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Rama Hussein (Ramahusseinph@gmail.com)
Damascus University
Imad Abokhamis
Damascus University

Article

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The association of Serum RANKL levels with disease activity and hematological parameters in Syrian patients with Rheumatoid Arthritis

Rama Hussein*[1], Imad Abokhamis[1, 2]

[1]Department of Microbiology, Hematology and Immunology, Faculty of Pharmacy, Damascus University, Syria.
[2]Assistant Professor, Instructor in Department of Hematology and Immunology, Faculty of Pharmacy, Damascus University, Syria.

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 and symptom duration. No correlation was observed with ESR, TJC, SJC, RF, VAS, CRP. There was a moderate inverse correlation between RANKL levels and BMD. By ROC curve, our results displayed that the best cutoff value of serum 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.

Key words: Rheumatoid Arthritis, RANKL, Disease activity, Parameters.

Introduction:

Rheumatoid arthritis (RA) is a chronic autoimmune and obdurate disease[1,2] that affects approximately 0.5–1.5% of the world population. It is more common about 2–3 times in women than men with an augmented occurrence in the age of 40–50 years [3]. It is demonstrated with a synovial inflammation, joint pain, damage of articular cartilage, bone erosions and extra-articular manifestations [4,5]. If the disease is detected late, it will lead to an important health problem associated with functional disability and increase in a mortality [6]. Bone erosions and articular deformities are one of the most important manifestations of rheumatoid arthritis and osteoporosis is estimated approximately in 32% of RA patients [6,7]. Starting with early treatment and reaching to the target is one of the most significant steps in order to prevent an irreversible joint damage and thus disease progression [8,9]. The inflammatory parameters currently available for established rheumatoid arthritis such as (ESR, CRP) are non-specific for disease [9,10]. Other biomarkers, anti-cyclic citrullinated peptide antibodies (ACPAs) and rheumatoid factor (RF) antibodies have been used in RA diagnosis according to American College of Rheumatology (ACR 2010) criteria [1,9,11]. 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[1]. In addition, RF antibodies elevate in some inflammatory conditions, other autoimmune diseases and some healthy people. About (33%) of RA patients are ACPA-seronegative [1] and 30% to 45% are RF seronegative despite the appearance of symptoms [9]. The sensitivity of ACPA and RF in established RA range from 56-80% and 60 to 86% respectively [1,12]. While for early RA, the sensitivity of RF is 57% and its low specificity ranges between 70-85%[12]. On the other hand, it is estimated that about 70% of joints may be destroyed during the second year of disease onset [2]. 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 of pathological alterations earlier [13,14]. The research has recently concentrated on RANKL/RANK/OPG pathway that stimulates osteoclasts and switches the normal balance towards bone resorption in rheumatoid arthritis thus preventing bone formation [7,15,16]. 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]. RANKL (receptor activator of NF-κB ligand) is a fundamental protein for 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]. The binding of RANKL with its receptor (RANK) drives to bone resorption [22]. The action of RANKL is inverted by OPG which prevents bone destruction by blocking the RANKL-

RANK interactions and thus inhibits osteoclastogenesis [23–25]. Recent research has indicated that RANKL is over expressed in the synovium and supports bone erosions in RA patients. It is up-regulated by pro-inflammatory cytokines such as TNF-α, interleukin (IL-1) and (IL-6) [26,27,28]. sRANKL can be detected not only in the synovial fluid, but also in the sera of RA patients [17,25,28]. 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 [17,29]. The goal of our study was to evaluate the serum levels of RANKL and the possibility of their association with disease activity and hematological parameters in a group of RA newly diagnosed Syrian patients.

Materials and methods

Study design. Our cross-sectional study was done during the period from January 2020 to March 2021. The participations 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 outpatient clinic of rheumatology departments at the clinic of Almoujtahed Hospital and Al-Mowasat Hospital in Syria. They were newly diagnosed according to the 2010 ACR/EULAR diagnostic criteria. They weren't subjected to any treatment and the symptoms onset was ≤2 years previous to diagnosis. Only newly diagnosed with early RA patients and their ages from 18 to 65 years were included in the research.

