Journal of
Babol University of Medical Sciences
JBUMS
e-ISSN: 2251-7170 p-ISSN: 1561-4107

# The Predictive Role of Some Immunological Markers and Testosterone in Iraqi Male Patients with Rheumatoid Arthritis

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**Article Type**

**Research Paper**

**Received:**
**Jun 10th 2024**
**Revised:**
**Aug 15th 2024**
**Accepted:**
**Sep 14th 2024**

## ABSTRACT

**Background and Objective:** Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammatory arthritis and extra-articular involvement. Roles of interleukin-18 (IL-18), Galectin-1 (Gal-1), and testosterone markers in pathogenesis RA are unknown. Therefore, the present study was conducted to detect these markers in patients with RA.

**Methods:** This case-control study was conducted on 100 men aged 39 - 80 years. 60 blood samples were collected from patients who visited the teaching hospital of Baqubah, Diyala governorate from Nov. 2022 to Jan. 2023. RA patients were diagnosis by the consultant doctor in advisory units based on symptoms and laboratories tests (CRP, ACCP, ESR, RF). 40 blood samples were collected from healthy individuals (control group). Levels of CRP and RF markers in samples were evaluated by RosheCobas e411, while Galectin-1, IL-18, ACCP, and testosterone were measured by (ELISA) (CUSABIO Company).

**Findings:** The present results revealed that the age groups of 40-49 and 50-59 scored the highest (31.7% and 28.3%) compared to the age groups of <40 and >60 years, which scored the least (11.7% and 10.0%). The positivity of rheumatoid factor (RF) and C-reactive protein (CRP) markers scored the highest percentage in patients (70.0% and 78.3%) compared to healthy subjects (12.5% and 20.0%). Gal-1 and testosterone levels were low in patients (5.23±2.07 and 176.20±42.41) compared to healthy subjects (22.73±8.42 and 341.30±64.59). In contrast, levels of IL-18 and ACCP markers were high in patients (323.70±40.90 and 34.88±6.75) compared to healthy subjects (169.58±35.61 and 5.55±2.39). The differences between age, RF, CRP, IL-18, ACCP, Gal-1 and testosterone and study groups were significant ($p<0.05$).

**Conclusion:** According to the results of the present study, disease severity increased with age. Galectin-1 and interleukin-18 are markers that are known to be prognostic in the screening of patients with RA. Testosterone is good marker in screening RA males with sexual dysfunction.

**Keywords:** *Rheumatoid Arthritis, Galectin-1, IL-18 and Testosterone, RF, CRP, ACCP.*

**Cite this article:** Hameedi BH, AL- Naqeeb AA, Bajilan SI, Turki SG. The Predictive Role of Some Immunological Markers and Testosterone in Iraqi Male Patients with Rheumatoid Arthritis. *Journal of Babol University of Medical Sciences*. 2025; 27: e38.

## Introduction

Rheumatoid arthritis is a multi-factorial disease branded by generation of autoantibodies, activation of fibroblast-like synoviocytes and infiltrating the joints of inflammatory cells (1). It is described by chronic and painful inflammatory arthritis, which result in advanced destruction of both the cartilage and bone and lead to functional disability (2, 3). In spite of considerable improvements in rheumatoid arthritis (RA) appreciative pathogenesis, there are still persistent and abundant unmet requirements. An extensive diversity of mechanisms of pathogens is involved in disparity in response to extremely directed therapies (4). The disease is more common in women compared to with a 3:1 ratio. Although the disease may develop at any age, the peak disease onset age is in the 40s (5). The environmental and genetic risk factors are of vital roles in the pathogenesis of the disease (6). The inheritance RA probability is assessed to be about 60% (3).

