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# Investigation of the Association between Serum Concentrations of Tumor Necrosis Factor-alpha (TNF-α) and Hematological Parameters in Rheumatoid Arthritis Patients in AL-Najaf Governorate

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## Abstract

**This study was performed to determine whether the serum concentrations of tumor necrosis factor-alpha (TNF-α) are elevated in patients with rheumatoid arthritis (RA) and to investigate the relationship between TNF-α levels and hematological parameters in RA patients. Serum samples were obtained from 70 patients with RA who had visited the Division of Rheumatology at AL-sadder Teaching Hospital in AL-najaf City and 25 age- and sex-matched healthy controls, and the clinical parameters of disease were assessed, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF). Blood pictures were performed using automated hematology analyzer Mythic™. Serum concentrations of TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA).**

**Serum concentrations of TNF-α, was significantly elevated in patients with RA compared to those of healthy controls. Although there was no significant relationship between TNF-α levels and RBC, Hb, HCT and red cell indices, the TNF-α levels of patients with RA showed a significant correlation with total leukocyte count (TLC), differential leukocytes and platelets.**

**It has been concluded that the serum concentrations of TNF-α was significantly elevated in patients with RA and strongly correlated with hematological alterations. These findings suggest a possible role for TNF-α in the pathogenesis of RA.**

## INTRODUCTION

**Rheumatoid arthritis (RA) is a chronic disease that causes inflammation mainly in the synovium and produces destruction and deformity of the joints. The etiology of RA remains unclear, but it is known to be associated with genetic and environmental factors.[1]**

**Various proinflammatory cytokines, such as tumor necrotic factor (TNF)-α, IL-6, interleukin (IL)-1β, and interferon (IFN)-γ, , are increased in the synovial tissue or synovial fluid of patients with RA.[2,3] Increased levels of proinflammatory cytokines lead to the proliferation of synovial tissue, and thereby cause damage in the articular cartilage and bone destruction in the adjacent area.[4,5] Tumor Necrosis Factor (TNF) is a multifunctional cytokine with potent proinflammatory effects, and is implicated in many inflammatory and autoimmune diseases and is a member of a group of cytokines that stimulate the acute phase reaction [6].**

**TNF is thought to be produced primarily by macrophages, but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. Large amounts of TNF are released in response to lipopolysaccharide, other bacterial products, and Interleukin-1 (IL-1). It has a number of actions on various organ systems, generally together with IL-1 and Interleukin-6 (IL-6)[7].**

**Tumor necrosis factor alpha (TNFα) plays an essential role in the pathogenesis of rheumatoid arthritis (RA), in view of the fact that anti-TNF treatment is successful in controlling chronic inflammation in RA [8,9]. TNF-α mediates a proinflammatory and immunostimulatory activities. Cytokines such as interleukin- 1(IL-1) and TNF are key mediators of the inflammation which induces bone and joint destruction in RA. The proinflammatory cytokine TNF-α is a major factor involved in the RA inflammatory state[6].**

**The current study was aimed to examine whether serum levels of TNF-α is increased in patients with RA and also aimed to compare the serum concentrations of TNF-α in and the hematological alterations of RA.**

## MATERIALS AND METHODS

### Subjects and clinical assessment

**The study was conducted in 70 patients who had visited the Division of Rheumatology at AL-sadder Teaching Hospital in AL-najaf City between February and May 2011, and who fulfilled the American College of Rheumatology (ACR) 2010 revised criteria for the diagnosis of RA.[10] Twenty five age-and sex-matched healthy adults without any evidence of chronic inflammatory disease served as the controls.**

**The patients underwent thorough clinical and laboratory evaluation, including complete medical history, seropositivity test for rheumatoid factor (RF), C-reactive protein (CRP), and estimation of erythrocyte sedimentation rate (ESR).**

**At the time of clinical assessment for disease, six milliliters of blood samples were collected intravenously from each patient, 10µl placed in EDTA tubes for measurement of hematological parameters, and 1ml for evaluation of ESR, whereas blood samples for serum preparation were collected in glass tubes without anticoagulant, stored for one hour at room temperature, centrifuged (2500 r.p.m. for 10 minutes at 4°C) and then aliquoted in plastic tubes before being stored at -20°C until analysis.**

### Study design

**The group of patients enrolled in the natural history protocol was classified according to the duration of RA disease [11] as follows:**

**1.Group I (GI): the group of patients with disease length of (less than one year) was considered as the group with very early disease duration.**

**2.Group II (GII): the group of patients with disease length of (1-5) years was considered as the group with early disease duration.**

**3. Group III (GIII): the group of patients with disease length of (5-15) years was considered as the group with median disease duration.**

**4.Group IV (GIV): the group of patients with disease length of (15-25) years was considered as the group with long disease duration.**

**5.Group V (GV): the group of patients with disease length of (more than 25) years was considered as the group with very long disease duration.**

