

The Role of Dickkopf-1 and Musculoskeletal Ultrasound in detecting Bone Loss in Rheumatoid Arthritis

Shaimaa M. Abdel Aziz¹, Mohamed G. Zaki¹, Henaz F. Khaled¹, Nayera Z. Saber^{1,*} and Nahla M. Zakaria²

¹Physical Medicine, Rheumatology and Rehabilitation, Faculty of Medicine Ain Shams University, Egypt

²Clinical and Chemical Pathology, Faculty of Medicine Ain Shams University, Egypt

*Corresponding author: Nayera Z. Saber, Physical Medicine, Rheumatology and Rehabilitation. Faculty of Medicine Ain Shams University, Egypt, Tel: +201117288825; E-mail: dr.nayera_abdelmoaty@med.asu.edu.eg

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Abbreviations: RA: Rheumatoid Arthritis; RANKL: Receptor Activators of Nuclear Factor-Kb Ligand; ACR: American College of Rheumatology; EULAR: European League Against Rheumatism; M-HAQ: Modified-Health Assessment Questionnaire; PBS: Phosphate

Buffered Saline; MCP's: Meta Carpo Phalangeal; GE: General Electric; AS: Ankylosing Spondylitis; DAS: Disease Activity Score; MSK US: Musculoskeletal Ultrasound; PD: Power Doppler; DEXA: Dual Energy X-ray Absorptiometry

Abstract

Aiming to study the possible association of Dkk-1 to bone loss detected by MSK ultrasound imaging and DEXA scanning rheumatoid arthritis patients by measuring its serum levels in relation to disease activity and severity. We conducted this study on 30 RA patients and 18 apparently healthy individuals serving as a control group. All patients and controls were subjected to history taking, clinical examination, laboratory investigations, and measurement of serum levels of DKK-1. Assessment of disease activity by using modified DAS 28 score and functional disease activity by MHAQ. Disease severity was assessed by Larsen score and musculoskeletal US to detect erosive arthritis and bone mineral density was assessed by DEXA scanning. Most of RA patients had active disease with mean DAS 28-ESR was 4.96 ± 1.08 with 26 cases had erosive arthritis (86.7%). serum DKK-1 levels were significantly higher in patients than control group ($P < 0.001$). Serum DKK-1 levels were higher in patients with more severe RA and positively correlated with joints erosions as assessed by Larsen score ($r = 0.954$, $p < 0.001$) and ultrasound Joint erosions score ($p < 0.001$). Serum Dkk1 in osteoporotic patients was significantly higher than in control group ($P < 0.001$). At serum DKK1 titre (1960pg/dl) it was a reliable diagnostic test of erosions in RA with sensitivity 100%.

Conclusion Serum DKK-1 may be a reliable biomarker of erosive arthritis detected by US, bone erosions and osteoporosis in RA. Moreover, it was highly associated with disease activity and severity.

Keywords DKK 1, Rheumatoid arthritis, bone erosions, Ultrasound, osteoporosis

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease primarily characterized by bilateral symmetrical polyarticular arthritis, which is often erosive. It is a common autoimmune disease (about 1% of the world population), affects three times as many women as men, and usually appears in middle age [1]. This disease is characterized by chronic synovial inflammation and synovial cell proliferation producing the pannus, which is responsible for bone and cartilage destruction [2]. Seventy% of patients reveal an irreversible joint damage after 2 years of disease, but 28% of RA patients already shows erosions at disease onset which is one of the hallmarks of RA [3]. Research on the mechanisms by which RA induces bone erosions has focused on the osteoclast's roles in shifting the normal balance between bone formation and resorption [4]. This imbalance is generated by key molecules that regulate osteoclast differentiation, such as cross-talk between receptor activators of nuclear factor- κ B ligand (RANKL) and Wingless (Wnt) signaling pathway, which is important for the growth and differentiation of