Galectins (Gals) are lectins that explicitly attach to carbohydrates as β-galactoside in the extracellular matrix and on the cell surface. Despite their extensive distribution in different organs and tissues, a well-known predilection for the immunity system was eminent. Currently, Gals are being documented as immune potent modulators in auto-immune and inflammatory disorders (7). While few family members have pro-inflammatory functionality, i.e., Gal-3, others such as Gal-1 are principally anti-inflammatory. Gal-1 has anti-inflammatory properties because of its pro-apoptotic influence in activated lymphocytes; this eases the swing of T helper 1 (Th1 to Th2) polarized immune response and Th17 to Trig, and prevents the pro-inflammatory cytokines (IL-2, IFNγ and TNFα) secretion (8). Gal-1-deficient mice showed augmented susceptibility to (CIA) and usage with recombinant Gal-1 amended the expressions (9, 10), indicating that Gal-1 is playing important role in pathophysiology of RA.

In RA pathophysiology, cytokines are of a noteworthy function (11). A chief cytokines class identified as interleukins is of a noteworthy function in the immunological reactions triggering inflammation. Interleukin IL-18 is expressed powerfully in the tissue as synovial, serum, and synovial RA fluid of patients, and is connected with the disease activity (12). Structurally, interleukin IL-18 is a pro-inflammatory cytokine similar to IL-1 and fits to the IL-1 super-family. IL-18 might be of crucial function in the RA progression (13). The disease severity appears to be linked to the concentrations of IL-18 in the affected joints and serum. With no external stimulation, patient's synovial cells in RA produce interleukin-18 (14).

Androgens, like testosterone, are of an anti-inflammatory range of effects *in vivo*, decreasing inflammatory cytokines secretion, i.e., TNF, IL-1 and IL-6, through inhibiting β cell lymphopoiesis and monocytes and antibody production (15). Serum androgen levels in inflammatory rheumatic diseases are frequently declined due to the stimulation of inflammatory cytokines, i.e., TNF, IL-1 and IL-6 of the aromatase enzyme in fibroblasts and immune cells (16, 17). Men with RA have low serum testosterone levels, maybe because of the deficiency in anti-inflammatory testosterone effects, lower levels of testosterone connected with serological markers and higher activity of disease (18). Another study showed that the testosterone levels and the presence of hypogonadism were not associated with the stage and activity of RA; however, testosterone deficiency was accompanied by a more frequent development of overweight and obesity, and a deterioration in purine and carbohydrate metabolism (19).

The purpose of the present work was to evaluate the role of Gal-1, IL-18 and testosterone in RA patients and to see their association with the activity of disease and their potential role as predictive markers.

## Methods

**Samples collection:** This case-control study included 100 random men aged 39 to 80 years old. 60 blood samples were taken from individuals who referred to the teaching hospitals of Baquba/Diyala governorate from Nov. 2022 to Jan. 2023. For patients with RA, examination and diagnosis were done by the consultant doctor in the advisory units based on symptoms and laboratories tests (CRP, ACCP, ESR, RF). Blood samples (n=40) were taken from healthy individuals (control group).
**Ethical Approval:** This research work has obtained the ethical approval from Ethics Committee of Baquba teaching hospital/Diyala province and the code is ScB20.
**Inclusion criteria:** The inclusion criteria of the present study include: age, race, disease severity (all patients in severe condition), and body mass index (all overweight patients).
**Exclusion criteria:** The exclusion criteria of present study include: RA patients with diabetes, heart diseases, and hyperlipidemia.
**Methods:** For serum isolation, 5 ml of blood were spun for 5 min at 3000 rpm. RF and CRP serum levels markers in the samples were measured by Roshe Cobas e411, while Galectin-1, IL-18, Anti-Cyclic Citrullinated Peptide (ACCP), and testosterone were measured by (ELISA) (CUSABIO Company). Such test was done based on the protocol procedure within the kit packing of the manufacturer company.
**Statistical analysis:** Galectin-1, IL-18, ACCP, and testosterone markers normality were first assessed (Kolmogorov-Smirnov and Shapiro-Wilk test). The parameters that passed the normality tests (non-significant different) were showed as Mean±SD, with student t-test utilized to define the significant differences (comparison between 2 groups). Others parameters (gender, age groups, RF, and CRP) appeared as numbers and percentages, while Pearson's chi-squared test was used to reveal significant differences in frequency. The nature and strength of the association between parameters was assessed by Pearson correlation coefficient. (ROC) curve was used to define sensitivity, (AUC), and specificity of parameters. $P \leq 0.05$ was considered significant. The data were analyzed utilizing SPSS v. 21.0 and GraphPad Prism v.6 statistical software.