**Methods**

**1. Erythrocyte sedimentation Rate (ESR)**

**The International Committee on Standardization in Hematology (ICSH) recommends the use of the Westergren method [12,13].**

**Procedure:**

1. **0.25ml of sodium citrate was prepared.**
2. **1ml of blood sample was mixed with sodium citrate.**
3. **The mixture was pipetted into Westergren tube.**
4. **Westergren tube was held in vertical position in a rick of ESR.**
5. **The ESR result was read after one hour (mm/hr.).**

**2. C - reactive protein (CRP) serology test**

**The CRP-latex test kit was used according to the manufacturing company instructions (SPINREACT, S.A. Spain).**

**Procedure:**

1. **One drop (50 µl) of the serum sample was mixed with a drop of CRP-latex.**
2. **The drops were mixed with a stirrer and spreading over the entire surface of the separate circle on the slide test.**
3. **The slide was placed on a mechanical rotator at 80-100 r.p.m. for 2 minuets.**
4. **The presence or absence of visible agglutination was examined macroscopically immediately after removing the slide from the rotator.**

**3. Rheumatoid Factor (RF) Latex serology test:**

**Rheumatoid factor (RF) Latex serology test kit was used according to the manufacturing company instructions (LTA s.r.l. Italy).**

**Procedure:**

1. **A drop of latex was mixed with 50µl of sample using disposable stirrer stick.**
2. **The mixture was spreading homogenously over the entire area enclosed by the separate circle on the test card.**
3. **Shake the card for 2 minute by a rotating motion at (100 r.p.m.).**
4. **The eventual agglutination was observed using artificial light.**

**4. Hematological Investigation**

**The hematological parameters were performed on EDTA blood using Mythic™ 18 (RINGELSAN CO., Turk) in Hematology Laboratory of AL-Sadder Teaching Hospital in AL-najaf City. Mythic 18 is a fully automated hematology analyzer performing complete blood count (CBC) on EDTA anticoagulated blood.**

**Procedure:**

1. **10 µl of EDTA blood sample was placed in the aspirator of the instrument.**
2. **The start key on the instrument was pressed and the blood sample was aspirated.**
3. **Results were provided within 1 minute on the LCD display, printed out on the printer and stored in the resident memory.**

**5. Measurement of TNF-α:**

**The serum concentration of TNF-α was measured using an AssayMax enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, USA) in Virology Laboratory of AL-Sadder Teaching Hospital in AL-najaf City.**

**Fifty microliters each of serum sample and assay diluent were placed in each well of a 96-well plate coated with a monoclonal mouse IgG against TNF-α. This mixture was incubated for two hours at room temperature, and each well was aspirated and washed five times with wash buffer. Subsequently, 50 µl of Biotinylated TNF-α Antibody was added to each well and incubated for two hours. Again, each well was washed five times with wash buffer. Following this, 50 µl of Streptavidin-Peroxidase Conjugate was added per well and incubated for 30 minutes and each well was aspirated and washed five times with wash buffer. Subsequently, 50 µl of substrate solution, which was prepared with equal amounts of stabilized hydrogen peroxide ($H_2O_2$) and tetramethylbenzidine, was added for a 20-minute reaction under dark conditions. The reaction was quenched by the addition of 50 µl stop solution (0.5 N of HCl). Within 30 minutes, the optical density was measured at a wavelength of 450 nm using the bioelisa reader ELx 800 (Molecular Device Co., biokit, CA, USA). The serum concentration of TNF-α was determined based on a standard concentration curve. The correlation coefficient (r) of the standard concentration curve was 0.990.**

**Statistical analysis**

**Data analyses were performed with GraphPad Prism version 5.04 Software (GraphPad Software Inc., San Diego, CA, USA). All of the descriptive variables were expressed as the mean ± standard error (SE). The correlations between the concentrations of TNF-α and hematological parameters were tested using Pearson's correlation test. The group analyses were performed using one-way ANOVA and Tukey's post-hoc analyses. For all tests, a *p* value less than 0.05 was considered statistically significant.**

## RESULTS

### Clinical characteristics of the study subjects

**The demographic and clinical data of the subjects are shown in Table (1). The mean age of the 70 patients with RA was 47.9 ± 3.32 years (range: 27-75 years), and the patient group was comprised of 13 males and 57 females. The mean disease duration from symptom onset was 11.52 ± .085 years (range: 0.25-31 years). Patients were divided into five groups according to the disease duration: 10 in the very early duration 0.43±0.06 year (range: 0.25-0.58 year), 19 in the early duration 2.28±0.22 (range: 1-4 years), 20 in the median duration 7.8±1.8 (range: 5-14years), 11 in the long duration 19.4±0.57 (range: 18-21 years), and 10 in the very long duration 27.7±1.6 (range: 25-31 years).The mean age of the healthy controls was 42±2.03 years (range: 24-75 years), and the control group was comprised of 6 males and 19 females.**

