Biomedical, Garden Grove, CA, USA) were analysed by enzyme-linked immunoabsorbent assay (ELISA) in accordance with the instructions of the manufacturers. Serum levels of 25-hydroxy-vitamin D_3 (25-OH-D_3) were determined by high pressure liquid chromatography (HPLC) using the Jasco HPLC system (Jasco, Tokyo, Japan) and Bio-Rad reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). According to the recommendation by Dawson-Hughes et al (27), patients with <75 ng/ml 25-OH-D_3 levels had hypovitaminosis. Serum parathyroid (PTH) hormone, osteocalcin (OC), C-

terminal telopeptides of type-I collagen (CTX) and procollagen type I amino-terminal propeptide (P1NP) were also assessed with a chemiluminescent enzyme-labeled immunometric assay (Roche Diagnostics GmbH, Mannheim, Germany).

Due to the circadian rhythm of the production of bone biomarkers (primarily CTX), blood samples were taken in the morning hours (at 2-hour intervals) in the patients.

3.3.2 Rheumatoid arthritis and ankylosing spondylitis

Blood was drawn after overnight fasting. Serum high sensitivity C reactive protein (hsCRP; normal: ≤ 5mg/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). ACPA (anti-CCP) autoantibodies were detected in serum samples using a second generation Immunoscan-RA CCP2 ELISA test (Euro Diagnostica; normal: ≤ 25 IU/ml). The assay was performed according to the manufacturer's instructions. Serum calcium (Ca; Roche Diagnostics; normal: 2.1-2.6 mmol/l) and phosphate (P; Roche Diagnostics; normal: 0.8-1.45 mmol/l); parathyroid hormone (PTH; Roche Diagnostics; normal: 1.6-6.9 pmol/l); 25-hydroxy-vitamin D3 (DiaSorin; normal: ≥75 nmol/l); osteocalcin (OC; Roche Diagnostics; normal: <41 μg/l), P1NP (Roche Diagnostics; normal: <75 μg/l), βCTX (Roche Diagnostics; normal: <0.57 μg/l), osteoprotegerin (OPG; Biomedica; median: 2.7 pmol/l); sclerostin (SOST; Biomedica; median: 24.14 pmol/l), DKK-1 (Biomedica; median: 36 pmol/l), soluble RANKL (Ampli-sRANKL; Biomedica; median: 0.14 pmol/l) and cathepsin K (cathK; Biomedica; median: 8.7 pmol/l) were determined by ELISA at baseline, 6 and 12 months after treatment initiation. The above mentioned reference values for biochemical data were generated by the local laboratory.

3.4 Bone mineral density measurements

BMD were measured by DXA (Prodigy GE Lunar, GE-Lunar Corp., Madison, Wisc., USA) at the lumbar spine (L1-L4 BMD) with anteroposterior projection and at the femoral neck (FN

BMD). BMD values were calculated in g/cm^2 and the results were expressed as T-score. Osteoporosis was defined as a lumbar spine or FN BMD T-score $\leq$ 2.5 SD according to the established WHO criteria. All BMD measurements were carried out by the same two experienced technicians.

At the time of DXA assessments, all patients and controls were also evaluated for total, trabecular and cortical BMD of the dominant forearm by pQCT (Stratec XCT-2000, Stratec Medizintechnik GmbH, Pforzheim, Germany). Data analysis was performed using the XCT6.00B software (Stratec) with measuring mask set to radius and threshold density to 269 mg/mm^3 to define trabecular bone. BMD values are expressed as mg/cm^3.

3.5 Statistical analysis

Data analysis were performed using the SPSS Statistics software, version 22.0 (IBM Corps, Armonk, NY, USA). For descriptive statistics, data were presented as frequency, range, median, mean ± standard deviation (SD). For comparisons between means, Student's t-test was used. For comparison between qualitative variables, independent t-test and Mann-Whitney test were used. Continuous variables were evaluated by paired two-tailed t-test and Wilcoxon test. Nominal variables were compared between groups using the chi-squared or Fisher's exact test, as appropriate. Correlations were determined by calculating the Pearson's correlation coefficient. Univariate and multiple regression analysis using the stepwise method was used to determine correlations and independent associations between parameters. DXA and pQCT parameters were the dependent variables and other parameters were independent variables. The β standardized linear coefficients showing linear correlations between two parameters were determined. The B (+95% CI) regression coefficient indicated independent association between the dependent and independent variable during changes. P values < 0.05 indicated statistical significance.