## Results

**1. Age groups of RA:** Findings of the current work displayed that there are significant differences ($p<0.05$) between age groups of patients; the 40-49 and 50-59 years scored the highest (31.7% and 28.3%), while <40 and >60 years scored the lowest (11.7% and 10.0%) (Figure 1).

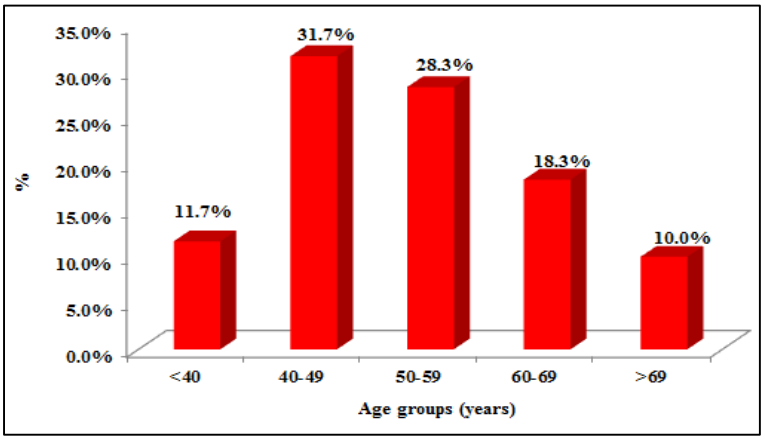


**Figure 1. Age groups of patients**

**2. Correlation between RF and CRP markers in study groups:** The current study results showed significant differences ($p<0.05$) in positivity of RF and CRP markers between study groups. The positivity of RF and CRP markers scored highest percentage in patients (70.0% and 78.3%) compared to healthy subjects (12.5% and 20.0%) (Table 1).

**Table 1. Comparison of positivity of RF and CRP markers between study groups**

| | Groups | | Total Number(%) | p-value |
|---|---|---|---|---|
| | Patients Number(%) | Healthy Number(%) | | |
| **RF** | | | | |
| Positive | 42(70.0) | 5(12.5) | 47(47.0) | <0.001*** |
| Negative | 18(30.0) | 35(87.5) | 53(53.0) | |
| **CRP** | | | | |
| Positive | 47(78.3) | 8(20.0) | 55(55.0) | <0.001*** |
| Negative | 13(21.7) | 32(80.0) | 45(45.0) | |

**3. Correlation between physiological and immunological markers in study groups:** Present outcomes revealed that there is a significant difference ($p<0.05$) between Galectin-1, IL-18, ACCP, and testosterone markers and study groups. The levels of Galectin-1 and testosterone markers were low in patients (5.23±2.07, and 176.20±42.41) compared to healthy subjects (22.73±8.42, and 341.30±64.59). In contrast, the levels of IL-18 and ACCP markers were high in patients (323.70±40.90 and 34.88±6.75) compared to healthy subjects (169.58±35.61 and 5.55±2.39) (Table 2 and Figure 2).

**Table 2. Comparison of mean levels of physiological and immunological markers between study groups**

| Groups | N | Mean±SD | p-value |
|---|---|---|---|
| **Galectin-1** | | | |
| Patients | 60 | 5.23±2.07 | <0.001*** |
| Healthy | 40 | 22.73±8.42 | |
| **IL-18** | | | |
| Patients | 60 | 323.70±40.90 | <0.001*** |
| Healthy | 40 | 169.58±35.61 | |
| **ACCP** | | | |
| Patients | 60 | 34.88±6.75 | <0.001*** |
| Healthy | 40 | 5.55±2.39 | |
| **Testosterone** | | | |
| Patients | 60 | 176.20±42.41 | <0.001*** |
| Healthy | 40 | 341.30±64.59 | |

