The association of serum RANKL levels with disease activity and hematological parameters in Syrian patients with rheumatoid arthritis

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\textbf{A B S T R A C T}

Our study aims to detect whether the serum RANKL could be a novel potential biomarker for activity and diagnosis of rheumatoid arthritis (RA). It included fifty-eight of RA patients and thirty of equal age and sex matched controls. Disease activity was determined by using DAS28-ESR. Serum Levels of RANKL were assayed by ELISA and compared with parameters such as ESR, CRP, Rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA). The serum RANKL levels were higher in RA patients compared to controls. There was an increase in its levels mean among post-menopausal patients compared to post-menopausal healthy group. RANKL levels were also higher in ACPA positive patients than ACPA negative. Our study found a correlation between RANKL levels and some parameters: DAS28, ACPA, CRP, and symptom duration. There was a moderate inverse correlation between RANKL levels and BMD. By ROC curve, our results displayed that the best cut-off value of RANKL was 178.99 pg/ml (sensitivity 79.31%; specificity 90%) to differentiate between RA patients and controls. In conclusion, elevated serum RANKL can be used as an indicator of disease activity and a diagnostic new biomarker in patients with early RA.

\textbf{1. Introduction}

Rheumatoid arthritis (RA) is a chronic autoimmune that affects approximately 0.5–1.5% of the world population [1,2]. The disease is demonstrated with synovial inflammation, joint pain, damage of articular cartilage, bone erosions, and extra-articular manifestations [3,4]. If RA diagnosis is detected late, it will lead to an important health problem associated with functional disability and increase in a mortality [5]. Bone erosions and articular deformities are one of the most important RA manifestations. Osteoporosis is estimated approximately in 32% of RA patients [5,6]. Starting with early treatment is one of the most significant steps in order to prevent an irreversible joint damage and disease progression [7,8]. The inflammatory parameters currently available for established rheumatoid arthritis are (ESR, CRP). Although ESR and CRP measurements are imperfect, both of them play a significant role in RA diagnosis, but the guidelines do not specifically recommend routine monitoring of them in all RA patients. CRP levels vary with age, sex, and race and ESR is affected by many factors such as age, sex, fibrinogen levels, hyper-gammaglobulinemia, and anemic conditions [7,9,10]. Other biomarkers, anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF) have been used in RA diagnosis according to American College of Rheumatology (ACR 2010) criteria [2,7,11]. ACPA appear in serum several years before the onset of the disease and show a high specificity (up to 95%). However, ACPA antibodies presented positively in some cases such as tuberculosis and scleroderma [2]. In addition, RF antibodies elevate in some inflammatory conditions, other autoimmune diseases, and some healthy people. About (33%) of RA patients are ACPA sero-negative [2] and (30–45%) are RF sero-negative [7]. The sensitivity of ACPA and RF in established RA range from 56 to 80% and 60–86% respectively [2,12]. While for early RA, the sensitivity of RF is 57% and its low specificity ranges between 70 and 85% [12]. For this reason, there is necessary to evaluate disease activity by searching for a new specific dependable biomarkers that may interfere in rheumatoid arthritis pathogenesis, lead to diagnosis and recognize patients who have a high risk [13,14]. The research has recently concentrated on RANKL/RANK/OPG pathway that stimulates osteoclasts and switches the normal balance towards bone resorption [6,15,16]. This pathway is highly implicated in inflammatory bone resorption [17]. It is composed of three key proteins that attribute to the superfamily of the TNF-α: Osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK), and soluble RANKL (sRANKL) [17,18]. Receptor activator of NF-κB ligand (RANKL) is a fundamental protein for

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differentiation, survival, and activity of osteoclasts. It is not only expressed in both osteocytes and osteoblasts; but also in other cells such as B cells, synovial cells, activated T cells, and natural killer cells [19, 20]. It is up-regulated by pro-inflammatory cytokines. RANKL-RANK interaction drives to bone resorption [5]. While the action of RANKL is inverted by OPG which prevents bone destruction thus inhibits osteoclastogenesis [21–23]. Recent research has indicated that RANKL is over expressed in the synovium and supports bone erosions in RA patients [5]. It can be detected not only in the synovial fluid; but also in the sera of RA patients [17,23,24]. Also, high levels of RANKL have been related with ACPA, RF antibodies, disease activity, and bone mineral density (BMD), especially in newly diagnosed patients reflecting its role in RA pathogenesis [17,25]. The goal of our study was to evaluate the serum level of RANKL and its association with disease activity and hematological parameters in RA newly diagnosed Syrian patients.