4 Results

4.1 Systemic sclerosis

4.1.1 Clinical characteristics of SSc patients

We examined a total of 44 patients with SSc (36 women, 8 men), whose average age was 64.1±9.0 years and the average disease duration was 18.0±5.9 years. Thirty-one patients (70.4%) were menopausal, the mean menopausal age was 46.1±3.2 years. The mean duration of menopause at the time of the study was 21.5±7.8 years. Only one patient (2.2%) was a long-term smoker and five patients (11.3%) reported habitual alcohol consumption. SSc patients had a mean BMI of 25.4 ± 3.9 kg/m^2. Thirty-three patients (75%) had lcSSc, and 11 patients (25%) had dcSSc. Regarding the cumulative clinical features of SSc patients, interstitial lung disease (ILD) was most frequently seen (n=35; 79.5%), followed by cardiac involvement (n=29; 65.9%), dysphagia and GERD (n=25; 56.8%), malabsorption syndrome (n=13; 29.5%), digital ulcers (n=13; 29.5%) and PAH (n=3; 6.8%). None of the patients had notable renal involvement. The prevalence of ANA positivity was 75% (n=33); ACA was present in 7 cases (15.9%) and 11 patients (25%) were positive for anti Scl-70 antibodies.

Of the 44 SSc patients, 17 (38.6%) had ever been treated with CS, however we did not have an exact data on the cumulative CS dose. Among the 17 patients, 13 (29.5%) received CS for less than 6 months. We did not include those patients, who had been on long-term (≥1 year) CS therapy. Cyclophosphamide IV pulses were administered to 8 SSc patients with interstitial pneumonitis or with rapidly progressive skin symptoms at doses of 750 mg/m^2 body surface area monthly for 6-12 months. Other immunsuppressive drugs, such as oral methotrexate (MTX; 10-20 mg/week for a duration of 6-36 months) and azathioprine (AZA; 2 mg/kg) were used in 13 patients (29.5%). One patient (2.2%) received rituximab therapy for rapidly progressive skin disease and severe arthritis.

With respect to the history of fractures, 19 patients (43.2%) had altogether 23 vertebral and non-vertebral osteoporotic fractures (hip, ankle, wrist). Of the 19 patients, 17 patients (89%) belonged to the limited group, while 2 (11%) belonged to the diffuse cutaneous group. Among the patients with osteoporotic fracture, 11 patients (57%) had gastrointestinal involvement, in 4 patients only osteopenia was detected based on the T-score determined by DEXA examination, and occurrence of hip fracture in the family history was determined in 4 cases (9%).

4.1.2 Bone turnover metabolism and bone densitometry assessments by DXA and QCT

Serum levels of calcium (2.41±0.14 vs. 2.32±0.11 mmol/l; p=0.001) and PTH (5.47±2.84 vs. 4.14±1.38; p=0.008), were significantly higher in SSc patients than in controls. Other bone markers, such as osteocalcin, CTX and P1NP did not differ significantly between the study groups, however, we observed a significant relationship between gastrointestinal involvement and OC, P1NP and CTX levels. All three markers were found to be significantly higher in case ofgastrointestinal tract involvement (P1NP: 58.62±27.34 vs. 37.56±13.36, p=0.014; OC: 25.21±10.08 vs. 18.39±7.42, p=0.02; CTX: 0.425±0.196 vs. 0.274±0.156, p=0.007). Of the bone metabolism markers, only the level of OC showed a significant difference between the lcSSc and dcSSc subgroups, the OC was significantly higher in the diffuse cutaneous group (20.45±8.41 vs 27.69±11.16, p=0.035). We did not observe any significant differences in the bone metabolism markers between the groups receiving and not receiving vitamin D supplementation, as well as those taking and not taking antiporotic medication.