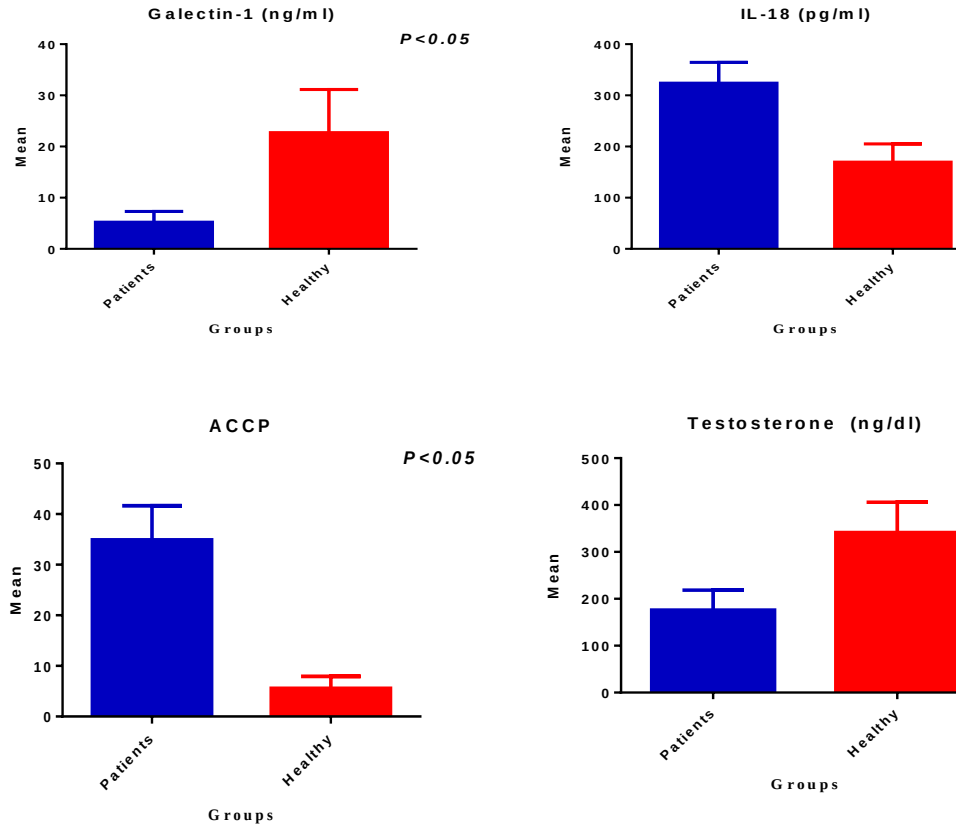


**Figure 2. Levels of physiological and immunological markers between study groups**

**4. Correlation between physiological and immunological markers in RA patients:** Additionally, table (3) shows that there are negative and positive correlations between physiological and immunological markers. Importantly, Galectin_1 is significantly and negatively correlated with ACCP (r= -0.364**, Sig.=0.004).

**Table 3. Correlation between physiological and immunological markers in RA patients**

| | Galectin_1 | ACCP | Testosterone |
|---|---|---|---|
| **Galectin_1** | | | |
| r | 1 | -0.364** | 0.064 |
| p | | 0.004 | 0.626 |
| **IL_18** | | | |
| r | -0.206 | 0.054 | -0.387** |
| p | 0.114 | 0.680 | 0.002 |
| **Testosterone** | | | |
| r | 0.064 | -0.006 | 1 |
| p | 0.626 | 0.965 | |

**5. Receiver operating characteristic (ROC) curve of immunological markers:** In the current study, figure (3) showed that Galectin-1, IL-18 and testosterone markers scored the highest sensitivity (100%, 100%, and 97%) and specificity (97%, 95%, and 90%) at cut off (15.23, 280.87, and 279.89) respectively, in screening patients with RA.

