ACR Open Rheumatology
Vol. 7, No. 1, January 2025, e11742
DOI 10.1002/acr2.11742

American College of Rheumatology
Empowering Rheumatology Professionals

Circulating Baseline CXCR3$^+$Th2 and Th17 Cell Proportions Correlate With Trabecular Bone Loss After 48 Weeks of Biological Treatment in Early Rheumatoid Arthritis

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Objective. The high prevalence of osteoporosis in rheumatoid arthritis (RA) is due to inflammation that stimulates differentiation of osteoclasts, a process involving circulating monocytes and T cell–derived factors. The aim of this study was to evaluate relations between circulating monocytes, T cell subsets, and changes in bone characteristics before and after treatment with biological disease-modifying antirheumatic drugs (bDMARDs) in RA.

Methods. Thirty patients with untreated early RA who met the American College of Rheumatology/EULAR 2010 criteria were included. Data were collected before and 48 weeks after treatment with methotrexate (MTX) together with one of three bDMARDs (abatacept, tocilizumab, or certolizumab pegol). Disease activity was measured using the Clinical Disease Activity Index, swollen or tender joint counts, C-reactive protein levels, and erythrocyte sedimentation rates. Proportions of monocyte and CD4$^+$ T cell subsets in blood samples were analyzed by flow cytometry. Bone densitometry was performed using high-resolution peripheral quantitative computed tomography (HR-pQCT).

Results. HR-pQCT revealed an overall decrease in cortical ($P = 0.009$) and trabecular ($P = 0.034$) bone mineral density, although a subset of patients showed no bone loss after 48 weeks of treatment. The overall bone loss was not associated with age, body mass index, sex, intraarticular glucocorticoid injections, or baseline disease activity. Loss of trabecular bone volume fraction correlated with high proportions of circulating CXCR3$^+$Th2 cells ($r = -0.38$, $P = 0.04$) and CXCR3$^+$Th17 cells ($r = -0.36$, $P = 0.05$) at baseline. Similarly, no loss of trabecular bone volume fraction correlated with high proportions of regulatory T cells ($r = 0.4$, $P = 0.03$) at baseline. However, the associations were not significant when corrected for confounders and multiple testing.

Conclusion. MTX together with bDMARDs efficiently reduce disease activity but only prevent bone loss in a subset of patients with RA after 48 weeks of treatment. The correlations of circulating baseline T helper cell and regulatory T cell populations with trabecular bone changes suggest a potential novel role for these cells in systemic bone homeostasis during early RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease manifested by inflammation in the synovium and risk of subsequent development of erosions of the bone adjacent to the joint.[1] RA is also associated with generalized bone loss, in which physical inactivity and systemic inflammation shift the balance from bone formation toward bone resorption.[2–5] Bone loss

ClinicalTrials.gov identifier: NCT01491815.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supported by grants from the Swedish Research Council (grants 2016-01192 and 2020-0185 to Dr Islander and 2019-01035 to Dr Rudin), the Novo Nordisk Foundation Center for Basic Metabolic Research (grant 19928 to Dr Islander), the Swedish state under the agreement between the Swedish government and the county councils, the ALF-agreement (grants ALFGBG-716421 and ALFGBG-965238 to Dr Islander, ALFGBG-857161 to Dr Carlsten, and ALFGBG-717541 to Dr Rudin), the Association against Rheumatism (to Dr Islander) and (to Dr Carlsten), King Gustav V's 80 Years' Foundation (Drs Islander, Carlsten, and Rudin), the Nanna Svartz foundation (Dr Islander), the Emil and Wera Cornells foundation (Dr Islander), and the IngaBritt and Arne Lundberg Foundation (grants LU-2018-0008 and LU-2020-0010 to Dr Islander). The high-resolution peripheral quantitative computed tomography was funded by the IngaBritt and Arne Lundberg Foundation.

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detected by dual-energy x-ray absorptiometry (DXA) has been reported to be more pronounced during the first years after RA diagnosis,[6,7] whereas studies in patients with early RA treated with biological disease-modifying antirheumatic drugs (bDMARDs) show no decrease or a slower decrease of bone mineral density (BMD).[8,9] However, analysis of bone using DXA only provides a two-dimensional BMD, whereas high-resolution peripheral quantitative computed tomography (HR-pQCT) is a sensitive imaging technology that allows a three-dimensional assessment of the microstructure for both cortical and trabecular bones.[10] A few longitudinal HR-pQCT studies have investigated bone microstructure of periarticular sites in patients with early RA. They identified early changes in bone microstructure, lack of sustained Clinical Disease Activity Index (CDAI) remission, and high rheumatoid factor (RF) titers as predictors for periarticular bone erosion.[11,12] Still, there is a need for additional studies using sensitive imaging technology, such as HR-pQCT, to determine the impact of effective antirheumatic treatment modalities on generalized bone loss in sites other than periarticular sites.

Bone loss is the result of a shift toward increased bone resorption, with osteoclasts as the main contributing cell type. In RA, an enhanced monocyte-to-osteoclast transition was shown to increase osteoclastogenesis and subsequent bone erosion.[13] Blood monocytes are divided into three major populations based on the surface expression of CD14 and CD16: classical monocytes, intermediate monocytes, and nonclassical monocytes.[14] The intermediate monocyte subset is expanded in patients with established RA[15–18] and has been implicated to play an important role as osteoclast progenitors in inflammatory conditions.[19]