While the mean 25-OH-D_3 levels were also comparable in SSc and controls (53.96±36.80 vs. 53.46±16.35 nmol/L; p=NS), vitamin D deficiency (25-OH-D_3 levels <50 nmol/L) in SSc patients (60%) was significantly more common than in controls (39.3%; p=0.003). Moreover, vitamin D insufficiency (25-OH-D_3 levels <75 nmol/L) was rather prevalent in both groups

(73% vs. 91%; p=0.06). Of our 44 patients, 32 received vitamin D supplementation, 23 patients (71%) nevertheless had hypovitaminosis based on the results.

The mean FRAX score for hip fractures was significantly higher in SSc patients compared to controls (4.00±4.36 vs. 2.31±2.49; p=0.049). Similar differences were found with respect to major fractures (13.48±8.03 vs. 9.28±5.13; p=0.009).

Regarding DXA, SSc patients exerted significantly lower L2-4 BMD (0.880±0.108 vs. 0.996±0.181 g/cm^2; p=0.019), as well as FN BMD (0.786±0.134 vs. 0.910±0.090 g/cm^2; p=0.007) as determined by DXA. Furthermore, L2-4 (-1.64±1.48 vs. -0.50±0.92; p=0.005) and FN T-scores (-1.78±1.01 vs. -0.44±0.84; p<0.001) were also significantly lower in SSc compared to controls. According to the WHO classification (T-score <2.5), the prevalence of OP in SSc was 22.7% both at the L1-4 and FN region. In contrast, none of the control subject had OP at any measurement site.

Evaluation by pQCT indicated significantly lower mean cortical bone density in SSc patients (328.03±103.32 mg/cm^3) compared to controls (487.06±42.45 mg/cm^3; p<0.001) and lower mean trabecular density in SSc (150.93±61.91 mg/cm^3) versus controls (184.76±33.03 mg/cm^3; p=0.037). Similar observations were made with regards to the total volumetric BMD at the radius (D100) (248.42±70.94 vs. 347.94±40.16 mg/cm^3; p<0.001).

4.1.3 Comparison of SSc subsets by qualitative variables

When assessing bone density and bone biomarkers in different SSc subsets, women had lower FN BMD as determined by DXA, as well as lower total and cortical density as measured by pQCT compared to men (p<0.05). SSc patients with pulmonary involvement (ILD) had lower pQCT total, trabecular and cortical density vs those without pulmonary involvement (p<0.01). Patients with digital ulcer and those with anti-Scl70 positivity exerted lower pQCT total and cortical density in comparison to digital ulcer and anti-Scl70 negative patients (p<0.05).

Multiple linear regression analysis was performed in order to identify factors associated with low BMD assessed by DXA and QCT in SSc patients. In our cohort, age inversely (p=0.005; p=0.027) and BMI positively (p=0.002; p=0.015) correlated with L2-4 and FN BMD, respectively, as determined by DXA. With respect to the pQCT assessments, pulmonary manifestations inversely correlated with total (p=0.024), trabecular (p=0.035) and cortical density (p=0.015). Moreover, anti-Scl70 positivity inversely correlated with pQCT total density (p=0.015) and the presence of digital ulcers with cortical density (p=0.001). With regard to the BMD values measured with the DEXA test, we did not find a significant correlation with either gastrointestinal involvement or steroid use.

Among the 44 SSc patients, 19 had OP and 25 did not. When comparing OP and non-OP patients, those with OP were significantly older (69.4±10.4 vs. 61.6±10.1 years; p=0.016), had lower BMI (23.0±3.5 vs. 27.1±5.0 kg/m^2; p=0.007) and higher FN FRAX value (6.07±3.80 vs. 2.54±4.20; p<0.001) than those without OP.

Both vertebral and FN BMD as determined by DXA significantly correlated with pQCT total, trabecular and cortical density (p<0.05).