2. Materials and methods

Study design: Our cross-sectional study was done during the period from January 2020 to March 2021. The participants who registered in our study were 58 (47 females and 11 males) newly diagnosed of RA Syrian patients a with mean age (48.71 ± 10.45) years and 30 (23 females and 7 males) age and sex-matched persons as a healthy group. Patients were obtained from the Rheumatology Clinic in Damascus at Almoujtahed Hospital and Al-Mowasat Hospital. They were newly diagnosed according to the 2010 ACR/EULAR criteria.

Inclusion criteria: Only newly diagnosed with early RA patients and their ages from 18 to 65 years. They weren’t subjected to any treatment and the symptoms onset was < 2 years (early stage).

Exclusion criteria: patients with coexistence of other systemic autoimmune diseases except Sjogren’s syndrome, patients having chronic pathologies such as liver and kidney diseases, patients with metabolic bone diseases (osteoporosis, current fractures, thyroid diseases, Paget disease, multiple myeloma, diabetes mellitus, and malignancy), and patients under treatment distress bone metabolism (steroids, vitamin D supplementations and thyrinox).

Ethical approval: Our research was performed in accordance with Helsinki Declaration and agreed by the Ethics Commission (No.3/February 26, 2020). Written informed consent was acquired from all patients.

Clinical Data collection: The following clinical tests were ordered and collected for all patients: The tender joint count (TJC) is associated with the amount of inflamed synovial tissue, the duration of symptoms, VAS: visual analogue scale, Hb: hemoglobin, WBC: white blood cell, RF: rheumatoid factor (RF), Anti-CCP: anti-cyclic citrullinated peptide (Anti-CCP).

Assessment of disease activity: The disease activity was estimated based on disease activity score 28 (DAS28) examining ESR, the number of swollen joint count (SJC), tender joint count (TJC), patient’s general health (GH; patient assessment of disease activity using a 100 mm visual analogue scale (VAS) with 0 = best, 100 = worst), then (DAS28-ESR) was calculated:

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\text{DAS28-ESR} = 0.56 \sqrt{(TJC28)} + 0.28 \sqrt{(SJC28)} + 0.70 \text{Ln (ESR)} + 0.014 \text{ (GH)} [26].
\]

Disease activity was explained by the following: clinical remission DAS28 ≤ 2.6, low disease activity (2.6 < DAS28 ≤ 3.2), moderate disease activity (3.2 < DAS28 ≤ 5.1), or high disease activity (DAS28 > 5.1) [27].

Bone mineral density (BMD): It was done for only 40 of RA patients, expressed as (gr/cm²) and measured at the proximal femur and lumbal spine (L1–L4) using dual-energy X-ray absorptiometry (DXA) machine. The result was interpreted based on T-score and evaluated according to World health organization (WHO) as the following: normal T scores > 1, Osteopenia when T scores –1 to –2.5, osteoporosis when T-scores ≤ –2.5 [28].

Examination of the serum RANKL levels: Serum RANKL was carried out according to the instructions of “HumanTNFSF11/RANKL PicoKine™ ELISA Kit” which purchased from Boster Biological Technology (Pleasanton, USA) and the Catalog Number: EK0842, intra-assay coefficient of variation 4.8%, inter-assay coefficient of variation 5.2%). The sRANKL ELISA kit was completed to detect free sRANKL without the OPG bond. The assay was performed using two kinds of antibodies: the detection antibody and polyclonal antibody. In this method, the test samples, standards were subjoined to the wells. Then biotinylated detection antibodies were added and then followed by washing with PBS or TBS buffer. Avidin–Biotin– Peroxidase Complex (ABC-HRP) was added into appropriate wells and unbounded conjugates were washed away. The following step was TMB adding to visualize HRP enzymatic reaction and produce a blue color product (Its wavelength between 450 nm and linearly was proportional to the human RANKL in the sample captured in plate. A standard curve was created by blotting absorbance of each standard concentration against RANKL concentration. All samples’ concentrations were obtained from standard curve, then multiplied by 2 (dilution factor). The range of detection was 78–5000 pg/ml. The Sensitivity of the assay kit for sRANKL was < 10 pg/ml [29].