Dysregulation of self-tolerance plays a vital role in the onset of RA, and abnormal immunosuppressive function and numbers of regulatory T (Treg) cells can contribute to its progression and severity.[20,21] In early RA, as well as in highly active RA, a reduced proportion of circulating Treg cells has been observed compared to healthy controls, resulting in an imbalance of the Treg cell–to–T effector cell ratio.[22,23] T helper (Th) cells are known to be involved in RA by inducing joint inflammation, which, if kept untreated, can lead to joint destruction and periarticular bone loss.[24] Interferon-γ (IFNγ)–secreting Th1 cells in synovial fluid and blood are predominant in established RA, whereas in early RA, the profile of synovial fluid cytokines has a Th2 and Th17 bias.[25,26] This is confirmed by the dominance of Th2 and Th17 cells in the blood of patients with untreated early (ue) RA and points toward a pathogenic role for these cells in early stages of the disease.[27] Interleukin-17 (IL-17)–secreting Th17 cells are a population of proinflammatory $CD4^+$ T cells with the capacity to induce osteoclastogenesis by producing receptor activator of nuclear factor kappa beta ligand and tumor necrosis factor (TNF).[28] Conversely, Th2 cells and their cytokines IL-4 and IL-13 are generally regarded to exert bone protecting effects by inhibiting osteoclast differentiation.[29–31] The increased presence of both cytokines in the synovial fluid of early RA, but not in established RA, implicates that they are part of an early regulatory response that is lost as patients progress to fully established disease.[25] However, there is conflicting data showing a connection between bone loss and[32,33] IL-4, as Lewis et al[32] reported that overproduction of IL-4 in a transgenic mouse strain resulted in osteoporosis. Thus, the association between Th2 cells and bone loss in RA needs to be further clarified.

In recent years, $CXCR3^+$Th2 and Th17 cells have caught attention in RA research.[27,34] CXCR3 is a receptor expressed on certain B and T cell populations and allows the migration toward its ligands CXCL9, CXCL10, and CXCL11. In RA, increased concentrations of these ligands have been found in synovial fluid, and subsequent infiltration of $CXCR3^+$ T helper and B cells has been observed.[34–36]

We showed previously in a well-defined population of patients with ue RA that a subgroup of patients had elevated levels of intermediate monocytes at baseline compared to healthy controls.[37] In that study, the frequency of intermediate monocytes was not associated with bone density but correlated positively with $CXCR3^+$Th17 cells at baseline.[37]

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Data cannot be made publicly available for ethical and legal reasons. Such information is subject to legal restrictions according to national legislation. Specifically, in Sweden confidentiality regarding personal information in studies is regulated in the Public Access to Information and Secrecy Act (SFS 2009:400). The data underlying the results of this study might be made available upon request, after an assessment of confidentiality. There is thus a possibility to apply to get access to certain public documents that an authority holds. In this case, Region Västra Götaland is the specific authority that is responsible for the integrity of the documents with research data. Questions regarding such issues can be directed to Katarina Almehed (katarina.almehed@vgregion.se), head of the Department of Rheumatology, Sahlgrenska University Hospital, Gothenburg, Sweden.

Additional supplementary information cited in this article can be found online in the Supporting Information section (http://onlinelibrary.wiley.com/doi/10.1002/acr2.11742).

Author disclosures are available at https://onlinelibrary.wiley.com/doi/10.1002/acr2.11742.

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Submitted for publication February 9, 2024; accepted in revised form August 19, 2024.

Thus, the aim of this prospective longitudinal study in early RA was to investigate changes in bone microstructure after 48 weeks of treatment with methotrexate (MTX) in combination with one of the three following bDMARDs: CTLA-4 immunoglobulin fusion protein (CTLA-4Ig) (abatacept), anti–IL-6R (tocilizumab), or anti-TNF (certolizumab pegol). Moreover, we assessed connections between changes in bone characteristics after 48 weeks of treatment and subsets of monocytes and T cells at baseline.

PATIENTS AND METHODS

Patients. The patients in this study include a subgroup from three of the four randomized treatment arms in the NORD-STAR trial, a phase 4 investigator-initiated randomized observer-masked clinical trial.[38,39] The patients were all recruited at the rheumatology clinic at Sahlgrenska University Hospital in Gothenburg and were asked to participate in bone analyses with HR-pQCT. Thirty of the patients completed the analyses both at baseline and at 48 weeks after inclusion. The baseline characteristics of these patients are shown in Table 1.

All patients were treatment naïve at inclusion and fulfilled the American College of Rheumatology/EULAR criteria for RA. The inclusion criteria were the following: aged ≥18 years old; two or more swollen joints and two or more tender joints; RF positive, or anti–citrullinated protein antibody (ACPA) positive, or C-reactive protein (CRP) levels ≥10 mg/mL; having at least moderate disease activity (>3.2) measured by composite index Disease Activity Score in 28 joints (DAS28)–CRP; symptom duration <24 months (retrospective patient-reported pain in the joints); and no treatment with glucocorticoids or DMARDs. Disease activity in patients was assessed by swollen joint count of 66 joints (SJC66), tender joint count in 68 joints (TJC68), swollen joint count in 28 joints (SJC28), tender joint count in 28 joints (TJC28), CRP, erythrocyte sedimentation rate (ESR), CDAI, DAS28-CRP, and DAS28-ESR. ACPA positivity was determined by multiplexed anticyclic citrullinated peptide (anti-CCP) test (BioPlex from BioRad), and RF positivity was determined by nephelometry (Beckman Coulter). Patients with >3 IU/mL anti-CCP antibodies or >20 IU/mL RF in serum were considered ACPA positive or RF positive, respectively, according to the current cutoff levels in the clinical immunology laboratories. All patients signed an informed consent form. The study was conducted in compliance with the Helsinki 123 Declaration and was approved by the regional ethic committee of Gothenburg (Dnr. 691–12 and amendment T270-13).

Study design. A detailed description of the treatment protocol of the full NORD-STAR cohort has previously been published.[40] Briefly, patients who fulfilled the inclusion criteria were randomly assigned into four different treatment arms. Because the focus of this study was to analyze the effect of targeted treatments on bone parameters in early RA, only patients treated with one of the three bDMARDs were included. All patients received

Table 1. Clinical characteristics of patients with early rheumatoid arthritis at baseline and after 48 weeks of treatment*