4.2 Rheumatoid arthritis and ankylosing spondylitis

4.2.1 Clinical characteristics of patients

We examined a total of 53 patients (34 women, 19 men), whose average age was 52.0±12.1 years and the average disease duration was 8.5±7.9 years. Twenty-seven patients were postmenopausal. Among the RA patients, RF positivity was found in 26 patients (72%), while ACPA positivity was found in 21 patients (58%).

4.2.2 Clinical response to anti-TNF therapy in RA and AS

TNF-α inhibition was highly effective in RA and AS patients. In the RA cohort (n=36), ETN and CZP treatment resulted in significant decreases in DAS28 after 3 months (3.52±0.79; $p<0.001$), 6 months (3.13±0.84; $p<0.001$) and 12 months of treatment (3.02±0.96; $p<0.001$) compared to baseline (5.00±0.86) (data not shown). In RA, CRP at baseline (16.0±19.1 mg/l) was also significantly higher than after 3 months (8.5±11.3 mg/l; $p<0.001$), 6 months (7.0±7.1 mg/l; $p=0.005$) and 12 months (7.5±7.9 mg/l; $p=0.011$).

In AS (n=17), BASDAI significantly decreased from 5.79±1.19 at baseline to 2.04±0.89 ($p<0.001$), 2.00±1.03 ($p<0.001$) and 1.86±1.04 ($p<0.001$) at 3, 6 and 12 months, respectively. Moreover, CRP was also higher at baseline (12.5±12.0 mg/l) than after 3 months (5.7±13.6 mg/l; $p=0.026$), 6 months (6.3±13.5 mg/l; $p=0.041$) and 12 months of therapy (4.4±6.6 mg/l; $p=0.003$).

Fortunately, all RA and AS patients were responders and no biologic switch was necessary in any of these patients during the one-year treatment period.

4.2.3 Effects of anti-TNF therapy on bone mineral density

While anti-TNF-α treatment halted further generalized bone loss over one year in the whole cohort, there was no significant difference in mean L2-4 vertebral and femoral neck BMD or T-scores at baseline compared to 12 months (L2-4 vertebral BMD 0.890±0.027 vs 0.889±0.025 g/cm^2 and T-score -0.51±0.21 vs -0.46±0.21; femoral neck BMD 0.842±0.020 vs 0.838±0.020 g/cm^2 and T-score -0.84±0.17 vs -0.89±0.17, respectively). Similar observations were made in the RA and AS subsets.

4.2.4 Effects of biologics on bone biomarkers

Among the bone turnover (formation and resorption) markers, in the full RA+AS cohort, one-year anti-TNF therapy significantly increased P1NP after 3 months (51.8±22.5 μg/l; p=0.042) and 6 months (53.5±27.1 μg/l; p=0.040) compared to baseline (46.7±19.3 μg/l). Similar differences in P1NP levels could be observed in the RA subset after 3 months (52.2±24.0 μg/l; p=0.041) and 6 months (56.4±29.0 μg/l; p=0.026) compared to baseline (45.6±19.9 μg/l). In AS, P1NP levels significantly increased after 12 months (56.9±28.8 μg/l; p=0.035) versus baseline (49.2±18.4 μg/l). At the same time, OC and βCTX had normal levels at all time points. Neither OC (baseline: 20.3±8.8 μg/l; 12 months: 20.5±9.8 μg/l) nor βCTX (baseline: 0.34±0.18 μg/l; 12 months: 0.35±0.18 μg/l) changed significantly in the full RA+AS cohort.

With respect to the Wnt-β-catenin pathway and its regulators, in the RA+AS cohort, DKK-1 levels were higher at baseline (59.7±28.6 pmol/l) compared to the reference value described above. DKK-1 levels significantly decreased after 6 months of treatment (51.6±25.5 pmol/l; p=0.045) compared to baseline. SOST levels were also higher at baseline (94.6±45.3 pmol/l) compared to the normal reference value. Conversely to DKK-1, SOST levels significantly increased after 12 months (112.4±76.1 pmol/l; p=0.035) versus baseline. In RA, DKK-1 also significantly decreased after 6 months (52.1±26.1 pmol/l; p=0.042) versus baseline (60.6±28.9 pmol/l). On the other hand, SOST significantly increased after 12 months (128.5±83.7 pmol/l; p=0.038) versus baseline (107.0±47.5 pmol/l). In AS, SOST significantly increased after 12 months (81.3±46.9 pmol/l; p=0.034) compared to baseline (70.6±29.0 pmol/l).