osteoblast [5]. Dkk is an endogenous, secreted inhibitory factor in the canonical Wnt signaling by binding the Wnt coreceptor LRP5/65. There are 4 Dkk proteins: Dkk-1, Dkk-2, Dkk-3, and Dkk-4, of which the type 1 (Dkk-1) is especially significant in the bone [6]. Dkk-1 could increase the expression of the osteoclast differentiation factors and RANKL, and decrease the expression of OPG. Therefore, the questions of whether Dkk-1 is involved in bone destruction and inflammation in RA, or whether there are relations between Dkk-1 and clinical and laboratory characteristics of human RA, have not been thoroughly clarified. In this study, we explored the potential role of Dkk-1 in RA.

The aim of this work was to address the possible association of Dkk 1 to bone erosions detected by ultrasound imaging in rheumatoid arthritis patients by measuring its serum level and relating it to disease activity and severity

Patients and Methods

This study was conducted on thirty RA Patients fulfilling the American college of rheumatology (ACR) and European League against Rheumatism (EULAR) new classification criteria [7]. Also, eighteen apparently healthy individuals matched for age and sex were included as a control group recruited from Physical Medicine,

Rheumatology & Rehabilitation out-patient clinic and internal medicine departments of Ain Shams University Hospitals. Exclusion of patients that had Paget disease, Multiple myeloma, breast cancer, bone metastasis, diabetes mellitus, hyperthyroidism, hyperparathyroidism, chronic liver and renal diseases, or autoimmune rheumatic disorders other than RA. Patients on medication that influence bone metabolism as: glucocorticoid, heparin, anticonvulsant, thyroxin, hormone replacement therapy or any drug used in treatment of osteoporosis, and Patients who were under biological treatment during last six months.

The study was conducted in accordance with the World Medical Association Declaration of Helsinki for human subjects and the study was approved by the ethics committee of the faculty of Medicine, Ain Shams University and all patients were informed and gave their written consent

All patients were subjected to the following

I-Full medical history: with special attention to number of joints affected, redness, hotness, swelling, pain, morning stiffness and it's duration, and deformities. In addition to extra articular symptoms and the used medications.

II-Thorough clinical examination with particular attention to number of tender and swollen joint counts, Assessment of disease activity using modified disease activity score (DAS-28-ESR) [8]. Assessment of functional disease activity by Modified-Health Assessment Questionnaire (M-HAQ). It includes eight activities of daily living, with 0-3 scoring for each activity [9]

III-Laboratory investigations: CBC was measured by coulter counter, ESR was evaluated by the Westergren method, CRP was examined by immuno-nephelometry, and anti-CCP was tested by ELISA. Serum samples were taken from each subject and from control then stored at -70°C until the assays were performed. Serum Dkk-1 levels were examined by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plates (Corning, Schiphol, Netherlands) were coated overnight at room temperature with monoclonal mouse anti-human Dkk-1 capture antibodies (R&D Systems) in phosphate buffered saline (PBS). (Anthos Microsystems, Krefeld, Germany). All measurements were performed in triplicate for each sample, and the mean values were calculated.

IV-Radiological investigations: 1) Plain X-Ray on both hands, wrists and feet anteroposterior and lateral views. All radiographs were scored according to the Larsen method following guidelines for scoring [10]. Joints selected were 10 metacarpophalangeal joints and both wrists, and the second to the fifth MTP joints in the scoring, with a range of 0 to 100 [11].

2) The diagnostic Musculoskeletal Ultrasound (US) for second and fifth MCPs of both hands, fifth MTPs of both feet, and the most swollen PIP (one in each hand), for a total of eight joints per patient, on the basis of their likelihood of involvement in early RA as well as the easy accessibility with US probe [12]. Synovitis, bone erosions, and power Doppler signal in the synovial membrane of the preselected joints were evaluated based on the OMERACT definitions and classified on semi-quantitative scales [13]. The Global ultrasound indices were calculated by adding scores from all joints. Using Logiq 9 ultrasound equipment from GE (General Electric Healthcare, Chalfont St Giles, UK) with a linear array transducer (9L) working with 9-13 MHz frequency. Power Doppler (PD) settings were standardized with a pulse repetition frequency of 750 Hz, a color-mode frequency of 9.1

MHz and low wall filters. The color gain was increased to the highest value not generating PD signals under the bony cortex. All joint regions were assessed by GSUS and PDUS.