Characteristics (N = 30)	Baseline	48 weeks	*P* value
Age, median (range), y	56 (21–78)	–	–
Female, n (%)	20 (66.7)	–	–
BMI, median (range)	25.3 (19–32.5)	–	–
Smoker, n (%)[a]	3 (10)	–	–
Self-reported symptom duration, median (range), mo	6 (2–22)	–	–
ACPA+, n (%)	26 (86.7)	–	–
RF+, n (%)	17 (56.7)	–	–
ACPA+ and RF+, n (%)	16 (53.3)	–	–
ACPA− and RF−, n (%)	3 (10)	–	–
MTX$^+$anti-TNF, n (%)	13 (43.3)	–	–
MTX$^+$anti-CTLA4-Ig, n (%)	10 (33.3)	–	–
MTX$^+$anti-IL6R, n (%)	7 (23.3)	–	–
CRP, median (range), mg/L	7 (1–180)	1 (1–9)	<0.001
ESR, median (range), mm/hr	23 (7–98)	6.5 (1–44)	<0.001
SJC66, median (range)	8 (3–28)	0 (0–3)	<0.001
TJC68, median (range)	10 (2–35)	0 (0–29)	0.003
SJC28, median (range)	7 (2–24)	0 (0–3)	<0.001
TJC28, median (range)	4.5 (0–27)	0 (0–16)	<0.001
DAS28-CRP, median (range)	4.89 (2.7–8.3)	1.6 (1–5)	0.003
DAS28-ESR, median (range)	5.18 (2.6–8.7)	1.7 (0–5)	<0.001
CDAI, median (range)	25.9 (10.1–68.7)	2 (0–24)	<0.001

*ACPA, anticitrullinated protein antibody; BMI, body mass index; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; MTX, methotrexate; RF, rheumatoid factor; SJC28, swollen joint count in 28 joints; SJC66, swollen joint count in 66 joints; TJC28, tender joint count in 28 joints; TJC68, tender joint count in 68 joints. The Wilcoxon's matched-paired signed rank test was used for comparison.
[a]Current daily smoker.

MTX, escalating within four weeks to 25 mg/wk together with folic acid supplementation.

The patients also received one of the following treatments:

- CTLA-4Ig (abatacept; Bristol Myers Squibb) at 125 mg every week subcutaneously
- anti-IL-6R (tocilizumab; Hoffmann-La Roche) at 8 mg/kg every four weeks intravenously or 162 mg every week subcutaneously
- anti-TNF (certolizumab pegol; Union Chimique Belge) at 200 mg every other week subcutaneously (loading dose of 400 mg at weeks 0, 2, and 4)

Intraarticular glucocorticoid injections were allowed if needed up to week 12. Thereafter, up to 40 mg were allowed every 12 weeks. In all treatment arms, intraarticular glucocorticoid was prohibited in weeks 20 to 24 and 44 to 48 to minimize its influence on week 24 and week 48 outcomes. Thirty percent of the patients received intraarticular glucocorticoid injections, as shown in Supp. Table 1. No oral glucocorticoids were given. The patients were included in the study from 2013 to 2018, and blood samples were taken within 1 to 2 weeks after RA diagnosis and then after 4, 12, 24, and 48 to 52 weeks of treatment.

Bone densitometry. All patients recruited at the rheumatology clinic in Gothenburg were asked to participate in DXA and HR-pQCT measurements at baseline (n = 46) and at the one-year follow-up. Thirty of the patients with RA completed the analysis both at baseline and at 48 weeks after inclusion to the study.

Areal BMD (aBMD) was measured at baseline and 48 weeks after treatment at the total hip, femoral neck, and lumbar spine (L1–L4) using the Hologic Discovery A (S/N 86491) device. The coefficient of variation (CV) for these measurements were 0.8% (total hip), 1.3% (femoral neck), and 0.7% (spine). One patient was not measured for total hip and femoral neck, which is why these data are missing.

Volumetric BMD (vBMD) and bone microarchitecture were measured at the lower leg (tibia) on the same side as the nondominant arm using a high-resolution 3D HR-pQCT device (XtremeCT; Scanco Medical AG), according to a previously described protocol.[41,42] In short, the tibia was measured at the standard measuring site recommended by the manufacturer (ultradistal). The first image was acquired at 22.5 mm from the reference line (ie, a line placed at the articular plateau by the operator). A total of 110 cross-sectional images were obtained with an isotropic resolution of 82 μm, resulting in a three-dimensional model of the bone. Each three-dimensional model (110 images) took three minutes of scan time to obtain, and the effective dose was 3 μSv. Quality assessments of the images was performed and graded from 1 to 5, according to the recommendation provided by the manufacturer (Scanco Medical AG), in which 1 to 3 were regarded as acceptable quality and 4 to 5 were regarded as unacceptable quality. Only images with quality 1 to 3 were processed further. Each site was analyzed according to the standard HR-pQCT protocol, yielding the following parameters: the trabecular bone volume–to–total volume ratio (BV/TV) was derived from the BMD of the trabecular volume of interest and the assumption that compact bone has a matrix mineral density of 1,200 mg hydroxyapatite (HA)/cm^3 (whereas the marrow background is equivalent to 0 mg HA/cm^3), trabecular number (TbN) (mm, TbN – 1; inverse of the mean spacing of the mid-axes), trabecular thickness (TbTh) (mm, [BV/TV]/TbN), cortical volumetric BMD (mg/cm^3), cortical area (mm^2), and total vBMD (mg/cm^3). The CVs for measurement of trabecular parameters were 0.8% to 2.6%, and the CVs for measurements of cortical parameters were 0.1% to 0.9%.

Flow cytometry. Peripheral blood samples were analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood with Lymphoprep (Axis-Shield), and the cells were blocked with mouse serum and human serum from bloodtype AB. To define the intermediate monocyte subset, the cell surface of PBMCs was stained with fluorochrome-conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated anti-CD14 (clone M5E2; BD Biosciences) and fluorescein isothiocyanate (FITC)–conjugated anti-CD16 (clone NKP15; BD Biosciences). The gating strategy of the monocyte subsets has been shown in a previous publication.[37] In brief, monocytes were initially gated according to their forward scatter (FSC) area and side scatter area characteristics. Doublet discrimination was done using FSC-area and FSC-height. The monocyte population was then subdivided based on their expression of CD14 and CD16. Three monocyte subsets were distinguished: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), and nonclassical monocytes ($CD14^{+}CD16^{++}$), as previously described.[14] $CD4^{+}$ T cells were stained and defined, as previously described.[27] In brief, for surface staining, the following antibodies were used: FITC-conjugated anti-CD45RA (clone L48; BD Biosciences) and anti-CD127 (clone HIL-7R-M21; BD Biosciences); APC-H7-conjugated anti-CD4 (clone SK3; BD Biosciences); APC/AF647-conjugated anti-CD127 (clone HIL-7R-M21; BD Biosciences), anti-CXCR5 (clone RF8B2; BD Biosciences), and anti-CD25 (clone 2A3; BD Biosciences); Brilliant Violet 421–conjugated anti-CD25 (clone BC96; Biolegend) and anti-CXCR3 (clone G025H7; Biolegend); and PE-Cy7-conjugated anti-CCR4 (clone TG6/CCR4; Biolegend).