In the full cohort, the baseline concentrations of CathK (27.4±6.8 pmol/l) were higher compared to the normal reference value above. There was a significant decrease in CathK production after 12 months (25.8±5.5 pmol/l; p=0.006) compared to baseline. In RA, CathK

also significantly decreased after 12 months (26.9±5.6 pmol/l; p=0.012) versus baseline (28.7±6.2 pmol/l).

With respect to the RANKL pathway, in the full cohort or in RA or AS patients, sRANKL and OPG levels did not show any differences during anti-TNF therapy. In addition, Ca, P, vitamin D3 and PTH levels also did not change during the one-year period.

When calculating bone formation/resorption ratios (P1NP/βCTX, OC/βCTX and OPG/RANKL), which better reflect bone turnover changes, in the full cohort, the P1NP/βCTX ratio significantly increased at 6 months (187.5±85.5; p=0.032) compared to baseline (160.8±56.5). In RA, the P1NP/βCTX ratio significantly increased after 6 months (190.2±87.2; p=0.035). In addition, the OC/βCTX and OPG/RANKL ratios did not change significantly during the 12 months in the full cohort, RA or AS.

4.2.5 Correlations between bone mineral density and laboratory biomarkers

Univariate and multiple regression analyses were performed in order to determine associations between BMD at various sites (dependent variables) and laboratory parameters (independent variables). In the full cohort and in RA patients, baseline and/or 12-month BMD at multiple sites exerted inverse relationships with CRP and βCTX, as well as positive correlations with SOST. In the AS subset, L2-4 BMD after one-year biologic therapy inversely correlated with baseline βCTX. Femoral neck BMD rather showed inverse correlations with CRP.

5 Discussion

5.1 Systemic sclerosis

OP has been associated with a number of inflammatory rheumatic diseases including SSc. To our knowledge, this may be the most complex study applying standard DXA, forearm pQCT, FRAX, as well as bone biomarkers in order to evaluate bone density and bone turnover in SSc patients in comparison to healthy controls. With respect to clinical features, 70% of SSc patients were postmenopausal. In the past, more than 40% of the patients had fragility fractures. Menopause plays an important role in the acceleration of bone loss. Others reported significantly greater proportion of SSc patients in menopause than in controls, which occurred significantly earlier in that group. Some authors also noted a longer duration of menopause in SSc patients. In our study, the frequency of menopause was not significantly different in SSc patients and age-matched control group.

When the bone status of SSc patients was compared to that of controls, vitamin D deficiency was more prevalent in SSc versus controls. We and others have also found D-hypovitaminosis in SSc. Moreover, low vitamin D levels were associated with organ manifestations, such as skin involvement. It has been established, that vitamin D is not only involved in bone homeostasis, but also exerts important immunological effects. Thus, D-hypovitaminosis has been implicated in the pathogenesis of various autoimmune diseases including SSc. Impaired VDR signalling in SSc has been reported, which, together with a decreased level of vitamin D results in hypersensitivity of SSc fibroblasts to TGF-β signalling, leading to an uncontrolled activation of fibroblasts. Others found that low levels of vitamin D were associated with different disease activity markers in SSc (e.g. disease duration, ESR, CRP values, the presence of ACA, systolic PAH, pulmonary fibrosis and nailfold capillaroscopic pattern). Our further analyses revealed

no significant associations between low vitamin D level and any disease-specific measures, including disease subset, autoantibody positivity or any organ manifestations.

Hip FRAX scores were also higher in SSc vs controls. Increased fracture risk associated with various risk factors has been reported in SSc, however, we found only one report on FRAX assessment in SSc. In that study, the 10-year fracture risk was higher in SSc patients with low BMD.