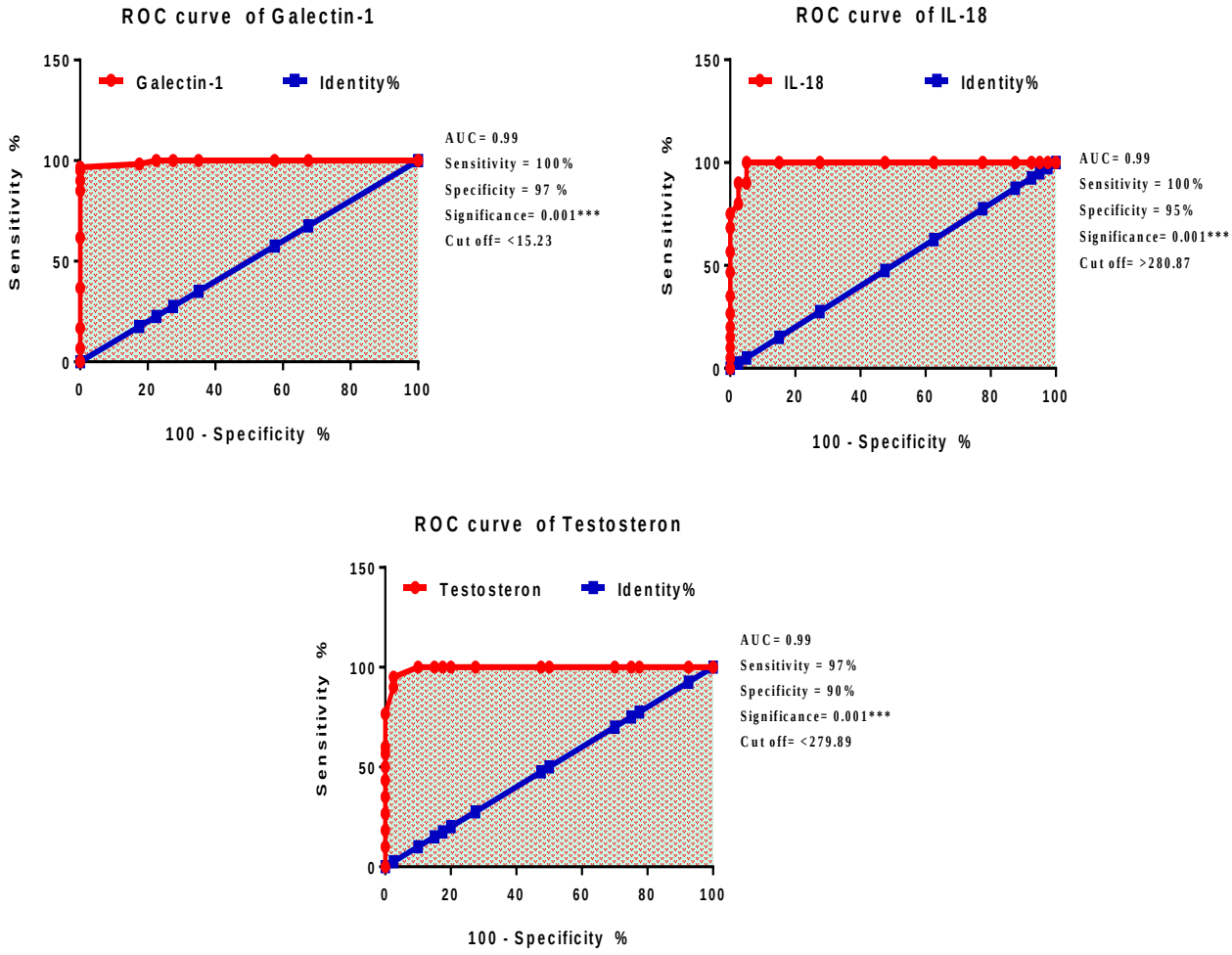


**Figure 3. ROC curve of Galectin-1, IL-18 and testosterone markers**

## Discussion

The purpose of the present work was to evaluate the role of Gal-1, IL-18 and testosterone in RA patients and to see their association with the activity of disease and their potential role as predictive markers. The results of present work showed that the occurrence of RA increases with age, especially ages higher than 40 years. These results are consistent with the results revealed in Egypt by Maged et al. (10) which showed that the mean age of patients with RA was more than 45 years, and it was attributed to several factors such as aging, organ dysfunction, chronic disorders, and impaired immune status, as well as sample size. Increasing age is associated with humoral immunity decrease and immune defensive mechanism resulting in upsurge pre-disposition to autoimmunity (20, 21). One of the major age-related modifications in β cell compartments is auto-reactive β lymphocytes buildup, which is recognized as

age-associated β cell and difference in gene expression and β cells receptor (22). A recent study showed that there exists a bi-directional relationship between depression and RA particularly in old individuals who have a higher risk of mortality. This raises the necessity for clinicians to monitor and be alert for the occurrence of such conditions (23).

