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Relation between bone mineral density and IL-17 serum levels in Serbian patients with early Rheumatoid arthritis

Abstract: Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and destruction of joint cartilage and bone. Different cytokines play important role in the processes that cause articular destruction and extra-articular manifestations in RA. The contribution of cytokines representing the Th1 (INF-γ), Th2 (IL-4) and IL-17A to the pathogenesis of early RA and bone mineral density (BMD) loss in still poorly understood. Serum samples of 38 early RA patients were evaluated for erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), C-reactive protein (CRP), anti-cyclic citrullinated peptide antibodies (anti-CCP) and for the tested cytokines (IL-17A, IL-4 and INF-γ). BMD was evaluated by dualenergyX-ray absorptiometry (DXA). Disease activity score (DAS28) calculation was assessed for all patients. Control serum samples were obtained from 34 healthy volunteers. The levels of tested cytokines were significantly higher (IL-17A, $p<0.001$; INF-γ, $P<0.001$; IL-4, $P<0.01$) in patients with early RA, compared to the healthy controls. In early RA patients, strong correlation of serum IL-17A was found with DAS28, ESR and CRP. Also, a significant negative correlation was found between serum INF-γ levels and the DAS28 score. Significantly positive correlation of BMD values and CRP, DAS28 IL-17A were also demonstrated. DXA analysis revealed that the most common site for osteoporosis was the lumbar spine followed by the femoral neck. BMD values significantly correlated with CRP, DAS28 score and IL-17A serum levels. The mean serum IL-17A levels, in patients with early RA, corresponded with disease activity, severity and BMD loss, indicating the potential usefulness of serum IL-17A in defining the disease activity and bone remodeling.

Keywords: Early rheumatoid arthritis, IL-17A, IL-4, INF-γ, BMD

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1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by severe synovitis, leukocyte infiltration, synovial membrane hyperplasia and cartilage destruction. The developed chronic inflammation is responsible for destructive mechanisms in the joint which finally induces structural damage and leads to functional disability [1]. Different cytokines play important role in the processes that cause articular destruction and extra-articular manifestations in RA [2]. Until recently, RA was considered to be Th1 and not a Th2 associated disorder. However, the pathophysiological concept of RA was radically changed by describing a new T-cell subset, named Th17 cells [3, 4].

IL-17A, a main cytokine produced by Th17 cells, induces the production the proinflammatory mediators, such as IL-1 and TNF-α from several joint cells, including synovial fibroblasts, macrophages and chondrocytes [4]. Moreover, IL-17A induces RANK (activator of NF-kB

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ligand) expression which is crucial for osteoclastogenesis and bone resorption [5]. In addition, recent report documented that TH17 cells, but not Th1 cells, cooperated with synovial fibroblasts in a pro-inflammatory feedback loop that leads the chronic destruction in RA [6], indicating that Th17 cells and IL-17A receptor signaling has been identified as a crucial pathway in turning an acute synovitis into a chronic destructive arthritis [7]. On the other hand, several studies have demonstrated elevated serum levels of IL-17A in patients with RA [8-10], as well as our recent study in Serbian patients with early RA [11]. However, even Th17 involved in RA pathogenesis has been attributed to IL-17-stimulated osteoclastogenesis [12], similar study has not been conducted in Serbian patients with early RA. Therefore, the current study was design to evaluate the serum levels of main TH1, Th2 and Th17 cytokines and bone mineral density (BMD) in patients with early RA and to analyze their potential correlation with different clinical and laboratory parameters.

2 Material and methods

2.1 Ethics Statement

The study was approved by the Medical Ethical Committee of Institute for Treatment and Rehabilitation "Niska Banja", Niska Banja, Serbia and Medical Ethical Committee of Blood Transfusion Institute in Nis, Serbia (number 03-5376/1). All subjects were informed of the details of the experiment prior to the taking of a sample of peripheral venous blood. A written informed consent document has been obtained from each participant.