After surface staining, the cells were fixed and permeabilized with a FoxP3/transcription factor staining buffer set (eBioscience), and intracellular staining for FoxP3 was performed using phycoerythrin (PE)–conjugated anti-FoxP3 (clone PCH101, eBioscience) with PE-conjugated streptavidin (BD Biosciences) antibodies. The T cell subsets were gated according to the gating strategy presented in Supp. Fig. 1. The phenotypes of defined T cell subsets were confirmed by lineage specifying transcription factor

expression analysis by real-time quantitative polymerase chain reaction and cytokine secretion analysis by Cytometric Bead Array (BD Biosciences), as previously shown.[27] Stained samples were analyzed using a FACSCanto II equipped with FACS Diva software (BD Biosciences), and the resulting data were analyzed with FlowJo software (Tree Star).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 9, SPSS (version 25; SPSS, Inc), and SIMCA 16 software (Umetrics). Wilcoxon's signed rank test was used for comparison between two dependent groups with non-Gaussian distributions, and a two-tailed Mann-Whitney U-test was used for comparisons between two independent groups with non-Gaussian distributions. Multivariate factor analysis by principal component analysis (PCA) and orthogonal partial least-squares (OPLS) analysis was used to analyze the associations between proportions of baseline immune cells and percentage change in bone parameters from baseline to 48 weeks of treatment. For SIMCA, log transformation was applied to normalize data. All data were scaled to unit variance by dividing each variable by 1/SD so that all the variables were given equal weight regardless of their absolute value. The quality of the OPLS models was assessed based on the parameters R2 (ie, how well the variation of the variables is explained by the model) and Q2 (ie, how well a variable can be predicted by the model). Additionally, the cross-validated analysis of variance (CV-ANOVA) *P* value was calculated as a measure of significance for the observed group separation. Only the variables that contributed most to the OPLS models were further analyzed by univariate analysis. Univariate correlations were performed using two-tailed Spearman's rank-order correlation. Linear regression models were performed and presented with unstandardized β values. There, all nonnormally distributed variables were log-transformed for inclusion in these linear regressions, and the models were adjusted for confounders, as described in the legends. To account for multiple testing, Bonferroni correction was applied, and associations with *P* values smaller than $P_{corrected}$ were defined as significant. The nine T cell subsets shown in Supp. Fig. 1 and the intermediate monocyte population were used in the PCA, OPLS, and linear regression analysis.

RESULTS

Clinical characteristics of patients with early RA. To investigate changes in bone characteristics, we analyzed data from 30 patients with RA at baseline and after 48 weeks of treatment (Table 1). At baseline, all patients were treatment naïve and newly diagnosed with RA. The patients were randomly assigned to treatment with MTX in combination with either anti-TNF (n = 13), anti-CTLA4-Ig (n = 10), or anti-IL-6R (n = 7). However, in this study, the patients were not analyzed separately by treatment because the subgroups were too small. The inflammation markers CRP and ESR, the disease activity composite scores DAS28 and CDAI, as well as TJC and SJC, were significantly reduced in the patients after 48 weeks of treatment resulting in CDAI remission in 57% of the patients (Table 1, Figure 1A–C).

Overall loss in bone density and microstructure after 48 weeks of treatment in early RA. HR-pQCT was used to investigate changes in vBMD and bone microstructure. An overall bone loss was observed for total vBMD (–0.84%, *P* = 0.004), cortical vBMD (–0.66%, *P* = 0.009), and trabecular bone volume fraction (–0.60%, *P* = 0.03) (Table 2). Changes in bone parameters were not associate with age, body mass index (BMI), sex, ACPA positivity, intraarticular glucocorticoid injections (Supp. Table 2), or disease parameters (Supp. Table 3) at baseline. However, after 48 weeks of treatment, negative associations were found for the changes in total and cortical vBMD with CDAI, SJC28, and SJC66, but after correction for multiple testing, the associations for total vBMD were no longer significant (Supp. Table 3). Furthermore, a wide distribution of bone changes was observed among the patients (Figure 2A). The majority of patients had bone loss after 48 weeks of treatment with respect to total vBMD (60%) and cortical vBMD (63.33%), but not to trabecular bone volume fraction (50%) (Figure 2B). Clinical characteristics at baseline of patients losing bone in comparison to patients not losing bone for total vBMD, cortical vBMD, and trabecular bone volume fractions are shown in Supp. Table 4. No major differences between patients losing bone and not losing bone were observed for any of the bone parameters.

Additionally, all patients underwent bone analysis by DXA to measure aBMD of the spine and femoral neck at baseline and

Table 2. Changes in cortical and trabecular bone parameters assessed by high-resolution peripheral quantitative computed tomography*

Parameter (N = 30)	Baseline	48 weeks	*P* value	Percentage change
Total vBMD, mean (SD), mg/cm^3	272.4 (50.56)	268.7 (49.67)	**0.004**	−0.84
Cortical vBMD, mean (SD), mg/cm^3	836.2 (63.1)	830.5 (63.1)	**0.009**	−0.66
Cortical area, mean (SD), mm^2	116.3 (31.4)	114.9 (30.5)	0.056	−0.95
Trabecular bone volume fraction, mean (SD), %	0.130 (0.026)	0.129 (0.026)	**0.034**	−0.60
Trabecular number, mean (SD), 1/mm	1.87 (0.30)	1.86 (0.34)	0.68	−0.69
Trabecular thickness, mean (SD), mm	0.070 (0.012)	0.071 (0.013)	0.67	0.83

*The Wilcoxon matched-paired signed rank test was used for comparison. Significant *P* values are shown in bold. Percentage change between baseline and after 48 weeks of treatment. vBMD, volumetric bone mineral density.