2.1. Characteristics of RA patients

Our study demonstrated that 48 (82.8%) of RA patients were ACPA or RF antibodies positive, while 42 (72.5%) of RA patients were both ACPA and RF positive antibodies. There were four (6.9%) ACPA and RF seronegative RA patients. According to DAS28-ESR, 34 (58.6%) of RA patients had the highest disease activity, 22 (37.9%) had a modest activity, and 2 patients (3.4%) had a little disease activity at diagnosis.

Table 1

| Variables            | Mean ± SD | Range (min, max) |
|----------------------|-----------|------------------|
| Age (years)          | 48.71 ± 10.45 | 43 (24–67)       |
| TJC                  | 9.66 ± 4.56  | 18 (2–20)        |
| SJC                  | 5.43 ± 2.60  | 12 (0–12)        |
| DAS28-ESR            | 5.39 ± 0.98  | 4.23 (7.4–3.17)  |
| ESR (mm/h)           | 44.79 ± 16.62 | 65 (10–75)       |
| CRP (mg/L)           | 20.55 ± 22.66 | 105.8 (0.2–106)  |
| VAS (%)              | 0.67 ± 0.20  | 0.80 (0.2–1.0)   |
| (g/dl) Hb            | 11.94 ± 1.18 | 6.1 (8.9–15)     |
| WBC (x10³/mm³)       | 8.11 ± 2.09  | 9.3 (3.5–12.8)   |
| Calcium (mg/dl)      | 8.88 ± 0.74  | 3.9 (6.10–10)    |
| RF (IU/L)            | 121.47 ± 133.78 | 706 (4.710)     |
| Anti-CCP (U/L)       | 375.02 ± 345.75 | 1316.5 (3.50–1320.0) |
| Symptom’s duration (months) | 8.05 ± 3.63 | 13 (3–16) |

TJC: tender joint count, SJC: swollen joint count, ESR: Erythrocyte sedimentation rate, DAS28: Disease activity score in 28 joints, CRP: C-reactive protein, VAS: visual analogue scale, Hb: hemoglobin, WBC: white blood cell, RF: rheumatoid factor (RF), Anti-CCP: anti-cyclic citrullinated peptide.
Comparison between the two study groups of RA patients in terms of sex, age and menopause.

| Variable | RA patients group (N = 58) | Control group (N = 30) | Chi-Square Tests (P-value) |
|----------|--------------------------|------------------------|---------------------------|
| Sex      |                          |                        |                           |
| Male     | 11 (19%)                 | 7 (23.3%)              | 0.630                     |
| Female   | 47 (81%)                 | 23 (76.7%)             |                           |
| Age categories |                  |                        |                           |
| Less than 40 | 9 (15.5%)             | 6 (20%)                | 0.491                     |
| Between 40 and 49 years | 22 (37.9%)           | 14 (46.7%)             |                           |
| 50 and above | 27 (46.6%)           | 10 (33.3%)             |                           |
| Menopause |                        |                        |                           |
| Pre-menopause | 31 (65%)              | 18 (78.3%)             | 0.132                     |
| Post-menopause | 16 (35%)              | 5 (21.7%)              |                           |

Comparison of serum RANKL between RA patients and control according to menopause.

| Serum RANKL (pg/ml) | RA group Postmenopausal N = 16 | Control group Postmenopausal N = 5 | Mann-Whitney Test P |
|---------------------|---------------------------------|-----------------------------------|---------------------|
| Mean ± SD           | 240.89 ± 159.73                 | 148.31 ± 14.63                    | 0.002               |
| Median              | 201.21                          | 145.02                            |                     |

The difference of RANKL levels in RA patients group according to DAS28.

| Variable | RANKL Levels (pg/ml) | Kruskal-Wallis Test P |
|----------|----------------------|-----------------------|
| Low (n = 2) | Mean ± SD            | 207.75 ± 38.7         | 0.963                 |
| Moderate (n = 22) | Mean ± SD         | 258.16 ± 158.73       | 205.14                |
| High (n = 34)  | Mean ± SD            | 243.65 ± 101.66       | 219.19                |

Comparison of serum RANKL in RA patients according to RF antibodies.

| Serum RANKL (pg/ml) | RA patients (RF Neg) N = 10 | RA Patients (RF Pos) N = 48 | Mann-Whitney Test P |
|---------------------|-----------------------------|-----------------------------|---------------------|
| Mean ± SD           | 217.57 ± 32.72              | 254.24 ± 135.05             | 0.673               |
| Median              | 226.16                      | 206.75                      |                     |

Fig. 1. Comparison of serum RANKL between RA group (N = 58) and the healthy group (N = 30).