2.2 Subjects and samples

Early rheumatoid arthritis patients were diagnosed according to American College of Rheumatology [13] criteria (disease duration less than 1 year and no prior use of disease modifying antirheumatic drugs or corticosteroids). Thirty eight patients (16 males and 22 females) with early RA from the Institute for Treatment and Rehabilitation "Niska Banja", Niska Banja, Serbia and thirty four, age and sex matched, healthy volunteers obtained from Blood Transfusion Institute in Nis (control group), were enrolled.

Blood samples from all subjects were obtained after overnight fasting (12h), immediately centrifuged and the sera were collected and stored at -20°C prior to evaluation of cytokines. All sera analysis was performed within seven days of blood collection and storage.

2.3 Clinical and laboratory data

Standard demographic characteristics of all subjects are presented in Table 1. All subjects underwent extensive medical examinations and serological evaluations, including measurements of rheumatoid (RF) factor (Human, Wiesbaden, Germany), Disease Activity Score [14] based on the evaluation of 28 joints (DAS28) calculated with the number of tender and swollen joints, erythrocyte sedimentation rate (ESR), determined by the Westergren method, along with the patient's global assessment of disease activity on the visual analogue scale (VAS) of 100 mm [15]. In addition, the anti-CCP antibody was tested by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Euroimmun, Lubeck, Germany).

Table 1. Demographic characteristics of patients with early RA and healthy controls

		Early RA	Healthy controls
Demographic characteristics	Number of patients	38	34
	Number of females	22 (57.89%)	19 (55.88%)
	Number of males	16 (42.1%)	15 (44.11%)
	Age (years)	57.81±11.02	53.61±10.45
	Weight (kg)	70.52±11.13	72.41±7.82
	Height (cm)	166±6.78	167±8.57
	BMI (kg/m^2)	25.57±4.29	25.85±3.56

Abbreviations: RA-patients with early rheumatoid arthritis. Results were presented as number of patients (%) or as mean ± SD.

2.4 Cytokine measurements

Serum INF-γ and IL-4 levels of all early RA patients and controls were determined by using ELISA kit (Invitrogen, Carlsbad, CA, USA), while serum concentrations of IL-17A were evaluated by using ELISA kit (BioSource, Nivelles, Belgium), according to the manufacturer's instructions.

2.5 BMD evaluation

BMD of the lumbar vertebrae (L1–L4) and left femoral neck was measured by dual energy X-ray absorptiometry (DXA) using the Choplogic Discovery QDRC device. Obtained results were presented as absolute values (g/cm^2), T and Z score. Osteoporosis was defined as a T score≤–2.5 SD the mean value of the controls, while reduced bone mass as a Z score ≤–1.0 SD the mean value of the controls, as described earlier [16, 17].

2.6 Statistical analysis

Results are presented as mean ± SD. Descriptive statistics parameters have been calculated on the database containing records for patients and control subjects. The distribution normality was analyzed using the Shapiro-Wilk test. Student's t test was used to compare between two means. Correlation between variables was performed using Pearson's correlation test. Values of $p<0.05$ were considered significant.

3 Results

The study included 38 patients with early RA and 34 healthy controls. Demographic, clinical and laboratory characteristics of patients and controls are shown in Table 1 and 2. There were no significant differences between the examined groups.

By using commercially available ELISA assay, we evaluated levels of cytokines representing the Th1 (INF-γ), Th2 (IL-4) subpopulations and IL-17A in the sera of the patients with early RA and healthy controls. As shown in Figure 1, patients with early RA showed significantly higher ($p<0,001$) mean serum levels of IL-17A, INF-γ and IL-4 ($p<0.01$) compared to the healthy controls. The most dramatic increase was observed for IL-17 in the sera of patients with early RA while RA/healthy ratio varied from

Table 2. Clinical and laboratory characteristics of patients with early RA

n=38	Parameter	Value
Clinical characteristics	Disease duration (months)	6.85±3.19
	DAS28 score	5.85±0.99
	VAS (mm)	50.39±12.59
Laboratory characteristics	ESR (mm/h)	49.26±25.93
	CRP (UI/L)	42.68±22.6
	Anti-CCP (U/ml)	65.79±36.45
	Number of RF positive patients (%)	21 (55%)
	RF (UI/ml)	130.28±100.84

Abbreviations: DAS28-disease activity score in 28 joints with sedimentation; VAS-visual analogue scale; ESR-eritrocyte sedimentation; CRP-C-reactive protein; Anti-CCP- antibody to citrulinated polypeptide; RF-rheumatoid factor. Results were presented as mean ± SD.