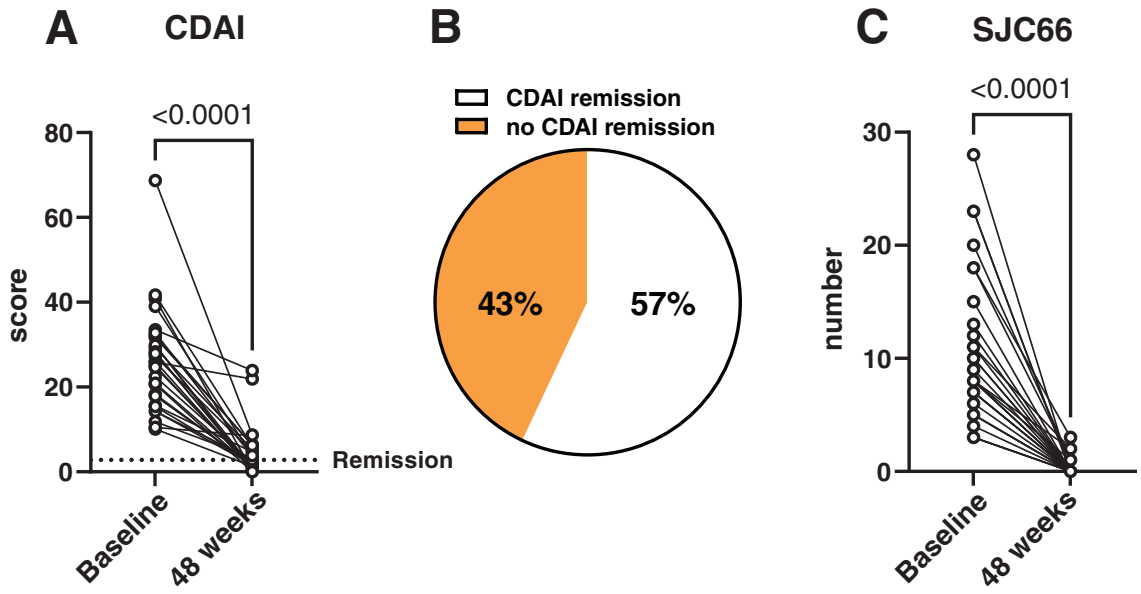

Figure 1. Differences in disease activity for patients with early rheumatoid arthritis between baseline and 48 weeks of treatment. Rheumatoid arthritis disease activity was assessed by the CDAI and the SJC66. (A) CDAI scores at baseline and after 48 weeks of treatment. The dotted line represents the limit for remission (CDAI ≤ 2.8). (B) The percentage of patients achieving remission or no remission after 48 weeks of treatment. (C) SJC66 scores at baseline and after 48 weeks of treatment. The Wilcoxon matched-paired signed rank test was used for comparison (N = 30). CDAI, Clinical Disease Activity Index; SJC66, swollen joint count in 66 joints.

after 48 weeks of treatment. Spine aBMD was significantly reduced (–0.97%, P = 0.01) after 48 weeks of treatment. The T score for spine was reduced (P = 0.02), and an increase in patients with osteopenia was observed. The Z score for spine was not changed after treatment. For the femoral neck aBMD, T score and Z score did not change during follow-up (Supp. Table 5).

Connections between baseline immune cells and overall bone change. To assess whether baseline levels of circulating immune cells (nine T cell populations [Supp. Fig. 1] and the intermediate monocyte population) were associated with overall change of bone characteristics after 48 weeks of treatment, we performed a cluster analysis by PCA. The bone

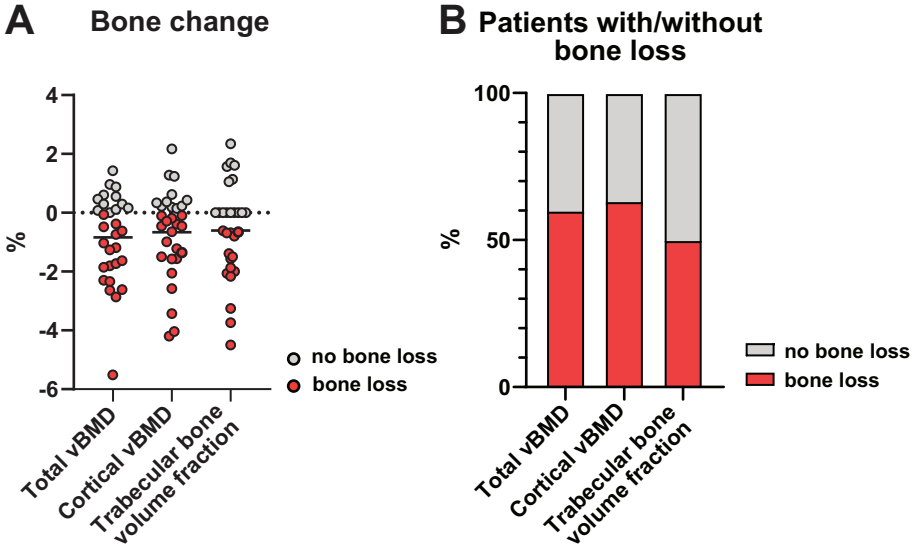

Figure 2. Distribution of bone change in patients with or without bone loss. (A) Percentages of bone change for total vBMD, cortical vBMD, and trabecular bone volume fraction. Bone loss is defined as <0% of change between baseline and 48 weeks of treatment. Means are shown with a bar, and each dot represents one patient. (B) The percentage of patients having no bone loss or bone loss after 48 weeks of treatment (N = 30). vBMD, volumetric bone density.

parameters were projected in three different quadrants, whereas the immune cell populations were spread over all four quadrants (Figure 3A). Next, we performed multivariate OPLS analysis to investigate associations between changes in individual bone parameters after 48 weeks of treatment with the baseline proportions of $CD4^+$ T cell subtypes and intermediate monocytes (nine T cell populations [Supp. Fig. 1] and the intermediate monocyte population). We found associations with several $CD4^+$ T cell populations for all bone parameters (Figure 3B–D). However, the low Q^2 values for total vBMD (Figure 3B; $Q^2 = 0.09$, *P* [CV-ANOVA] = 0.27) and cortical vBMD (Figure 3C; $Q^2 = 0.09$, *P* [CV-ANOVA] = 0.27) indicate poor predictive power of the model, which was also confirmed by the nonsignificant *P* values for the CV-ANOVA. For trabecular bone volume fraction, the Q^2 value was higher, indicating a better predictive power, as shown by the significant *P* value for the CV-ANOVA (Figure 3D; $Q^2 = 0.28$, *P* [CV-ANOVA] = 0.01).