Patients were excluded if they had the following conditions: patients with coexistence of other systemic autoimmune diseases except Sjögren's syndrome, patients having liver and kidney diseases, metabolic bone diseases such as osteoporosis, current fractures, thyroid diseases, Paget disease, multiple myeloma, diabetes mellitus and malignancy, patients under treatment distress bone metabolism such as steroids, vitamin D supplementations and thyroxin.

Ethical approval. Our research was performed in accordance with Helsinki Declaration and agreed by the **Scientific Research Ethics Committee - faculty of Pharmacy - university of Damascus** (No.3/ 26.2.2020). Written informed consent was acquired from all patients.

Clinical Data collection. A clinical test was conducted for patients and all information was collected in their own files that included: tender joint count (TJC), swollen joint count (SJC), visual analog scale (VAS), the duration of symptoms, morning stiffness.

Biochemical analysis. Blood samples were collected from all 88 Participations at confirmed diagnosis before therapy, then the serum samples were centrifuged at 4000 rpm for 4 minutes, separated and stored at -20°C until calibration is performed. The laboratory parameters were assessed and conducted using standard laboratory procedures for all RA patients: complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and rheumatoid factor (RF), 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:

DAS28-ESR = 0.56√ (TJC28) + 0.28√ (SJC28) + 0.70 Ln (ESR) + 0.014.

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).

Bone mineral density (BMD). It was done for only 40 of RA patients, expressed as (gr/cm2) and measured at the proximal femur and lumber 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 when T scores > -I, Osteopenia when T scores -1 to - 2.5, osteoporosis when T- scores ≤ -2.5.

Examination of the 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 was completed to detect free sRANKL without the OPG bond. The assay was performed using two kinds of antibodies: the detection antibody which is a biotinylated antibody specific for RANKL and polyclonal antibody from goat as the capture 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 with PBS or TBS buffer. The following step was TMB adding to visualize HRP enzymatic reaction and produce a blue color product that changed into yellow after adding acidic stop solution. The absorbance of yellow product using a plate reader was measured at 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 to 5000 pg/ml. The Sensitivity of the assay kit for sRANKL was <10 pg/ml.

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 ($\chi 2$) test was used for comparison of variables including categorical data.

variables. The ROC (receiver operating characteristic) curve was used to determine the standard cutoff of sRANKL and assess the sensitivity, specificity, positive predictive value (PPV) and negative

predictive value (NPV) of serum RANKL. 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).

Characteristics of patients. Our results 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 sero-negative patients.

According to DAS28-ESR, 34 (58.6%) of the RA patients had a high level of disease activity which the (Mean ± SD) of DAS28-ESR was 6.003 ± 0.66 (Table 3).

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 to 178.55 pg/ml) with a mean (166.57±13.6 pg/ml) and a statistically significant difference was (P=0.0001) (Fig1).

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 ± 14.63pg/ml), p-value was 0.002 (Table 4).

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 5).

In addition, the mean levels of RANKL 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), (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 6).

RANKL correlations with laboratory parameters in the 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) and disease duration (r=0.34, p=0.008). No correlation was observed with ESR, TJC, SJC, RF, VAS, CRP. 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) (Table7, Fig 3,4,5,6,7).

The ROC curve analysis: The ROC curve displayed that the best serum cutoff of RANKL concentration was 178.99 pg/ml with AUC of 0.902 (95% C.I., 0.841- 0.962; p < 0.001) to differentiate between RA patients and healthy groups (Table 8, Fig 8). 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 cutoff, also the odd ratio was 34.5 which indicates the probability of the RANKL positivity increases when suffering from rheumatoid arthritis. (Table 9, Fig 9)