*Synovitis was defined as a non-compressible hypoechoic intracapsular area (synovial thickening) 0=absent, 1=mild, 2=moderate, 3=sever synovitis [14].

*Bone erosions were defined as changes in the bone surface of the area adjacent to the joint (0=regular bone surface, 1=irregularity of the bone surface without formation of a defect seen in 2 planes, 2=formation of a defect in the surface of the bone seen in 2 planes, 3=bone defect creating extensive bone destruction) [14].

*Power Doppler signal was used to display flow signal in the synovium (0-3) (0=absent, 1=mild, 2=moderate, 3=severe) vascularity. The Global ultrasound indices were calculated by adding scores from all joints, with maximum score 24 [14].

3) Dual Energy, X-ray Absorptiometry (DEXA) scanning: used to assess bone mineral density according to WHO criteria for definitions of osteopenia and osteoporosis.

V-Data processing/Statistical analysis: The clinical, radiological and laboratory data were recorded on an investigative report form. These data were transferred to IBM card, using IBM-PC with statistical program SPSS-V-19.0 (IBM Corp, USA, 2010), for data analysis to obtain: Descriptive statistics, Analytical statistics (Student's "t" test Mann-Whitney U/Wilcoxon Rank Sum Test (Z test): Kruskal-Wallis (H) test groups regarding one non-parametric variable, Spearman rank order correlation "R": to study the relation between two nonparametric variables in the same group, Chi-Square test (X2): to analyze qualitative data). P-value=level of significance: P>0.05=Non-significant (NS), P<0.05=Significant (S) and P<0.001=Highly significant (HS). Sensitivity=True+ve/ (True+ve+False-ve) × 100, Specificity=True-ve/ (True-ve+False+ve) × 100 and ROC Curve.

Results

RA Patients group consisted of 28 females (93.3%) and 2 males (6.7%), their age ranged from 25.0-65.0 years with a mean ± SD of 46.70 ± 10 years and mean disease duration of 7 ± 6.02. and 18 apparently healthy individuals 14 females (77.8%) and 4 male (22.2%), their age ranged from 30.0 to 64.0 years with a mean of 47.32 ± 11.02 matched for age and sex served as control group. Descriptive and clinical data of the patients were expressed in (Table 1).

Clinical data	30 RA patients	
	Range	Mean ± SD
Disease Duration (years)	0.2-20	7 ± 6.02
Duration of morning stiffness (hours)	0-3	1.35 ± 0.85
Number of tender joint (28)	0.0-28.0	12.57 ± 7.36
Number of swollen joint (28)	0.0-5.0	1.53 ± 1.77
Modified DAS-28 ESR	2.50-7.14	4.96 ± 1.08
MHAQ	0.13-1.40	0.64 ± 0.39
Hg concentration(gm/dl)	7.4-15.2	11.5 ± 1.7
ESR(mm/h)	22.0-110.0	53.77 ± 20.86

CRP(mg/dl)	4-30	12.50 ± 1.300
RF Ab (IU/dl)	8-256	49.53 ± 12.07
Anti-ccp Ab(IU/dl)	2-283	71.38 ± 14.33

Table 1: Descriptive and clinical data of RA patients. DAS: disease activity score, MHAQ: modified health assessment questionnaire.