Regarding DXA, SSc patients had lower L2-4 and FN BMD and T-score values vs controls. Moreover, WHO-defined OP was more common is SSc. These data are in line with previous reports. Studies in the literature suggested that SSc is a risk factor for bone loss, however the prevalence of OP was within a wide range from 3% to 51%. This wide dispersion could be attributed to the heterogenity of the patients studied (e.g. age, gender, menopausal status, geographic location, disease subtype, organ manifestations and CS exposure). Other groups also reported lower BMD at multiple sites in SSc.

In our cohort, total, trabecular and cortical volumetric BMD as determined by pQCT was lower in SSc compared to controls. The difference was more pronounced in cortical BMD. To our knowledge, there has been only one study where bone pQCT was performed in SSc patients. In that study, Marot et al demonstrated significant alterations in the trabecular bone compartment, however, measures of the cortical compartment were not different in SSc patients and controls.

In our study, we also compared DXA and pQCT. The total, trabecular and cortical density values determined by QCT all significantly correlated with L2-4 and FN BMD measured by DXA. We obtained similar results in our recent study involving RA patients compared to controls.

Among bone markers, we found elevated PTH levels in SSc compared to controls. Others also reported higher PTH in SSc. In our cohort, OC, P1NP and CTX levels were similar in SSc

and controls. With respect to OC and P1NP, similar results were reported by others. Allanore et al found elevated CTX levels in 16 out of 33 patients. Moreover, we found significant associations between gastrointestinal involvement and levels of OC, P1NP and CTX. Similar relationship has not yet been reported in SSc.

When assessing, different SSc subsets, women had lower DXA FN BMD than men. In addition, SSc patients with pulmonary involvement, digital ulcers and anti-Scl70 positivity had greater bone loss as determined by pQCT in comparison to patients without these features. Moreover, dcSSc patients compared to lcSSc patients, as well as patients with compared to without gastrointestinal manifestations exerted higher bone turnover as indicated by bone biomarkers. In general, SSc patients with OP were older, had lower BMI and higher hip FRAX. Our study indicated significant, close correlations between BMD determined by DXA and volumetric bone density assessed by pQCT. Moreover, the multiple regression analysis indicated that older age and lower BMI were independently associated with lower L2-4 and FN BMD by DXA. On the other hand, disease specific measures, pulmonary manifestations, digital ulcers and anti-Scl70 positivity may determine pQCT volumetric density values. Our data suggested BMI as an independent influencing factor of FN and lumbar BMD by DXA. Others reported reduced lean body mass in SSc patients due to decreased physical activity, malnutrition and CS treatment, which may contribute to a reduced BMI. They were also able to show that lower BMI was an independent risk factor for low BMD at the hip and femoral neck. With regards to bone biomarkers, Allanore et al also found correlation between CTX levels and the presence of dcSSc vs lcSSc, higher Rodnan skin score, pulmonary manifestations and anti-Scl70 positivity. In the above mentioned French study by Marot et al the presence of ACA and digital ulcers were associated with low BMD by DXA at all sites and trabecular density by pQCT at tibia highlighting the suspected role of repeated vasospasm and subsequent systemic microangiopathy in the alteration and resorption of bone tissue

In conclusion, our study suggests that the existence of OP, as well as BMD determined by traditional DXA shows association with general features, such as age or BMI. On the other hand, volumetric bone density assessed by pQCT, as well as bone biomarkers rather showed associations with SSc-specific features including dcSSc vs lcSSc subtype, organ manifestations (pulmonary, digital ulcers, gastrointestinal) and anti-Scl70 positivity. These data suggest, that in dcSSc patients with extensive organ involvement and anti-Scl70 positivity, pQCT and bone biomarkers may have additional value during the assessment of bone status. Larger cohort studies are needed in order to determine the real place of these techniques in determining bone status in SSc patients.

5.2 Rheumatoid arthritis and ankylosing spondylitis

RA and AS have been associated with secondary osteoporosis and increased fracture risk. Inflammatory bone formation is also a hallmark of AS. Anti-TNF biologics may inhibit bone loss, but they exert limited effects on inflammatory bone formation. Furthermore, biological therapy may affect the production of bone markers in RA and AS. With respect to mode of action, TNF inhibitors decrease RANKL and increase OPG expression in inflammatory diseases. TNF inhibition also results in blockade of DKK-1 and stimulation of Wnt-dependent bone formation.