DISCUSSION

Bone erosions and cartilage degeneration are the most important clinical manifestations of rheumatoid arthritis, these erosions may

be observed around the bone surrounding peri-articular joints, resulting in an increase the mechanism of bone resorption, especially in the sites of synovitis[30,31]. Recently, studies have focused on that RANKL is implicated in causing bone loss in RA and thus participating in the pathogenesis [6,32]. RANKL is produced by osteoblasts and stromal cells. It binds to its receptor (RANK) inducing osteoclast differentiation and inhibiting apoptosis [18,33]. In our study, the increase of serum RANKL levels that we found in RA patients compared to the control group can be explained by the effect of inflammatory cytokines in inflamed joints such as TNF-α, IL-1, IL-6, IL-17 which play an important role in the pathogenesis of rheumatoid arthritis and support increased production of RANKL by different cells [34]. Synovial cells, osteoblasts, epithelial cells, B cells and T cells are the most important cells that lead to increase levels of RANKL, supporting osteoclast activation [3,25,35]. Our study is consistent with several studies: Półtorak et al. study 2021 which contained 50 RA patients and showed the elevation of serum RANKL levels in RA patients compared to healthy subjects (N=26) [3]. Bruno et al. study in Portugal 2021 (128 RA patients and 26 healthy individuals) and Boman et al. 2017 study in Sweden (407 RA patients and 71 healthy controls) indicated that the detrimental effect of inflammatory cytokines are capable of activating osteoclasts through increased expression of RANKL [19,34]. It also approved with the study of Motawa et al. in Egypt 2020 which sRANKL levels mean in RA patients (N=55) was 7.7 ± 1.9 ng/ml and 0.8 ± 0.2 ng/ml in healthy subjects (N=25), ($P<0.05$). Their study have indicated the role of activated T- cells in secreting soluble RANKL in addition to their role in the expression of membrane-associated RANKL through the effect of TNF-α [36]. In

contrast, there was no statistical difference in serum RANKL levels between the patients and healthy group in the 2016 Remuzgo et al. study. This could be due to the small number of RA patients (26 patients) and the healthy group (N=10) in his study [19]. Sousan et al. study in Iran 2017 did not find a statistically significant difference (P=0,49) in serum RANKL levels between patients and healthy controls because all of their patients and controls were only women [37]. Our result also contrasted with the Carmen study et al. 2016 in Spain, which showed that serum RANKL values were below the detection limit in 85% of studied RA patients (N=93). The reason for the discrepancy is that 76% of their patients were in the clinical remission or in the phase of low disease activity, as TNF-α antagonists and some DMARDs used in the RA treatment have inhibited the expression of RANKL by synovial cells [38]. Our study found an elevation in RANKL levels in post-menopausal patients compared to post-menopausal healthy group, this confirms its importance as a biomarker interferes in the bone loss and pathogenic mechanism of rheumatoid arthritis. This is what we found a similar in the study of Çakırca et al. 2012 in Turkey that included a similar number (N=30) of postmenopausal patients and the healthy groups [39]. There was a significant increase in serum RANKL levels in ACPA seropositive RA patients' group (N=48) compared with ACPA seronegative (N=10). It could be explained by the direct effect of these auto-antibodies in upregulation of RANKL by certain immune cells or osteoclasts. Both ACPA and RANKL have a synergistic role in affecting osteoclasts by supporting its differentiation or increasing their number by binding on their surface to stimulate TNF-α production [39]. It can be argued that both ACPA and RANKL positivity are important markers of the

destructive mechanisms of rheumatoid arthritis. Our result was documented by the study of Boman et al. 2017 in Sweden conducted on 407 patients and showed that plasma levels of RANKL were higher in ACPA positive patients than ACPA negative patients[40].

Our study showed that serum RANKL levels positively correlated with each of the variables: DAS28, Anti-CCP and disease duration. No correlation was observed with ESR, TJC, SJC, RF, VAS, CRP. Similarly, a positive association between serum RANKL levels and DAS28 was found in some studies done by Moura et al. study in Brazil 2021 which demonstrated a positive correlations between RANKL and DAS-28 [41] and Motawa et al. study in Egypt 2020 consisted of 55 RA patients ($r= 0.56$, $P <0.001$) [36]. These studies and ours confirm that increased RANKL level may reflect RA activity and may be added with DAS28 as an assistant biomarker to assess disease activity. On the other hand, Kolahi et al. study in Iran 2017 [42] and Boman et al. study in Sweden 2017 did not observe a correlation between serum RANKL levels and DAS28 in patients with RA [19]. According to the correcation with CRP, a study by Bruno et al. study 2021 in Italy [44] and Burska et al 2021 study in the United States did not find a significant association between serum RANKL and CRP [45]. On the other hand, the study of Çakırca et al. 2012 in Turkey presented a positive association between serum RANKL levels with both ESR and CRP, this may be explained that the inflammatory mechanism may reflect disease activity and associate with bone loss in RA patients [39]. Plasma RANKL levels were also associated with ESR in Antonia et al. study in Sweden suggesting an association of RANKL level with inflammation. We found a moderate inverse correlation between