The most frequently affected joints in the form of tenderness or swelling were PIPs in 25 patients (83.3%) followed by wrists, elbows, knees, and MTPs in 24 patients (80.0%), then MCPs and shoulders in 23 patients (76.7%) then ankles in 21 patients (70%), while the least affected joints were the toes interphalangeal joints in only 8 patients (26.7%).

Among the 30 RA patients, 11 patients (36.7%) had different forms of extra-articular manifestations as myalgia and painful calf muscle (36.7%), ocular dryness, dry mouth, malaise (26.7%) and carpal tunnel syndrome (20%). None of RA patients had cardio-pulmonary, gastrointestinal, genitourinary, organomegaly, or neurological manifestations except of carpal tunnel syndrome. Larsen X-ray score values ranged from 10-35 units with a mean of 22.10 ± 7.31 units.

There were no clinically relevant changes in routine hematology and biochemistry findings except for mild to moderate anemia in 9 patients and 3 patients (10%) had thrombocytosis, mean serum Dkk-1 levels were significantly higher in patients (3165.33 ± 1415.53 pg/ml) than in control group (1144.44 ± 1028.539 pg/ml) with Z=4.23, P<0.001 (Figure 1).

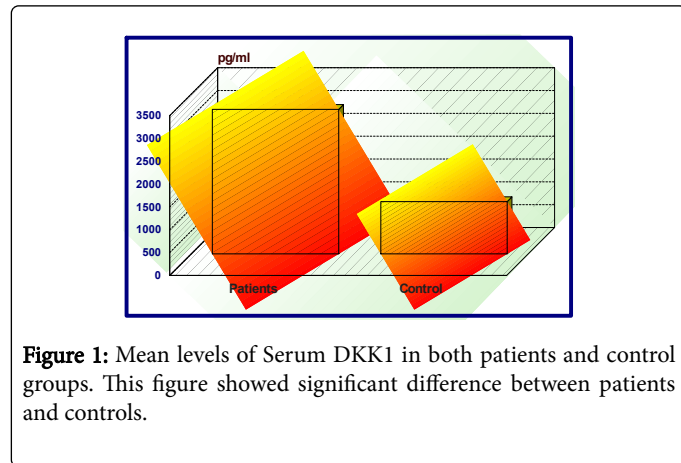


Figure 1: Mean levels of Serum DKK1 in both patients and control groups. This figure showed significant difference between patients and controls.

Relation of serum DKK-1 to clinical and laboratory data

Dkk-1 levels were highly positive correlated with Modified DAS28. A highly significant relation was found between severity of DAS-28 grades and increased titre of Dkk-1 (H=14.43, p<0.001).

In RA patients, Dkk-1 levels were positively correlated with ESR and CRP (r=0.371, p<0.05, r=0.488, p<0.001) respectively. Also serum Dkk-1 was correlated with RF and anti-CCP (r=0.105, p<0.001, r=0.263, p<0.001) respectively.

Conventional radiological and US findings

US detected erosive disease in 6 patients (20%) not detected by X-rays. Nevertheless, US of the 8 targeted joints failed to detect erosive disease in 2 patients (6.7%) that were detected by X-rays of these

patients as erosions located on other joints (third and fourth MCP joints). Combination of two techniques, revealed 26 cases of erosive arthritis (86.7%) (Figure 2). Furthermore, US Joint erosions were highly positively correlated with ESR, Modified DAS28, Larsen score and US vascularity signals (+0.488, +0.585, +0.683 and +0.546) respectively.

According to US OMERACT grading, we found that 53.3% of RA patients had G2 erosions, and 56.7% had G2 synovial hypertrophy, while 53.3% of patients had G1 of Doppler vascularity signals denoting that more than half of the patients had ongoing disease activity (Table 2).