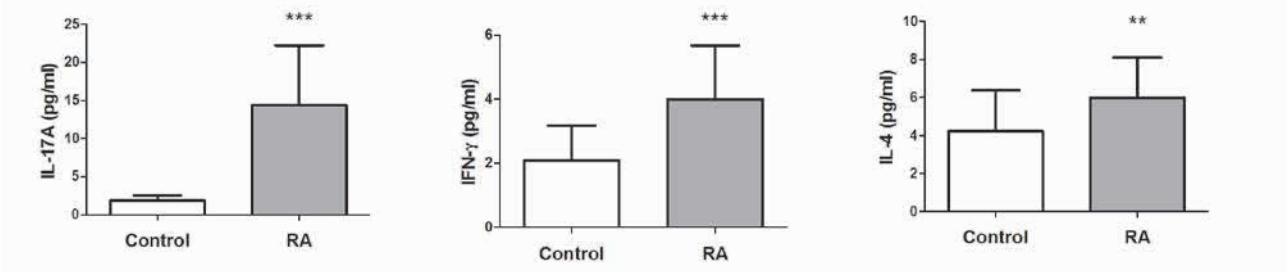

Figure 1. Serum levels of IL-17A, IL-4 and INFγ in patients with early RA and healthy controls

Abbreviations: RA-patients with early rheumatoid arthritis; IL-17A-interleukin-17; IL-4-interleukin 4; INFγ-interferon gamma;

approximately 2 for INF-γ to 1.4 for IL-4. The mean serum levels of tested cytokines in RA patients with negative RF (IL-17: 12.65±5.86; INF-γ: 4.31±1.65; IL-4: 5.58±2.05) did not significantly differ from those with positive RF (IL-17: 16.55±9.49; INF-γ: 3.62±1.69; IL-4: 6.5±2.13). We obtained significant positive correlations between serum levels of IL-17A and some inflammatory markers as ESR (r=0.524, p<0.01) and CRP (r=0.457, p<0.01). Significant correlations were demonstrated between serum levels of IL- 17A and DAS28 (r=0.462, p<0.01) and INF-γ and DAS28 score (r=-0.415, p<0.05). No significant correlations were found between serum levels of tested cytokines and patients' age, disease duration and VAS (Table 3).

The frequencies of osteoporosis (T score ≤–2.5) and reduced bone mass (Z score ≤–1), according to the BMD measurement site, are presented in Table 4. In the total patient group, osteoporosis occurred in 10% and osteopenia in 34% in the lumbar spine while 5% and 18% patients had osteoporosis and osteopenia in femoral neck, respectively. Reduced bone mass occurred in 15% patients in lumbar spine and 8% patients in femoral neck. As shown in Table 4, most common site for osteoporosis was lumbar spine followed by femoral neck. BMD values in lumbar spine significantly correlated with CRP (r=0.38, p<0.05) and DAS28 score (r=0.398, p<0.01), as well as with serum levels of IL-17A (r=0.468, p<0.01) (Table 3). There was no statistically significant correlation between patients' age, disease duration, ESR, and BMD at any anatomical site.