The two most relevant positive and negative associations for each bone parameter were further analyzed by correlation tests. The change in total vBMD did not correlate with Th1Th17, $CXCR3^+$Th17, or Th2 cells at baseline (Supp. Fig. 2A–C), but a significant negative correlation with Th0 cells was observed (Figure 3E; r = –0.38, *P* = 0.04). Similarly, the change in cortical vBMD did not correlate with $CXCR3^+$ Th17, Th1Th17, or Th2 cells (Supp. Fig. 2D–F), but a significant negative correlation with Th0 cells at baseline was found (Figure 3F; r = –0.43, *P* = 0.02). The change in trabecular bone volume fraction did not correlate with Th0 cells at baseline (Supp. Fig. 2G), but a significant positive correlation with Treg cells of CD4 (Figure 3G; r = 0.4, *P* = 0.03), as well as significant negative correlations with $CXCR3^+$Th2 cells (Figure 3H; r = –0.38, *P* = 0.04) and $CXCR3^+$Th17 cells (Figure 3I; r = –0.36, *P* = 0.05) at baseline, were observed. OPLS analysis showed no associations between baseline intermediate monocytes and change in bone parameters after 48 weeks of treatment, although the proportion of intermediate monocytes was reduced after treatment compared to baseline (Supp. Fig. 3).

To identify additional possible confounders for the change in bone parameters, we performed Spearman correlation analysis between the baseline immune cell populations and baseline disease parameters. Correlations were found between CRP, CDAI, SJC28, and SJC66 for various immune cells (Supp. Table 6), and the subsequent analysis was also corrected for these parameters. To further validate our findings, we performed linear regression analysis between change in bone parameters after 48 weeks of treatment and baseline immune cell subpopulations (nine T cell populations [Supp. Fig. 1] and the intermediate monocyte population). The analysis was corrected for age, sex, glucocorticoid injections, CRP baseline score, CDAI baseline score, and SJC28 and SJC66 baseline scores. As shown in Table 3, baseline proportions of Treg cells associate positively with change in trabecular bone volume fraction (β = 0.4, *P* = 0.04). Furthermore, Th0 associated negatively with changes in total vBMD (β = –0.06, *P* = 0.03) and cortical vBMD (β = –0.07, *P* = 0.01). Changes in trabecular bone volume fraction were negatively associated with $CXCR3^+$Th2 cells (β = –0.15, *P* = 0.04) and $CXCR3^+$Th17 cells (β = –0.16, *P* = 0.04) at baseline. However, after correction for multiple testing ($P_{corrected}$ = 0.005), the associations were no longer significant.

Taken together, high baseline proportions of $CXCR3^+$Th2 cells and $CXCR3^+$Th17 correlated with trabecular bone loss, whereas high baseline proportions of regulatory T cells correlated with no trabecular bone loss in patients with early RA after 48 weeks of treatment. The correlations were confirmed as

Table 3. Linear regression between percentage change of bone parameters and immune cells at baseline*

	Percentage change, total vBMD		Percentage change, cortical vBMD		Percentage change, trabecular bone volume fraction	
Parameter (N = 30)	β	*P* value	β	*P* value	β	*P* value
Intermediate monocytes (baseline)	0.367	0.724	0.992	0.336	−1.322	0.255
Th0 (baseline)	−0.059	**0.033**	−0.068	**0.013**	0.036	0.286
Th1 (baseline)	0.106	0.141	0.020	0.784	0.114	0.163
Th1Th17 (baseline)	0.088	0.374	−0.001	0.988	0.204	0.059
Th2 (baseline)	−0.084	0.258	0.000	0.999	−0.102	0.233
$CXCR3^+$Th2 (baseline)	0.029	0.672	0.109	0.109	−0.154	**0.041**
Th17 (baseline)	−0.003	0.959	0.023	0.652	−0.060	0.286
$CXCR3^+$Th17 (baseline)	0.007	0.924	0.088	0.213	−0.161	**0.038**
Tfh (baseline)	0.111	0.123	0.052	0.481	0.116	0.167
Treg of CD4 (baseline)	0.170	0.331	−0.113	0.520	0.398	**0.039**

*Linear regression analysis with percentage change of bone parameters from baseline (dependent variable) and proportions of intermediate monocytes, Th0, Th1, Th1Th17, Th2, $CXCR3^+$Th2, Th17, $CXCR3^+$Th17, Tfh, and Treg cells of CD4 at baseline (independent variables). β values are unstandardized coefficients. Adjusted for age, sex, baseline C-reactive protein level, baseline Clinical Disease Activity Index score, baseline swollen joint count of 28 joints score and baseline swollen joint count of 66 joints score, and glucocorticoid injections. Significant *P* values after linear regression are shown in bold (*P* <0.05). Significant *P* value after Bonferroni correction for multiple testing: $P_{corrected}$= 0.005. Tfh, follicular helper T; Treg, regulatory T; vBMD, volumetric bone mineral density.