serum RANKL levels and BMD at the level of both lumber spine and femoral neck. A 2012 study by Çakırca et al. demonstrated a negative association between serum RANKL levels and BMD at the level of the lumber spine (r=-0.369) and femoral neck (r=-0.361) [39]. A similar negative correlation was found between RANKL levels and bone density in the Fadda study in Egypt 2014, (r = -0.4, P =0.01)[46]. These findings suggest that RANKL may be involved in the activation of osteoclasts and the consequent promotion of bone resorption, which decreases BMD in RA patients. The ROC curve analysis exhibited that the best serum cutoff of RANKL concentration was 178.99 pg/ml with AUC of 0.902 (the sensitivity was 79.31% and specificity was 90%. This confirms the possibility of using serum levels of RANKL in established diagnosis of rheumatoid arthritis. In Burska study 2021, the cut-off of serum RANKL was 500 pmol/L, AUC was 0.680 (95% CI 0.619–0.740, $p<0.0001$ with a specificity of 79%, sensitivity of 47%, PPV was 70% and NPV was 59%, an odds ratio of 2.23) to classify RA patients (N=151) /non-RA (N=147) [45]. This discrepancy in the results may be due to sensitivity of the used kit in assaying of serum RANKL. The study of Burska has addressed the importance of studying serum levels of RANKL and the possibility of adding it as an additional marker to the diagnostic criteria for rheumatoid arthritis.

Conclusion: Our results demonstrated that measurement of sRANKL could become a novel biomarker for diagnosis and disease activity in RA patients.

Data availability statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

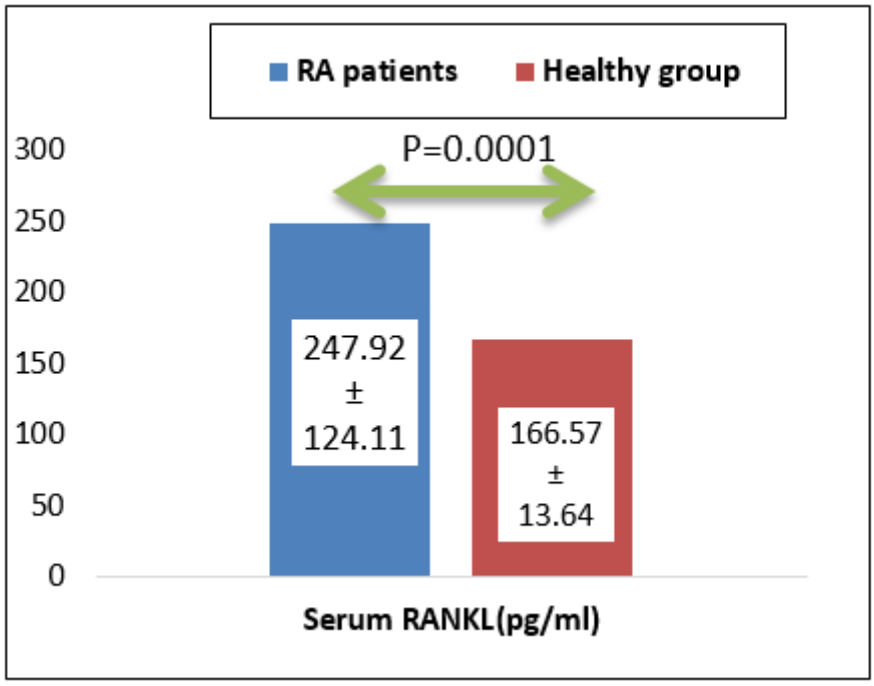

Figure 1

Comparison of serum RANKL between RA group (N=58) and the control group (N=30)