This higher RF+ve proportion of patients compared to RF-ve patients is attributed to the fact that these antibodies are pentameric IgM antibodies which are binding to the fragment crystallizable region (Fc) portion of immunoglobulin G, which raises in inflammation or auto-immune disease, as well as in healthy patients. RA is an inflammatory disease, so this will cause this protein to be elevated (24). (RF) are detected in diverse diseases, including auto-immune and non-auto-immune diseases, as well as (RA). It has been shown in 4% of young people, healthy and elderly people (25). According to our results, the positivity of the rheumatoid factor IgM test is 70 percent. The results of the current work are consistent with those of a preceding work which detected sensitivity to rheumatoid factor IgM in the range of 60-80 percent (26).

C-reactive protein has a strong moderate association with the activity of disease. Nevertheless, noteworthy pitfalls are there in such biomarker use in RA, and necessitating careful interpretation of CRP results. The findings of previous work emphasize the RA heterogeneity and the necessity to improve biomarker panels, to improve stratifies RA, and to recognize the group for whom inflammatory activity cannot be accurately detected with CRP (27). A recent study showed that in a large group of RA patients with no preceding cardiovascular actions, a 20 mg/L upsurge in CRP concentrations was linked to 1% upsurge in 10-years risk of cardiovascular events. This proposes that active targeting of residual inflammatory risk more than RA-specific and conventional risk factors may additionally decrease cardiovascular events in RA patients (28). Considering the CRP positivity in RA patients, the present results showed that 78.3% of patients have positive CRP and these results are close to results in Al-Najaf province, Iraq by Al-Kefaee et al (29), which showed that 70% of patients have positive CRP.

Gal-1 is able to recalibrate homeostatic mechanisms, resulting in inflammatory replies control. Gal-1 principally has anti-inflammatory activities on both the adaptive and innate immune system (30). In the present work, serum Gal-1 levels were lower in RA patients compared to controls. Scarce reports were documented on Gal-1 in RA patients in Argentina (31) and Egypt (10).

In a preceding work directed in Mexico, synovial Gal-1 levels were low compared to that in control and RA patients (30) Likewise, reduced Gal-1 expression was confirmed in patient's tissue as synovial with long standing JIA in comparison to healthy ones. There was declined apoptosis evidence in the same patient's sub-set, signifying that Gal-1-related down-regulation of apoptosis takes place in patients with long standing JIA (31). Alternatively, other reports confirmed higher serum Gal-1 levels in RA patients compared to controls (31). In RA patients, synovial Gal-1 level was greater compared to patients with crystal arthropathy and osteoarthritis (10).

The impact of genetic variants in Gal1 gene (LGALS1) on Gal-1 levels was beforehand established (32). The differences in results could also be attributed to the polyvalent Gal-1 function and its discrepancy expression via several pathological and normal tissues (33). Galectin-1 mostly has anti-inflammatory properties. In particular circumstances, however, it is also able to have paradoxical pro-inflammatory influences, indicating that galectin production and function are differently controlled based on the time, tissue, or context. This lectin structure, the glycosylation process, in addition to the glycosylated ligand's structure and availability are able to be affected by numerous pathophysiologic contexts (8). It is remarkable that faulty function of glycosylation has been related to a variety of rheumatic illnesses, including RA (34).

Xibillé-Friedmann et al (35) detected no-significant association between serum Gal-1 and ACPA or RF antibodies positivity. Alternatively, Xibillé-Friedmann et al (35) stated that the decline in synovial Gal-1 is associated with a significant upsurge in anti- Gal-1 auto-antibodies and ACPA antibody titers. They proposed that auto-antibodies are able to limit the Gal-1 amount and maybe block its biological influence; nevertheless, no indication of an association with RF was detected.