4 Discussion

Rheumatoid arthritis is a chronic systemic autoimmune inflammatory disease characterized by joint inflammation, T cell infiltration of synovium, synovial hyperplasia

Table 3. Correlations of serum cytokine concentrations and BMD with clinical and diagnostic parameters of patients with early RA

Parameters	Serum cytokines			BMD
	IL-17A	INFγ	IL-4	
DAS28	r=0.462, p<0.01	r=-0.415, p<0.05	n.s.	r=0.398, p<0.05
VAS	n.s.	n.s.	n.s.	n.s.
ESR	r=0.524, p<0.01	n.s.	n.s.	n.s.
CRP	r=0.457, p<0.01	n.s.	n.s.	r=0.380, p<0.05
Anti-CCP	n.s.	n.s.	n.s.	n.s.
Age of patient	n.s.	n.s.	n.s.	n.s.
Disease duration	n.s.	n.s.	n.s.	n.s.
IL-17A	n.t.			r=0.468, p<0.01

Abbreviations: DAS28-disease activity score in 28 joints with sedimentation; VAS-visual analogue scale; ESR-eritrocyte sedimentation; CRP-C-reactive protein; Anti-CCP- antibody to citrulinated polypeptide; BMD-bone mineral density in lumbar spine; n.s.-not significant, n.t.-not tested.

Table 4. Frequency of osteopenia, osteoporosis and reduced bone mass in patients with early RA

	t score		z score
	Osteopenia	Osteoporosis	Reduced bone mass
Lumbar spine	34% (13)[a]	10% (4)[c]	15% (6)[e]
Left femur	18% (7)[b]	5% (2)[d]	8% (3)[f]

Abbreviations: Osteoporosis was defined as a T score≤–2.5 SD below the mean value of young, healthy persons, and reduced bone mass as a Z score≤–1 SD below the mean value of healthy age- and sex-matched persons. a- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 17.68±8.71; IL-4: 6.5±1.61. b- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 15.001±6.4; IL-4: 5.88±2.42. c- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 8.43±3.72; IL-4: 5.28±2.66. d- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 11.34±7.71; IL-4: 4.66±1.45. e- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 14.005±3.62; IL-4: 6.67±2.05. f- Mean serum levels of IL-17A and IL-4 in the subgroup is as follows: IL-17A: 10.94±1.94; IL-4: 6.23±2.42. Values are expressed as mean ± SD.

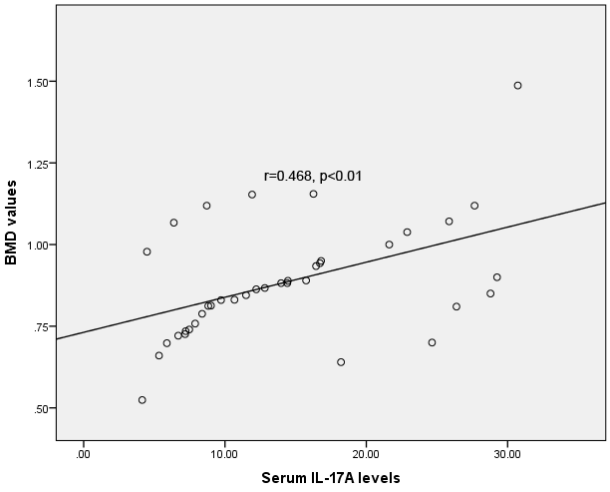

Figure 2. Correlation between serum IL-17A levels and BMD values

with cartilage and bone erosion and progressive joint destruction [18].

Results presented in our study showed markedly elevated levels of IL-17A mean serum levels in patients with early RA compared to the healthy controls, results consistent with our recent report [11]. Also, these results are in line with previous studies, demonstrating the increased serum IL-17 level in patients with well-established RA [8, 9] and early RA [10]. Further, among evaluated diagnostic parameters, mean serum IL-17A levels correlated positively with CRP, ESR and DAS28 score. Production of pro-inflammatory mediators from different joint cells, including chondrocytes, fibroblasts and macrophages, is induced by IL-17A [4]. This cytokine decreases collagen synthesis by synovium, as well as cartilage and proteoglycan synthesis in cartilage, indicating the ability of IL-17A to increase the bone destruction and reduce its formation [19]. Positive correlation of CRP, ESR and DAS28 in our study is consistent with recent reports [11, 20], indicating that IL-17A might be a potent inducer of CRP from human smooth muscle cells and hepatocytes [21]. Different reports indicated a strong correlation between IL-17A and disease activity and systemic inflammation, in patients with early RA, as assessed by DAS28 score [22, 8]. In line with those results, other report note that increased IL-17A serum levels do not correlate with ESR and CRP in patients with well-established RA, suggesting that serum IL-17A is of limited use to indicate disease activity in well-established RA [22]. These findings suggest that serum IL-17A levels may represent possible marker for development and onset of RA.