In this study, we assessed the effects of ETN or CZP therapy on disease activity, bone loss and bone biomarkers in RA and AS. As expected, one-year anti-TNF treatment was effective in both RA and AS patients as it significantly decreased DAS28 and BASDAI, respectively. These effects of ETN and CZP were observed as early as after 3 months. The anti-inflammatory effects of anti-TNF therapy was accompanied by inhibition of bone loss in both diseases. L2-4 and femoral neck BMD remained unchanged after one-year ETN or CZP therapy in the entire cohort, as well as in RA and AS. These effects have previously been reported by others. Baseline CRP inversely correlated not only with baseline L2-4 and femoral neck BMD, but also

with BMD after one-year treatment. Thus, at baseline, high-grade inflammation correlated with low BMD. Moreover, we found that baseline CRP may predict further bone loss after one-year anti-TNF therapy.

The first publications on the effects of TNF inhibitors on bone and osteoporosis were published in the 2000's. In most studies, effects on the bone have been associated with favourable clinical responses. A great amount of data have been published on infliximab, ETN, adalimumab and some data also on golimumab. We have not found any reports on the bone effects of CZP.

Regarding bone biomarkers, one-year anti-TNF therapy significantly increased P1NP over time in our full cohort, in RA and AS. Other markers of bone turnover, such as OC or βCTX did not change upon anti-TNF therapy. The P1NP/βCTX ratio increased over time in the full cohort and in RA. Bone resorbing activity as indicated by βCTX at baseline was associated with low baseline BMD and we showed that baseline βCTX may also predict further bone loss after one-year anti-TNF therapy. In line with our findings, previous studies found that TNF inhibitors increased serum P1NP levels and, in some cohorts, suppressed βCTX levels in RA and AS. These findings were not always consistent as some other studies showed no effects of TNF-α inhibitors on OC, P1NP and βCTX levels. Furthermore, anti-TNF agents have been shown to increase OPG/RANKL, OC/βCTX and P1NP/βCTX ratios. In most studies, changes in bone biomarkers by anti-TNF therapy were associated with improvements in disease activity in RA and AS.

With respect to DKK-1 and SOST, upon one-year ETN or CZP treatment, DKK-1 levels transiently decreased in the whole cohort and in the RA subset. On the other hand, SOST levels significantly increased in the entire cohort, as well as in the RA and AS subsets after 12 months of treatment. When comparing RA and AS, SOST levels were lower at all time points in AS compared to RA. Baseline SOST correlated with L2-4 and femoral neck BMD at baseline, as

well as after 12 months in the whole cohort and in RA. This association was further confirmed by regression analyses. Interestingly, higher baseline SOST was associated with higher BMD at all sites. Moreover, high baseline SOST predicted higher BMD after 12 months of anti-TNF treatment. Similar to our observations, other studies in RA also reported that TNF-α inhibitors suppressed DKK-1 leading to increased bone formation. In addition, in one study anti-TNF treatment increased SOST production in RA. The seemingly controversial effects of anti-TNF biologics on DKK-1 and SOST need further discussion. In general, DKK-1 and SOST were thought to have unidirectional effects on the bone. Both molecules were described to block Wnt-β-catenin-dependent bone formation. TNF-α may induce both DKK-1 and SOST production. DKK-1 may directly drive SOST expression by osteocytes and DKK-1 blockade also inhibits SOST production. Moreover, low SOST and DKK-1 production have been associated with syndesmophyte formation in AS. SOST levels remain low after TNF-α blockade, which may partly explain the inefficacy of TNF inhibitors on bone formation in AS. Interestingly, low SOST and DKK-1 levels in AS result in increased CRP production. Indeed, SOST levels increased in our study in both AS and RA, however the absolute serum concentrations of SOST were lower in AS vs RA at all time points. Our findings that TNF-α inhibitors may differentially regulate DKK-1 and SOST suggest that, apart from the direct stimulatory effect of DKK-1 on SOST, there may be other, indirect regulatory mechanisms between these two molecules. Finally, in our cohort high SOST correlated with high BMD at all sites. This finding supports previous observations that, while under non-inflammatory conditions, such as osteoporosis, SOST inhibits Wnt-mediated bone formation and contributes to bone loss and joint destruction, under TNF-dependent inflammatory conditions, SOST may preserve bone and attenuate joint damage. Furthermore, anti-TNF treatment may increase SOST levels and, as we demonstrated in this study, high SOST may correlate with high BMD.