Maged et al (10) showed the Gal 1 is considered as a predictive diagnostic marker in screening patients with RA (sensitivity 95% and specificity 83%), and these results matched with the present study (sensitivity 100% and specificity 97%). The present study showed negative correlation between Gal 1 and ACCP in RA patients because Gal 1 is an anti-inflammatory marker that decrease with disease severity while ACCP is an inflammatory marker that increase with disease severity.

RA is a mutual auto-immune disease that principally distresses females. Anti-inflammatory and inflammatory cytokines may be convoluted in enhancing or worsening the condition progression and might aid as early diagnosis biomarkers (15). Results of the current work exhibited high IL-18 levels in patients in comparison to healthy subjects, and such findings matched with results found in Baghdad, Iraq by Jasim et al (14). IL18 is of a physiological function in encouraging inflammatory disorders (36). Because of the role that IL18 plays in the RA pathophysiology, a superior thoughtful of the intricate connection between RA and microbial pathogens might one day help generate effectual methods to avoid the early disease stages, evading the clinical phase onset (14, 37).

IL-18, another IL-1 cytokine super-family member, is widely detected in the synovial issues of RA patients (38, 39). The mRNA levels of IL-1β IL-18 expression are differentially regulated. It has been detected that IL-18 distributes in the synovial membrane with a diverse pattern in comparison to IL-1β. While IL-1β is primarily localized through the interstitium, vasculature and lining layer, IL-18 is highly expressed in lymphocytic masses (12). The IL-18 expression in synovial tissue in vitro is related to the augmented IL-1β and TNF-α, and the IL-18 blockade by a neutralizing antibody was revealed to amend the disease in rodent RA models. Also, IL-18 contributes to degradation of cartilage by affecting proliferation of cell and the expression of metalloproteinase matrix and inducible NO synthase gene expression (40, 41).

In RA, IL-18 cytokine might contribute to inflammation by extravasation of leukocyte through the endothelial cell adhesion molecules up-regulation, directly act as monocyte, lymphocyte or neutrophil chemo-attractant and discharge chemokines from synovial fibroblasts (42). The IL-18 administration to mice with CIA or Freund's incomplete adjuvant eased the cartilage inflammation severity and development (43). This finding was similar to cases where IL-18-/- mice got IL-18 (44). In dissimilarity, low IFN-γ levels were noticed in RA synovitis (45). Such is elucidated to take place as IL-18 bears the Th1 phenotype nonetheless, and does not encourage IFN-γ production levels in cases of elevated inhibitory molecules expression, i.e., TGF-β and IL-10. The unique IL-18 role in encouraging the angiogenic factors discharge and up-regulation, i.e., MCP-1, SDF-1α, and VEGF in synovial tissue in RA through dissimilar pathways have been defined (15). Likewise, the IL-18 use for stimulating RA synovial fibroblasts in vitro encouraged the surface vascular CAM expression and neutrophil chemo attractant; additional IL-18 is then formed through synovial fibroblasts and by the TNF-α action created by synovial macrophages in a +ve mechanism of feedback (46). Meaningfully, higher IL-18 mRNA and protein levels were noticed in RA tissue as synovial; nevertheless, osteoarthritis patients' age was linked to joint disorder (46).

IL-18 was linked to the RA severity in the synovium, interim with IL-12 and IL-15 or myeloperoxidase to upsurge injury (47). Furthermore, it has been established that IL-18 is up-regulated in (SLE) gout and serum and might show a significant part in pro-inflammatory cytokine assembly (46). IL-18 up-regulates the membrane-bound expression (RANKL), (M-CSF), (GM-CSF), soluble RANKL, and (OPG) in cultured

fibroblasts. Inducing effects of IL-18 might encourage the osteoclasts maturation, resulting in degradation of bone in RA (12). Also, IL-18 was recognized as a cytokine formed in viral defense and is created by monocytes through inflammasomes activation (48). Preceding documents from our group exhibited that RA PBMCs encouraged with anti-CD28 and anti-CD3 prompt higher IL-18 levels in supernatants culture (49). Kudela et al (50) showed that IL-18 is playing an important role in screening patients with RA (specificity of 100% and a sensitivity of 60%), and these results are close to the present results which showed that the sensitivity of IL-18 was 100% and the specificity was 95%.