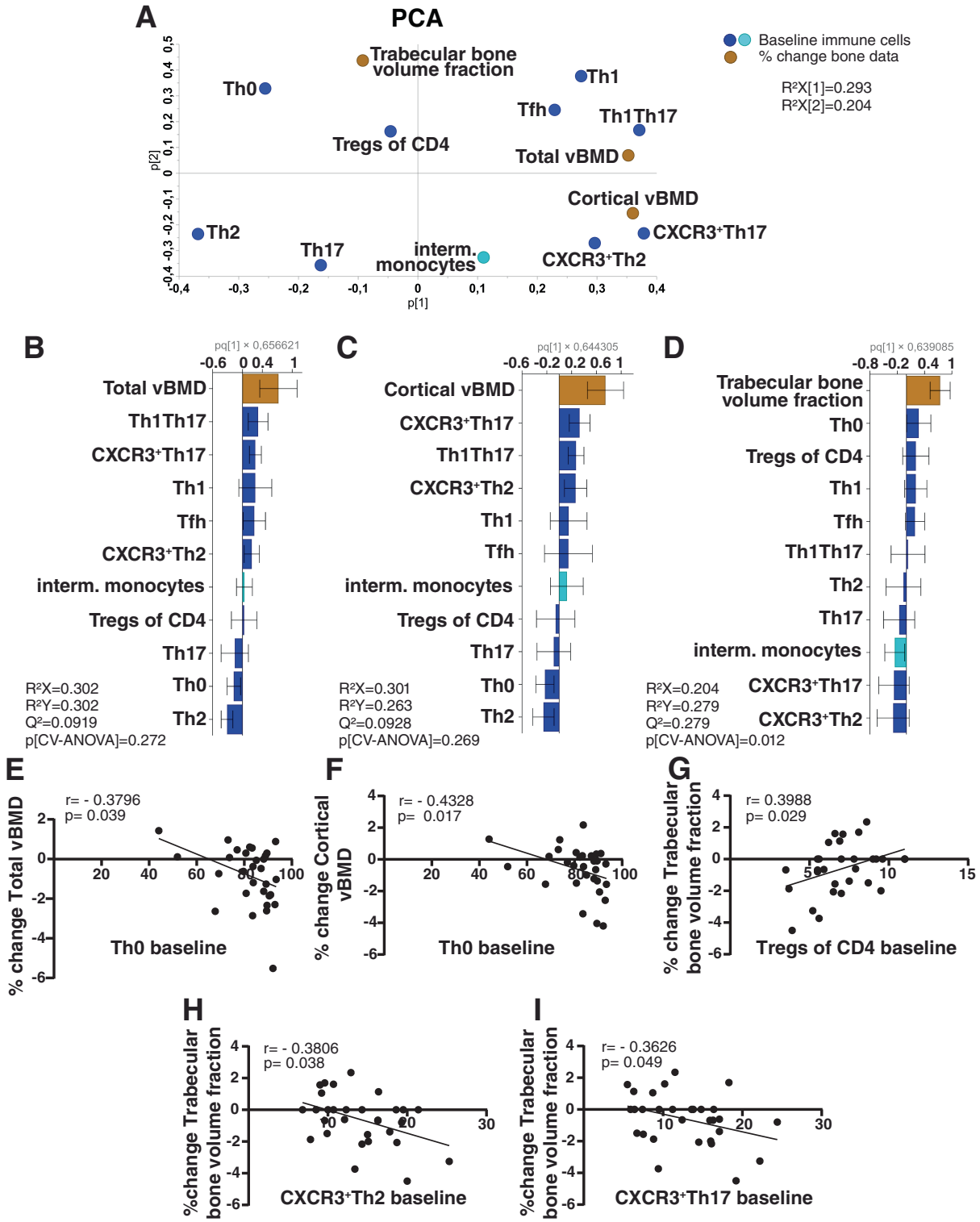

Figure 3. Relationship between baseline immune cells and change in bone parameters after 48 weeks of treatment. (A) PCA plot showing the association between baseline immune cells and changes in bone parameters after 48 weeks of treatment. Variables close to each other located on the same axis are positively associated, and variables on opposite sides of the axis are negatively associated. (B–D) OPLS column loading plots show the association between changes in bone parameters (y-variable) and baseline immune cells (x-variables). (E–I) Univariate correlation analysis between the proportion of indicated immune cells and the change in bone parameter changes. Two-tailed Spearman's rank-order correlation test was used, and regression lines are presented in the correlation plots (N = 30). CV-ANOVA, coefficient of variation analysis of variance; interm., intermediate; PCA, principal component analysis; OPLS, orthogonal partial least-squares; Tfh, follicular helper T; Treg, regulatory T; vBMD, volumetric bone mineral density.

significant associations after correction for confounding factors. However, the associations were no longer significant when corrected for multiple testing.

DISCUSSION

The immune system has major effects on bone homeostasis and RA is associated with the development of both periarticular and generalized bone loss. The effect of treatment with bDMARDs on generalized bone loss and associations between bone density, microstructure, and immune cells in patients with early RA has not been determined before. In this study, we used HR-pQCT to investigate such changes in patients with ue RA after 48 weeks of treatment with bDMARDs and explored relations between bone characteristics, intermediate monocytes, and CD4+ T cell subsets.

The results reveal an overall total bone loss from baseline to 48 weeks of treatment, including reduction of cortical vBMD and trabecular bone volume fraction, which was independent of age, sex, BMI, ACPA positivity, and glucocorticoid injections. However, it needs to be noted that the study population has a mean age of 58 years, indicating a high proportion of postmenopausal women in the group, in whom bone loss to a similar extent is expected.[43–46] Still, a direct comparison between the previous publications and the results from this study have several limitations. First, the previous studies were performed in cohorts of healthy women with mean ages spanning from premenopausal to postmenopausal. Second, except for the study by Johannesdottir et al,[46] in which HR-pQCT of the tibia was also performed, other methods to analyze BMD were applied. Finally, the time span between the bone measurements varied in the studies. Together, this makes it difficult to conclude whether the generalized bone loss measured in the tibia in this study is a result of menopause, age, and/or RA.

In comparison to the highly sensitive method HR-pQCT, we also performed the commonly used DXA measurement of spine and femoral neck at baseline and after 48 weeks of treatment. The DXA confirmed bone loss in spine, but not in the femoral neck. Previous studies of patients with early RA treated with bDMARDs also showed no or a slower decrease of BMD when measured by DXA.[8,9] Together, this indicates the need to use a more sensitive method to detect generalized bone loss in early RA.