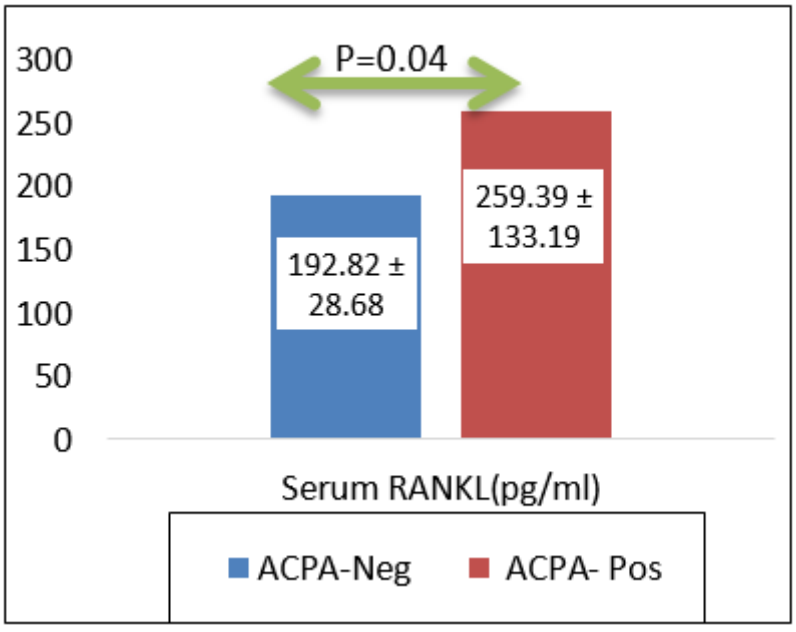

Figure 2

Comparison of serum RANKL in RA patients according to ACPA

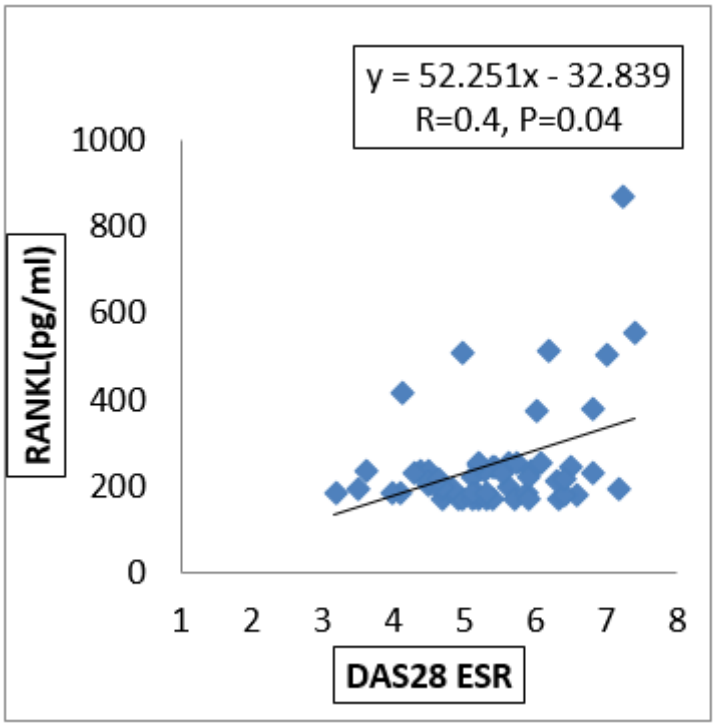

Figure 3

Correlation between RANKL levels and DAS28