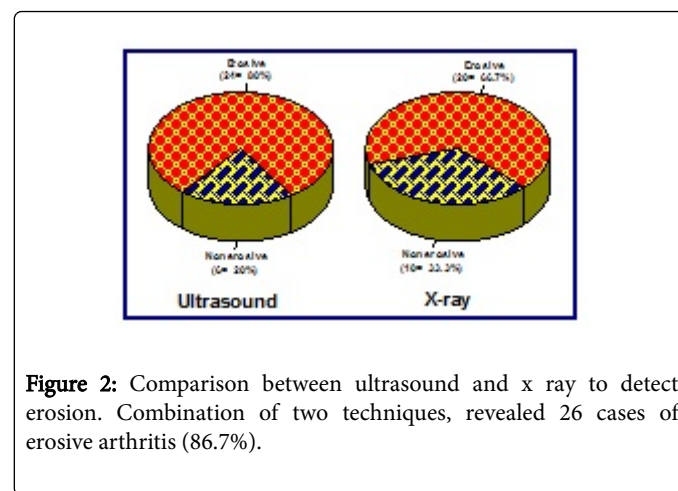


Figure 2: Comparison between ultrasound and x ray to detect erosion. Combination of two techniques, revealed 26 cases of erosive arthritis (86.7%).

Grades	Ultrasound Findings					
	Joint EROSIONS		Synovial hypertrophy		Power signals doppler	
	Frequen cy	%	Frequen cy	%	Frequen cy	%
G1	13	43.4	12	40	16	53.3
G2	16	53.3	17	56.7	14	46.7
G3	1	3.3	1	3.3	-	-

Table 2: Frequency of Ultrasound findings by OMERACT grading. G2 Synovial hypertrophy represented highest frequency of US findings.

Relation of serum DKK-1 levels to severe RA

Serum Dkk-1 levels were higher in patients with joints erosions and significantly correlated with radiological erosions assessed by Larsen score (r=+0.954, p<0.001) and also significantly correlated with ultrasound erosions score (r=+0.718, p<0.001). Moreover, a highly significant relation between increased US erosion grading as well as PDUS signals grading and increased levels of serum Dkk-1 (H=23.63, p<0.01, Z=3.10, p<0.001) respectively.

All patients underwent bone densitometry (DEXA) and they were classified according to total mean (T-score) into normal in 9 patients (30%), osteopenic in 11 patients (36.7%) and osteoporotic in 10 patients (33.3%). Serum Dkk1 levels in osteoporotic patients were significantly higher than in control group (t=4.704, P<0.001). Moreover Serum Dkk1 levels increased as BMD decreased but difference between DEXA grading (normal, osteopenic, osteoporotic)

didn't reach a statistical significant level ($H=0.91$, $P>0.05$). At serum Dkk 1 titre (1360 pg/dl) it was a reliable diagnostic test of RA with sensitivity 96.7%. At serum Dkk1 titre (1960 pg/dl) it was a reliable diagnostic test of erosions with sensitivity 100%.

Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that primarily attacks synovial joints, leading to articular destruction and functional disability [15]. Moreover, Fujita et al. found that Dkk-1 facilitates osteoclastogenesis by enhancing RANKL/RANK interaction [16]. Notably, Dkk-1 plays an important role in the promotion of synovial angiogenesis [17]. Therefore, we explored the potential role of Dkk-1 in RA in this study and its relation to disease activity and severity.

In the present study, the disease duration of 30 RA patients ranged from 0.2-20 years with a mean of 7 ± 6.02 years. We didn't select a specific disease duration, This is in accordance with Wang et al. who also didn't select a specific disease duration, the mean of their disease duration was 12 ± 7.2 years [18]. In the present study, mean serum Dkk-1 level, in patients was 3165.33 ± 258.441 pg/ml, while it was 1144.44 ± 1028.539 pg/ml in controls. There was a highly significant difference between them ($P<0.001$). This is in accordance with Orsolini et al., they showed that the mean serum level of Dkk-1 was significantly higher in RA patients than in healthy controls ($p<0.001$) [19]. In addition, authors previously assessed circulating Dkk-1 levels in patients with active RA compared with patients with OA and healthy individuals. They found that circulating Dkk-1 levels were higher in RA patients and lower in OA patients compared with healthy individuals [20]. Also, Long et al. they measured circulating Dkk-1 levels in 130 SLE patients, 100 RA patients and 50 healthy individuals, they reported that mean serum level of Dkk-1 was higher in RA patients compared to SLE patients and to healthy individuals [21]. Moreover, Wang et al. also found the serum concentration of Dkk-1 in RA patients was statistically significant higher than that in healthy controls and than in patients with OA ($P<0.0001$), AS ($P<0.0001$), SLE ($P<0.0001$), and SSc ($P<0.0001$) [18].