Osteoporosis development in patients with RA has previously been shown to be related to increased disease activity.[47,48] Not all patients included in this study had reached remission after 48 weeks of treatment with bDMARDs, as was also the case in the whole NORD-STAR trial.[39] In this substudy, the group of patients with bone loss after 48 weeks of treatment did not have higher disease activity at baseline compared to the group that had no bone loss. However, after 48 weeks of treatment, the patients who had an overall and cortical bone loss received higher scores for CDAI, SJC28, and SCJ66 at that time point. Even though this was not observed for the trabecular bone, these associations confirm a link between disease activity and generalized bone loss in our study.

Although it has been shown earlier that the presence of ACPA is associated with bone loss in patients with RA,[49] we could not find an association between ACPA positivity and bone loss. The contrasting result could be due to the small study population (N = 30) in this study. Even though we found that 86.7% of the patients were ACPA positive, it needs to be mentioned that we used a commercial CCP assay, which was shown to be less sensitive than a custom array based on citrullinated peptides and proteins detected in RA synovial tissue samples.[50]

Osteoporosis in RA has been associated with higher numbers of circulating osteoclast precursors originating from the monocytic lineage.[51] Specifically, the intermediate monocytes are expanded in patients with RA and are suspected to negatively regulate BMD.[16,19,52] However, we have previously shown that intermediate monocytes are not expanded and are not associated with bone characteristics in patients with ue RA.[37] In the present study, the population of circulating intermediate monocytes was significantly reduced after 48 weeks of treatment compared to baseline, but there was no association between baseline proportions of intermediate monocytes and bone change after treatment.

RA is defined as a prototypic $CD4^+$ T cell disease,[53] but relations between baseline proportions of circulating $CD4^+$ T cell subsets and changes in bone microstructure after 48 weeks of treatment have not been studied previously. A positive correlation between the proportion of Treg cells at baseline and changes in trabecular bone was found. However, after correction for confounding factors and multiple testing, the association could not be confirmed. An imbalance between proinflammatory Th17 cells and Treg cells, with increased Th17 cells and decreased Treg cells, has been identified in RA, and this dysregulation of self-tolerance could be causal for disease onset and progression.[21] Treg cells can inhibit osteoclast differentiation and by that bone resorption. This indicates that the reduced numbers or functions of Treg cells observed in RA could result in bone loss.[21] This is in line with data from this study, in which high proportions of circulating Treg cells at baseline correlated with no loss of trabecular bone after 48 weeks of treatment.

Negative correlations between proportions of baseline $CXCR3^+$Th2 and $CXCR3^+$Th17 cells and trabecular bone loss were found. However, after correction for confounding factors and multiple testing, the associations could not be confirmed. $CXCR3^+$Th2 and $CXCR3^+$Th17 cells are found in the circulation of patients with established RA, but their levels are increased in synovial fluid compared to blood. The accumulation of $CXCR3^+$ cells in the joints might be explained by the high levels of CXCR3 ligands found in the synovial fluid.[34]

$CXCR3^+$Th17 cells secrete IL-17 and IFNγ and both cytokines are increased in the plasma of patients with early RA.[54] IL-17 is a known driver of osteoclastogenesis and anti-IL17A therapy was shown to have a positive effect on bone and

cartilage damage in inflammatory arthritis.[55] The role of IFNγ for bone homeostasis in RA is more controversial because it can exhibit anti- as well as pro-osteoclastogenic functions. However, studies have shown a shift toward pro-osteoclastogenic effects under conditions of estrogen deficiency and inflammation, which are factors that also influence RA.[56] We previously described a positive correlation of intermediate monocytes with CXCR3$^+$Th17 cells in patients with ue RA. It is tempting to speculate that the possible pro-osteoclastogenic effect of CXCR3$^+$Th17 cells in early RA could be reflected by its correlation with elevated levels of intermediate monocytes, cells that are suspected to be directly linked to osteoclastogenesis.[37]

Although Th2 cytokines are generally thought to exhibit anti-inflammatory functions, IL-4 and IL-13 can induce the secretion of proinflammatory cytokines (eg, IL-6 in RA fibroblast-like synoviocyte cultures), indicating the pathogenic potential for RA.[34] Serum IL-6 is a major predictor of bone loss in women, particularly during the first decade after menopause.[57] IL-6 is also critical for inflammatory bone loss in RA.[58] Furthermore, because of the ability of IL-4 to induce osteoporosis,[32] speculation about a potential osteopenic function of CXCR3$^+$Th2 cells during bone loss in RA is tempting.

The limitations of this study include the low number of patients, which made it impossible to investigate whether bDMARDs with different targets have differential effects on bone development and joint erosions. Further, the expected average yearly bone loss in postmenopausal women is similar to that found in this study. This makes it hard to conclude whether the bone loss is induced by hormonal changes or RA. Additionally, one year is a short time frame for the evaluation of generalized bone loss. Thus, a longer follow-up time would have been informative. Although significant correlations were observed between baseline CD4$^+$ T cell populations and bone loss, significant associations could not be confirmed after sensitivity testing. Thus, the associations found are suggestive, possibly because of the low number of patients.

To the best of our knowledge, this is the first study using HR-pQCT–based bone characterization in patients with early RA before and after 48 weeks of bDMARD treatment to evaluate the development of generalized bone loss and alterations in bone microstructure. We found that age, sex, and baseline disease activity were independent of bone loss in patients with early RA after 48 weeks of treatment with MTX combined with bDMARDs. However, higher disease activity at 48 weeks associated with bone loss. Correlation analysis between circulating immune cells at baseline and changes in bone after treatment suggest CXCR3$^+$Th2 and CXCR3$^+$Th17 cells as negative regulators and Treg cells as positive regulators of trabecular bone in RA.

ACKNOWLEDGMENTS

We thank all the patients, investigators, nurses, joint assessors, and study teams involved in the NORD-STAR study. We thank the staff at the Clinical Immunology Laboratory of the Sahlgrenska University Hospital for excellent technical assistance in collecting the flow cytometry data. We also sincerely thank the research nurse Ulrika Hjertonsson for performing the bone measurements in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Islander confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Helsinki Declaration requirements.

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