Fig. 2. Comparison of serum RANKL in RA patients according to ACPA antibodies.

2.2. Statistical analysis

Statistical analysis was achieved using Statistical Package for the Social Sciences (SPSS) version 25 and Excel 2019 programs. Data were expressed as mean ± SD for quantitative and median and percentiles for quantitative non-parametric measures. Tests that have been relied upon 1). The Kolmogorov-Smirnov test to check whether the data within the normal distribution or not. 2). Mann Whitney test to verify the differences in two independent groups. 3). Kruskal–Wallis test: for comparison between more than two patients’ groups for non-parametric. 4). Ranked Spearman correlation test to study the relationship between each two variables for non-parametric data. 5). Chi-square (χ2) test was used for comparison of variables including categorical data variables. The ROC (receiver operating characteristic) curve was used to determine the standard cut-off of sRANKL and assess the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). P-value < 0.05 was considered statistically significant.

Results: The laboratory and clinical features of the RA patients are demonstrated in Table 1. There were no statistically significant differences in between the RA patients and the healthy control group in terms of sex, age and menopause state (Table 2).

2.3. Determination of serum RANKL levels in study groups

There was an increase in serum RANKL levels which ranged from 168.17 to 870.9 pg/ml with a mean (247.92 ± 124.1 pg/ml) in RA patients compared to healthy control, ranged from (133.1–178.55 pg/ml) with a mean (166.57 ± 13.6 pg/ml) and a statistically significant difference was (P = 0.0001) in Fig. 1. Our study found an elevation of RANKL levels mean in post-menopausal patients (240.89 ± 159.73 pg/ml) compared to post-menopausal healthy group (148.31 ± 14.63 pg/ml), p-value was 0.002 (Table 3). Our result showed that there wasn’t a real statistically significant differences in RANKL levels between RA patients’ groups according to disease activity (p = 0.963) (Table 4). In addition, the mean of sRANKL was higher (259.39 ± 133.19 pg/ml) in ACPA sero-positive patients than those ACPA sero-negative with a mean (192.82 ± 28.68 pg/ml, P = 0.04) as shown in Fig. 2. On the other hand, we didn’t find a significant difference in serum RANKL levels between the two groups of RA patients according to positive or negative RF antibodies (P > 0.05), (Table 5).
2.4. RANKL correlations with laboratory parameters in RA group

Our study had exposed a moderate positive correlation between serum RANKL levels with each of the following variables: DAS28 (r = 0.4, P = 0.04), ACPA (r = 0.32, p = 0.048), disease duration (r = 0.34, p = 0.008), and a weak correlation with CRP (r = 0.258, p = 0.05). No correlation was observed with ESR, TJC, SJC, RF, and VAS. With regard to BMD, we found a moderate inverse correlation between the serum RANKL levels and BMD at the level of lumber spine (r = 0.439, p = 0.005) and femoral neck (r = 0.406, p = 0.007) (Table 6, Fig. 3).

The ROC curve displayed that the best serum cutoff of RANKL concentration was 178.99 pg/ml with AUC of 0.902 (95% CI., 0.841–0.962; p < 0.001) to differentiate between RA patients and healthy groups (Table 7, Fig. 4). Our results pointed that 49 (46 true positive, 3 false positive) were RANKL positive, and 39 (27 true negative, 12 false negative) were RANKL negative according to the RANKL cut-off (Table 8).

Table 6
The correlation between RANKL levels and study variables.

| Serum RANKL Levels (pg/ml) | Spearman’s rho | Spearman’s rho P |
|---------------------------|----------------|-----------------|
| Variables                |                |                 |
| ESR (mm/h0)              | 0.161          | 0.227           |
| CRP (mg/l)               | 0.258          | 0.050           |
| RF (IU/L)                | 0.086          | 0.522           |
| Anti-CCP (U/L)           | 0.320          | 0.04            |
| TJC                      | 0.215          | 0.105           |
| SJS                      | 0.074          | 0.580           |
| VAS(%)                   | 0.157          | 0.238           |
| DAS28-ESR                | 0.4            | 0.04            |
| Symptom’s duration       | 0.346          | 0.008           |
| Lumbar spine BMD (g/cm²) | −0.439         | 0.005           |
| Femoral neck BMD (g/cm²) | −0.406         | 0.007           |