Diarra et al. assessed the binding of Dkk-1 to LRP6 receptor in serum samples in healthy individuals compared with patients with RA and Ankylosing Spondylitis (AS), a prototype erosive and a bone-forming disease, respectively. Dkk-1 levels were twice as high in RA compared with controls; on the contrary, Dkk-1 levels in AS were lower than controls. They proposed that Dkk-1 is a master regulator of joint remodeling, shifting the balance from bone resorption, when its expression is increased, to new bone formation, when its expression is inhibited [22].

In the present study there was a strong positive correlation between serum Dkk-1 levels and modified DAS-28 ($r=0.718$, $P>0.001$). This was in agreement with Diarra and coworkers, and Liu et al., who reported that there was a significant positive correlation between serum levels of Dkk-1 levels and DAS-28 in RA patients ($r=0.265$, $P<0.001$) [22,23]. Furthermore, in the present study a highly significant relation between severity of different DAS-28 score and increased titer of Dkk-1, suggesting a role of Dkk-1 levels in assessment of disease activity of RA patients.

In the present study, we revealed that, Dkk-1 levels were positively correlated with ESR and CRP. This was in accordance with Wang et al., who found that Dkk-1 levels were positively correlated with levels of ESR and CRP ($P<0.05$) which can be used to determine disease activity

and assess drug efficacy in patients with RA. Moreover, a significant positive correlation existed between serum Dkk-1, and RF anti-CCP concentration. As the presence of RF and anti-CCP predicted joint damage, So Dkk-1 could serve as a biomarker to identify RA patients at high risk of erosive changes. However, Wang et al., found no correlation between serum Dkk-1 levels and RF ($r=0.105$, $P>0.05$) or anti-CCP ($r=0.263$, $P>0.05$) [18]. This difference may be due to that their patients were steroid users; as it was previously explained by Hafstrom et al., who mentioned that the presence of RF and anti-CCP predicted radiographic progression in patients not treated with prednisolone but failed to predict progression in patients treated with this drug as prednisolone may modulate not only inflammation but also autoimmunity-associated pathogenic mechanisms [24].

Regarding radiological data, DEXA results showed that serum Dkk1 concentration in the osteoporotic patients group was significantly higher than control group ($P<0.0001$). This was in agreement with the main finding in Grandaunet et al., their study enrolled 238 patients with RA, that Dkk1 levels at baseline were significantly higher (2010 pg/mL) in patients developing per articular bone loss compared with patients without per articular bone loss (1332.2 pg/mL, $P<0.05$) [25]. There was also negative correlation between s.DKK-1 levels and forearm T-score, lumbar T-score and neck of femur T-score, moreover a difference was found in serum Dkk1 levels between DEXA grading (normal, osteopenic, osteoporotic) but didn't reach a statistical significant level ($H=0.91$, $P>0.05$). This was confirmed by Joseph et al., who studied serum DKK1 in 18 patients with a reduced BMD and 18 controls Serum Dkk1 expression was negatively correlated with lumbar and femoral T and Z-scores and in the same study serum Dkk1 concentration in the osteoporosis group was significantly higher than control group (941 ± 116 vs. 558 ± 47 pg/ml, $p<0.001$) [26].