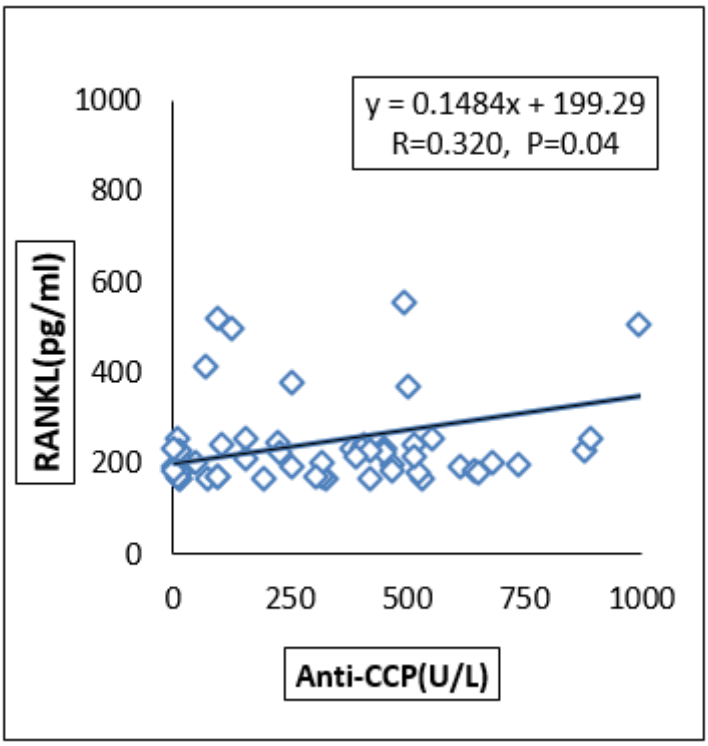

Figure 4

Correlation between RANKL levels and Anti-CCP

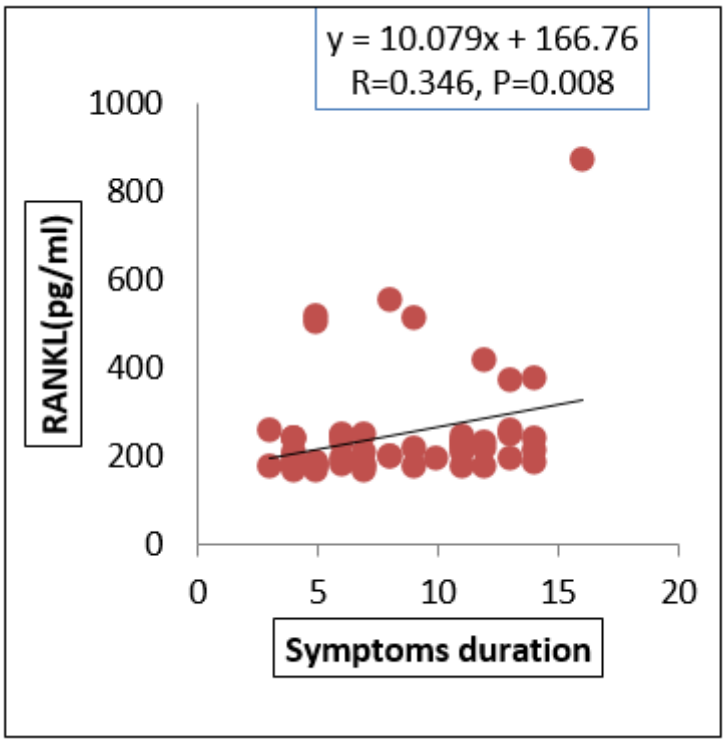

Figure 5

Correlation between RANKL levels and Symptoms duration