When comparing bone resorption indicated by βCTX with bone formation associated with SOST, only βCTX, but not OC, and only SOST, but not DKK-1 correlated with BMD. Thus, our data suggest that βCTX and SOST, in addition to CRP, may be the most important biomarkers and independent predictors of BMD. Moreover, most of these associations were seen in the entire cohort, where two-third of the patients had RA, and in the RA subset alone. Interestingly only βCTX and SOST, but not CRP associated with BMD in RA. In contrast, in our relatively small AS subset, only CRP, but not βCTX or SOST correlated with femoral neck BMD. In conclusion, in RA, SOST and βCTX, but not CRP are independent predictors of BMD (at baseline and after 12 months), whilst, in AS, CRP may serve as the best biomarker of inflammatory bone metabolism. As mentioned above, low SOST and DKK-1 levels in AS may stimulate CRP production, this may further explain the outstanding importance of CRP in AS-related bone changes.

CathK is a matrix-degrading enzyme that effectively digests collagen. CathK has been implicated in osteoporosis and a CathK inhibitor, odanacatib, has been tried in postmenopausal osteoporosis. In RA, increased CathK levels correlated with joint damage. CathK is strongly expressed at different spinal regions in AS. In our entire cohort and in the RA subset, but not in AS, TNF inhibition resulted in significantly decreased CathK levels after one year. In a study of 13 AS patients, infliximab, ETN or adalimumab treatment did not change CathK levels. We did not find any reports in the literature on the effects of biologics on CathK production.

In summary, despite its diverse effects on bone formation in RA and AS, anti-TNF therapy (ETN and CZP) slowed down bone loss in both diseases. Independent predictors of BMD were SOST and βCTX in RA, whilst CRP in AS. Further studies are needed to evaluate the potential beneficial effects of biologics on inflammatory bone loss.

6 New findings

1. D-hypovitaminosis and osteoporosis occur more frequently among our hungarian patients treated for systemic sclerosis than in the control group.

2. Using pQCT for the first time in Hungary in this group of patients, we established that volumetric BMD values can be associated with certain disease-specific parameters (diffuse cutaneous form, anti-Scl-70 positivity, presence of finger ulcers and pulmonary involvement).

3. CZP treatment (similar to other TNF-α inhibitor treatments) leads to the cessation of bone loss in RA by reducing inflammation.

4. Our study showed an increase in the level of sclerostin in both RA and AS, but the absolute serum concentration of sclerostin was lower in the AS group than among the RA patients, which may partially explain the ineffectiveness of TNF-α inhibitors regarding the prevention of syndesmophyte formation.

5. In RA, sclerostin and βCTX are independent predictors of BMD, while in AS, CRP is considered the best biomarker of inflammatory bone metabolism.

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8 List of publications

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Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. Gulyás, K., **Horváth, Á.**, Végh, E., Karancsiné Pusztai, A., Szentpétery, Á., Pethő, Z., Váncsa, A., Bodnár, N., Csomor, P., Hamar, A. B., Bodoki, L., Bhattoa, H. P., Juhász, B., Nagy, Z., Hódosi, K., Karosi, T., FitzGerald, O., Szűcs, G., Szekanecz, Z., Szamosi, S., Szántó, S.: Effects of 1-year anti-TNF-[alfa] therapies on bone mineral density and bone biomarkers in rheumatoid arthritis and ankylosing spondylitis.
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Total IF of journals (all publications): 73,673
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