Markedly elevated serum levels of INF-γ in patients with early RA, compared to the healthy controls, correlate negatively with DAS28 score. Similar results were obtained in our recent study [11] and other report [23], indicating that treatment of patients with INF-γ resulted in significant improvement of the clinical parameters [24]. Synovial T cells, from patients with well-established RA, increase synthesis and INF-γ production compared to the T cells from early RA, after in vitro stimulation [25]. In line with these findings, earlier results have suggested that INF-γ played a key role in the resolution of synovial inflammation [22], confirming that discrepancy between INF-γ levels in early RA and other early arthritis might be important for the transition to persistent inflammation [26]. On the other hand, even we found increased serum levels of IL-4 in patients with early RA, we were not able to find any correlations with some clinical and diagnostic parameters. These observations are confirming previous results from in vitro [27] and in vivo studies [11, 23]. However, the role of IL-4 in early RA is not yet clear. IL-4 has shown pro and anti-inflammatory effects in animal models of arthritis [26]. Also, in patients with arthritis IL-4 prevents bone and cartilage erosion, indicating the ability of tissue repair [28]. Furthermore, supplementation of IL-4 to synovial fibroblasts can modulate gene expression profile, resulting with highly modulated fibroblast function [29]. On the other hand, previous studies confirmed the significant role of TNF-α in bone lesions [30, 31], but these requires further analysis.

By using DXA analysis we showed that the most common site for reduced BMD and osteoporosis, in our patients with early RA, was lumbar spine, followed by femoral neck. These findings are in agreement with earlier study [32], showing that patients with RA have a higher risk of reduced BMD than normal age and sex matched population. Also, several studies found BMD reduction in lumbar spine and femoral neck [33] or only in the lumbar spine [34] while others were not able to find any BMD reduction [35, 36]. These differences might be due to differences in the baseline characteristics and disease characteristic, including the follow up period duration. Further, disease activity, assessed by ESR and DAS28, correlated with BMD reduction in the lumbar spine. Obtained correlations are supported by different studies in early RA [37, 38]. It is well known that lumbar spine consists of 75% trabecular bone and that the inflammation tends to increase the bone turnover [39]. Increased secretion of inflammatory cytokines such as TNF-α, IL1 and IL6 in RA, which leads to chronic inflammation, increases CRP levels and induces bone resorption via osteoclast up-regulation [40]. On the other hand, presented results showed that serum IL-17A levels correlated with reduced BMD in lumbar spine in patients with early RA. These findings correspond with

increased levels of IL-17A levels in patients with early RA. To our knowledge, this is the first study reporting the association between IL-17A serum levels and reduced BMD in Serbian patients with early RA. Th17 cells represent a typical pro-osteoclastogenic Th subset [41]. In animal studies with collagen-induced arthritis, IL-17A overexpression enhances receptor activator of nuclear factor κB ligand (RANKL) expression and up-regulates RANKL/osteoprotegerin ratio in the synovium [42]. Also, IL17A acts as a potent stimulator of osteoclastogenesis by inducing RANKL expression on osteoblasts [43], suggesting that IL-17A may play significant role on bone remodeling and BMD loss in patients with RA [44].

The study has a major limitation is that the number of early RA patients is relatively low, but our results are consistent, therefore, we recommend conducting studies with a larger number of early RA patients which can shed more light on IL-17 and BMD in early RA.

In summary, we observed that, out of tested cytokines, elevated serum IL-17A levels corresponds parallel with the degree of disease activity, severity and BMD, indicating that IL-17A might play a significant role on bone remodeling and BMD loss in Serbian patients with early RA. Also, further and larger analysis should be provided in order to consider IL-17A as a possible marker in the early course of disease as well as an ideal therapeutic strategy for ameliorating the bone destruction in patients with early RA.

Conflict of interest statement: Authors state no conflict of interest

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