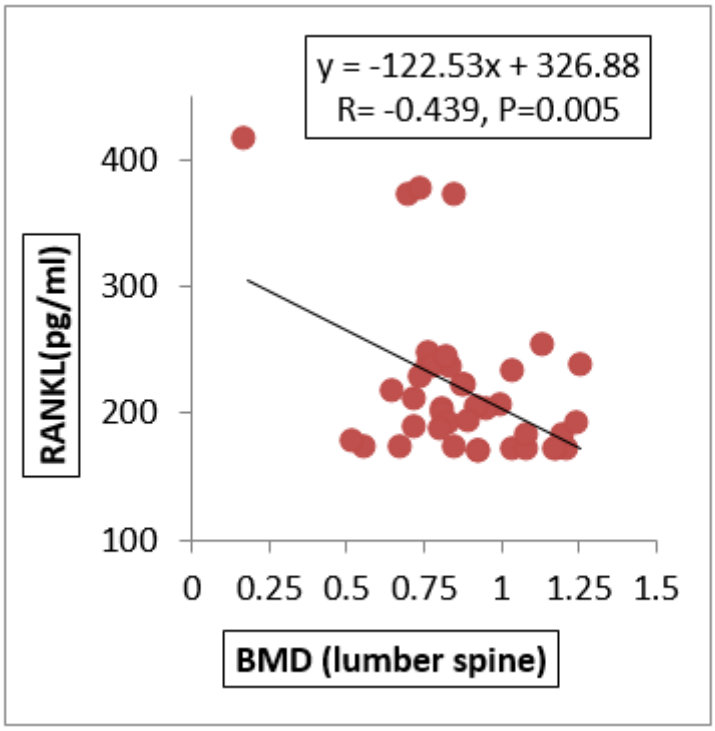

Figure 6

Correlation between RANKL levels and BMD (lumber spine)

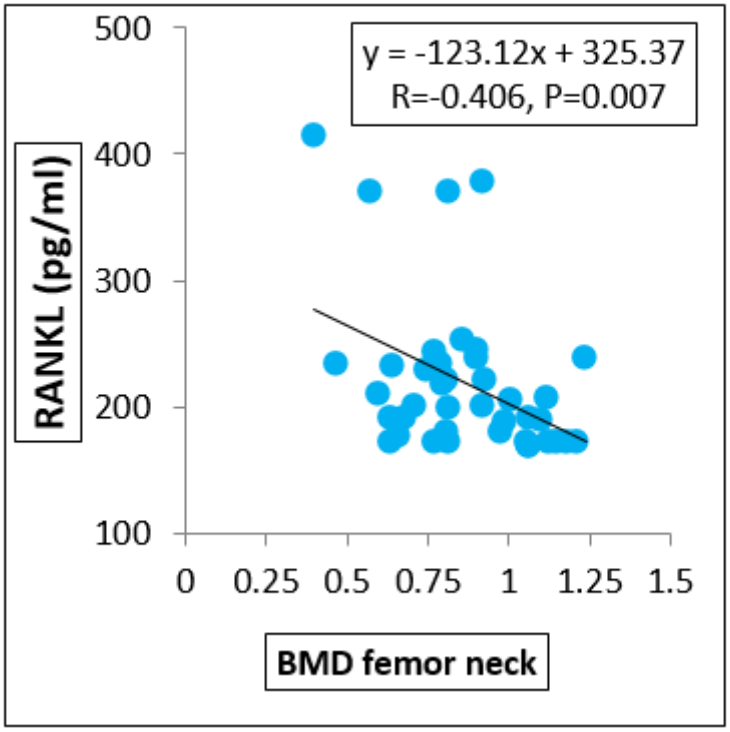

Figure 7

correlation between RANKL levels and BMD (femur neck)