3. Discussion

Bone erosions and cartilage degeneration are the most important clinical manifestations of rheumatoid arthritis [30,31]. Recently, studies have focused on that RANKL is implicated in causing bone loss in RA [5,32]. Application of a such novel biomarker is observed widely as a key plan for improving the success degree of RA diagnosis. RANKL has both clinical validation (linking with disease biology) and analytical validation that its testing uses a modest serum assay and can easily be achieved by routine laboratory and provided reliable data [33]. Some of studies had indicated that RANKL concentrations were augmented several years before symptom start [25], other study proposed that RANKL levels can predict RA diagnosis over clinical biomarkers mainly in sero-negative RA patients [34]. A positive association was found between serum RANKL levels and DAS28 in some studies [35,36]. These studies had confirmed that increased RANKL level may reflect RA activity and may be added as an additional biomarker to assess disease activity.

In our study, the increase of serum RANKL levels in RA patients compared to the control group can be explained by the effect of inflammatory cytokines that support increased production of RANKL [37]. Our study is consistent with Półtorak et al. study [38], Bruno et al. study, and Boman et al. study in Sweden [19,37]. In contrast, there was no
RANKL levels positively correlated with DAS28, Anti-CCP, and disease group compared with ACPA seronegative. It could be explained by the increase in serum RANKL levels in ACPA sero-positive RA patients similar in Çakırca et al. study in Turkey [41]. There was a significant post-menopausal healthy group, this confirms its importance in bone elevation in RANKL levels in post-menopausal patients compared to clinical remission or in low disease activity [40]. Our study also found an elevation in RANKL levels in post-menopausal patients compared to post-menopausal healthy group, this confirms its importance in bone loss and pathogenic mechanism of rheumatoid arthritis as found in a similar in Çakırca et al. study in Turkey [41]. There was a significant increase in serum RANKL levels in ACPA sero-positive RA patients’ group compared with ACPA seronegative. It could be explained by the direct effect of these auto-antibodies in upregulation of RANKL by certain immune cells or osteoclasts [41]. Our study presented that serum RANKL levels positively correlated with DAS28, Anti-CCP, and disease duration. A moderate inverse correlation was found between serum RANKL levels and BMD at the level lumber spine and femoral neck. A similar negative correlation was found by some studies [41,42]. The ROC curve analysis exhibited the best serum cut-off of RANKL level was 178.99 pg/ml with AUC of 0.902 (sensitivity: 79.31% and specificity: 90%). This confirms the possibility of using serum RANKL in established diagnosis of rheumatoid arthritis. In Burska et al. study, the cut-off of sRANKL was 500 pmol/L, AUC was 0.680 (specificity: 79%; sensitivity: 47%, PPV was 70%, and NPV was 59% (to classify RA patients/non-RA [34]. This discrepancy may be due to sensitivity of the used kits in assaying of serum RANKL.

Finally, we can conclude that the clinical validity of RANKL is related to its relationship with disease complications such as articular malformations, disease activity and related to BMD. The close follow-up of the serum RANKL in early RA patients may be a predictor of possible osteoporosis. So, finding such a sensitive indicator for inflammatory activity predicts the extent of tissue damage and bone degradation. Also, the clinical impact of RANKL assay is manifested in confirmed diagnosis of disease, and the initiation of treatment early. Clinical trials suggest that Denosumab, a human monoclonal antibody and antagonist of RANKL, may increase BMD, prevent progression of joint damage, and suppress bone erosions. In future, clinical utility of neutralizing RANKL may be is an effective, safe and cost-effective option for the treatment of RA in combination with Conventional synthetic DMARDs (csDMARDs) [43].

### 3.1. Limitation and powerful of the study

The powerful of our study is the use of high sensitivity ELISA method and RANKL concentration is measured in early onset of RA before therapy. The study is limited by the low number of patients and a lack of information such as other bone turn over markers. However, the overall results of the current study are generally consistent with many previous studies. It might be much more reliable if we avoided some limitations in the method, we applied such as the sample size.

### 4. Conclusion

Our results demonstrated that measurement of sRANKL could become a novel biomarker for both diagnosis and disease activity of rheumatoid arthritis.

### Declaration of competing interest

None declared.

### Data availability

Data will be made available on request.

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