ACPA are detected in RA patients that yield in post-translation modification versus citrullinated protein. ACPA are associated with destruction of joints and upsurge disease progression risk and death (51). The results of the current study agree with several previous studies which concluded a higher level of Anti-CCP antibody in the patient group in comparison to the control group with higher specificity for RA compared to other inflammatory marker such as RF and CRP, which is consistent with the previous research in Baghdad, Iraq by Oglah et al (22). Anti-CCP antibody is a specific marker for RA because its level does not increase in other inflammatory diseases which makes it more reliable and more specific than other markers used for severity assessment and confirmatory testing compared to other markers (52). Additionally, Iaremenko et al (53) showed that ACCP is considered as a predictive diagnostic factor in screening patients with RA. Oglah et al (22) showed that ACCP level was altered based on the activity of the disease. ACCP level in active disease is extremely noteworthy compared to the level in RA patients with activity of disease as moderate and low. A local study exposed that the level of serum ACCP in RA patients increases with growing disease severity, higher level of ACCP in moderate and severe activity of disease compared to mild severity (54).

Sexual difficulties are a noticeable problem in men suffering from RA. Sexual activity was noteworthy linked to the activity of disease, depression, life quality, with lower free and total testosterone levels. Previous work exposed that sexual activity was prominently affected in RA patients in comparison to healthy controls and also RA patients are suffering from more depression compared to healthy persons (55). Sexual activity was affected significantly by activity of disease, physical disability, and level of serum hormone. Such dysfunction might be owing to pain being persistent, frequent disease activity, physical incapacity and reliance on others, joint stiffness, psychological prominence, fatigue, and/or treatment with certain drugs (56, 57). AP study showed that RA negatively affected patient's sexual function and considerably impaired sexual function in comparison to controls. Sexual function measuring must be considered a crucial part of the health in RA patients according to comprehensive evaluation, which might provide clues to better treatment decisions and disease management (57, 58).

Hypoandrogenism might play a part in RA and/or seems as a chronic inflammatory reaction problem. The higher testosterone levels in young males might to a certain extent have protective roles versus RA (59). Clinical improvement following a fruitful treatment is tracked by an upsurge in the serum testosterone level in RA (60). It must be illustrious that patients undergoing long-term treatment with glucocorticoids might practice hypoandrogenism (61). In a case-control study in Sweden, out of 151 RA male cases, the RF was just detected in 73% of the cases; the serum testosterone level in RA patients was significantly lower compared to control group; and abnormal level of serum testosterone was lower meaningfully in RA patients and +ve RF. It means that hormonal variations might take place throughout the RA onset and can distress the phenotype of disease (62). Few findings are there in respect to the importance of androgen levels in the RA incidence, particularly in men. Lower testosterone levels may be due to primary gonadal dysfunction, and similarly might be because of dysfunction in hypothalamic-pituitary-gonadal axis, or be caused by an inflammation that results in lower testosterone secretion (18). Pikwer et al (62) informed that there were no significant differences in respect to the serum inflammation indicators or health eminence among the cases

with initial RA and control subjects. Consequently, it cannot be settled that secondary inflammation (CRP, ESR) in primary RA is the major reason for variances among the groups. Lower serum testosterone levels might be attributed to RA or might designate the role of androgens in the RA pathogenesis (63). Prakashini et al (59) showed that testosterone is a good factor in screening RA males with sexual dysfunction, and these results are consistent with the present study, which showed that testosterone is a good factor in screening RA males with sexual dysfunction (sensitivity 97% and specificity 90%).

It can be concluded that disease severity is amplified with progression of age. Gal-1 and IL-18 are respectable prognostics markers in screening RA patients. Testosterone is good marker in screening RA males with sexual dysfunction. Further investigation is required to detect the role of Gal-1 and Gal-2 indicators in pathogenesis RA in Iraqi male patients.

## Acknowledgment

We would like to thank the people who contributed in any way to the study.

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