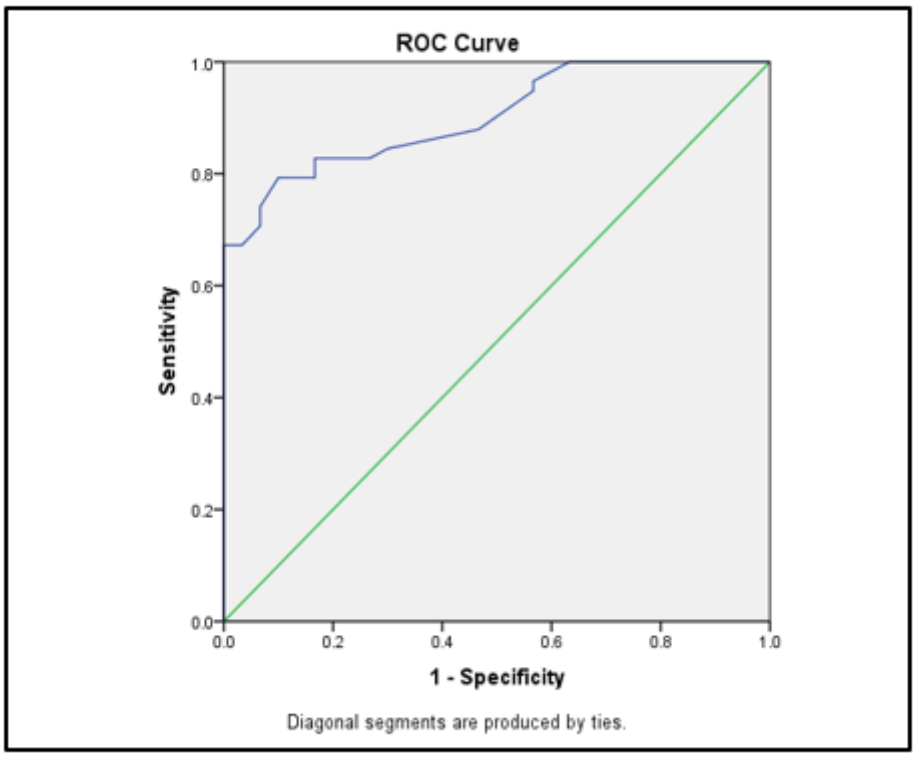

Figure 8

ROC curve analysis showing the diagnostic performance of sRANKL for discriminating RA patients from controls group

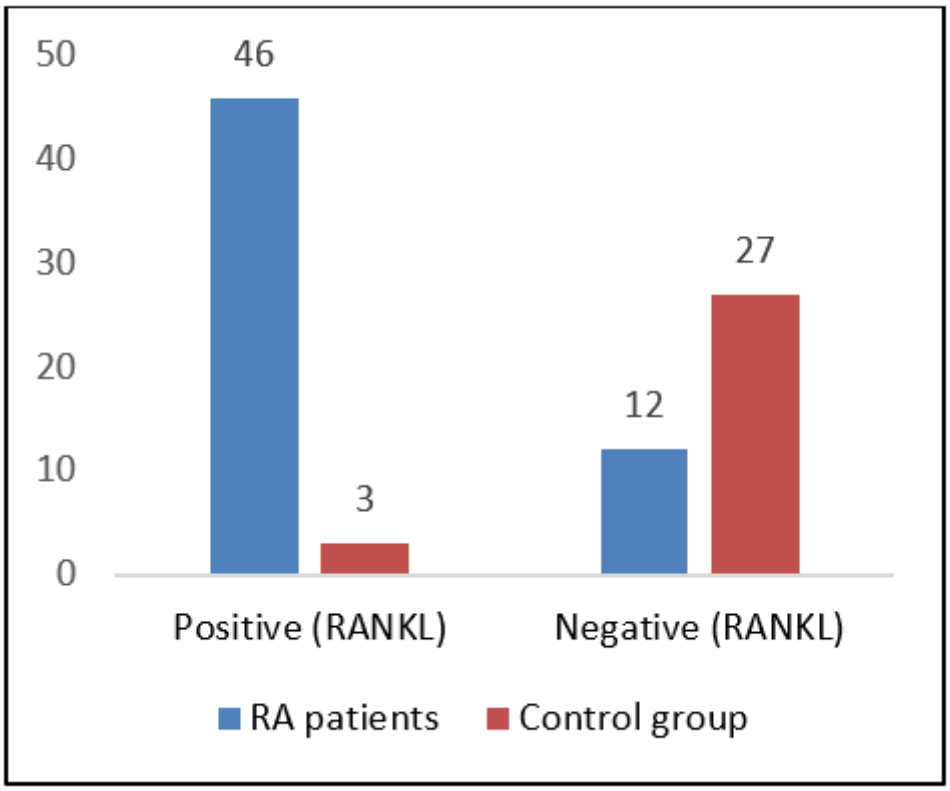

Figure 9

the distribution of RA patients and control group according to positive or negative of serum RANKL.

Supplementary Files

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- SupplementaryMaterial.docx