Among the present 30 RA patients Larsen X-ray score values ranged from 10-35 units with a mean of 22.10 ± 7.31 and serum Dkk-1 levels were highly significant positive correlated with erosion score ($r=0.954$, $P<0.001$). This was in accordance with findings of Garnero et al., who reported a higher rate of radiologic progression, assessed by Sharp score, in patients with increased DKK-1 levels; each standard deviation increase in DKK-1 levels associated with a relative risk of progression of 1.65 [27]. Also Liu et al. and Wang et al., reported that serum DKK1 was correlated with Sharp score ($p<0.05$, $p<0.001$) respectively [18,23].

Furthermore, analysis of musculoskeletal US scanning revealed that DKK-1 levels also showed a highly significant positive correlation with US joint erosions ($r=0.718$, $P<0.001$). This was in accordance with Orsolini et al., they reported that patients with erosions had serum levels of Dkk-1 slightly higher than those without erosions [19]. In the present study, there was a highly significant positive correlation between US score of bony erosions and Larsen score ($r=0.683$, $p<0.001$). Therefore, scored radiographs and ultrasound Joint erosions can be used as an outcome measure to assess the severity and progression of RA.

Due to work time limitations we did US examination on second and fifth MCPs of both hands, fifth MTPs of both feet, and the most swollen PIP (one in each hand), for a total of eight joints per patient. However, we detected bony erosions more than that by X-rays, US detected erosive disease in 6 patients (20%) not detected by X-rays. Nevertheless, US of the 8 targeted joints failed to detect erosive disease in 2 patients (6.7%) that were so detected by X-rays. In these patients, erosions located on other joints (third and fourth MCP joints). Combining both techniques, 26 cases of erosive arthritis (86.7%) were detected. These results are consistent with Wakefield et al., and

Brentano et al. their study showed that in 40 RA patients US performed on the MCP joints of the dominant hand, detected 6.5-fold more joints with erosions than that detected with X-rays. On other hand, X-rays detected erosion in another 10 (8.8%) patients not detected by US, so combined both techniques revealed 52 (45.6%) patients with erosive diseases [28,29]. So if performing US on a limited number of joints, this reduces the time for examination, it also decreases the capacity to detect erosions. Meanwhile, US cannot replace radiography for the detection of erosion, and when both the techniques are combined, they show complementary efficiency and display the best results. Dohn et al., confirmed that US acts almost as well as MRI and CT for the detection of erosions [30]. Hence, adding US to a clinical prediction rule in early RA raises the predictability of RA diagnosis [31].

We noticed that Dkk-1 serum levels significantly increased with severity of US vascularity which reflects that Dkk-1 was associated with active synovitis. *Ziswiler & Tamborrini* confirmed that US is a valid tool and is more sensitive than clinical examination in the detection of synovitis [32].

In the present study, US Joint erosions were high significant positively correlated with DAS28, ESR, CRP, RF and anti CCP ($p < 0.001$). However, no correlation was found between US Joint erosions and other clinical parameters, this points out that US is able to detect changes in clinically silent joints, this gives US more diagnostic importance as early predictor of joint damage. This also was in agreement with Ohrndorf et al., who mentioned that there was a significant correlation between changes in the 7-joint ultrasound (US7) score obtained by (GSUS and PDUS) and both DAS28, ESR and CRP. It is an appropriate supplementary instrument to DAS28, CRP and ESR for gaining information about the activity of joint processes and therapeutic follow-up of RA [33,34].

In conclusion, our studied results suggested that serum Dkk-1 is a novel osteoclastogenic marker, it played a role in RA-associated joint damage and is a promising diagnostic and prognostic biomarker for both diagnosis as well as predict disease severity and joint destruction detected by ultrasound imaging

Statement of Disclosures

All authors declare that there was no conflict of interest, no funding sources in preparing this work. All authors participate in clinical assessment, in manuscript editing, prepare patients data for statistical analysis

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