**Table 1: Clinical characteristics of the study subjects.**

| Character | | RA patients (n=70) | | | | |
|---|---|---|---|---|---|---|
| | Healthy Control | Very early Duration | Early Duration | Median Duration | Long Duration | Very long Duration |
| Number of subjects | 25 | 10 | 19 | 20 | 11 | 10 |
| Age (years) (range) | 42±2.03 (24-75) | 44±3.2 (31-54) | 44±3.1 (27-76) | 49±2.95 (34-73) | 48±5.1 (37-70) | 52±2.28 (49-59) |
| Female/male ratio | 19/6 | 9/1 | 14/5 | 16/4 | 9/2 | 9/1 |
| Disease duration (years) (range) | -- | 0.43±0.06 (0.25-0.58) | 2.28±0.22 (1-4) | 7.8±1.8 (5-14) | 19.4±0.57 (18-21) | 27.7±1.6 (25-31) |
| %RF positive patients | -- | 90% | 94.7% | 90% | 81% | 90% |
| ESR (mm/hour) | 19.9±0.5 | 59.1±9.3 | 52.5±4.8 | 52.1±6.67 | 64.18±5.6 | 46.6±4 |
| %CRP positive patients | -- | 90% | 89% | 90% | 90.9% | 80% |

### Hematological parameters

**The statistical analysis showed a significant decrease (P<0.0001) in RBC, Hb, hematocrit (HCT) and red cell indices among the patients of RA compared to the control (Table 2) whereas for total leukocytes (TLC) count, monocytes (MON), granulocytes (GRA) percentage and platelets count (PLT) of the study groups, statistically significant (P<0.0001) high values were determined at patients with RA compared to the control.**

**Table 2: Hematological parameters in healthy group and in the five groups of patients suffering from rheumatoid arthritis.**

| Parameters | Healthy control (n=25) | RA patients (n=70) | | | | |
|---|---|---|---|---|---|---|
| | | Group I (n=10) | Group II (n=19) | Group III (n=20) | Group IV (n=11) | G |
| RBC($\times10^{12}$/L) | 5.67±0.12 | $4.19\pm0.175_{a}^{***}$ | $4.49\pm0.13_{a}^{***}$ | $4.43\pm0.10_{a}^{***}$ | $4.21\pm0.17_{a}^{***}$ | 4.57 |
| Hb(g/dL) | 15.49±0.21 | $11.05\pm0.40_{a}^{***}$ | $11.27\pm0.29_{a}^{***}$ | $10.81\pm0.40_{a}^{***}$ | $11.08\pm0.44_{a}^{***}$ | 11.5 |
| HCT % | 46.67±0.75 | $33.71\pm0.84_{a}^{***}$ | $33.39\pm0.75_{a}^{***}$ | $32.75\pm1.13_{a}^{***}$ | $33.54\pm1.16_{a}^{***}$ | 33.9 |
| MCV(fL) | 91.51±1.10 | $76.38\pm2.48_{a}^{***}$ | $74.57\pm1.56_{a}^{***}$ | $75.64\pm1.79_{a}^{***}$ | $76.85\pm2.56_{a}^{***}$ | 71.5 |
| MCH(pg) | 31.36±0.53 | $25.89\pm0.87_{a}^{***}$ | $24.91\pm0.63_{a}^{***}$ | $24.61\pm0.78_{a}^{***}$ | $25.81\pm1.04_{a}^{***}$ | 25.7 |
| MCHC(g/dL) | 35.29±0.17 | $33.71\pm0.38_{a}^{ns}$ | $34.14\pm0.45_{a}^{ns}$ | $33.34\pm0.67_{a}^{**}$ | $33.06\pm0.30_{a}^{*}$ | 33. |
| TLC($\times10^{9}$/L) | 7.00±0.25 | $9.51\pm1.15_{a}^{ns}$ | $7.97\pm0.61_{a}^{ns}$ | $8.66\pm0.63_{a}^{ns}$ | $8.05\pm0.54_{a}^{ns}$ | 8.6 |
| LYM% | 33.06±1.01 | $26.33\pm2.38_{a,b}^{ns}$ | $30.74\pm2.96_{a}^{ns}$ | $28.58\pm1.92_{a,b}^{ns}$ | $28.68\pm0.84_{a,b}^{ns}$ | 20.7 |
| MON% | 3.95±0.21 | $12.61\pm3.13_{a}^{**}$ | $11.84\pm1.53_{a}^{**}$ | $9.42\pm1.37_{a}^{ns}$ | $16.51\pm2.78_{a}^{***}$ | 12.( |
| GRA% | 59.83±1.37 | $64.73\pm3.95_{a,b}^{ns}$ | $66.09\pm2.21_{a,b}^{ns}$ | $63.57\pm2.55_{a}^{ns}$ | $65.8\pm0.78_{a,b}^{ns}$ | 74.4 |
| PLT($\times10^{9}$/L) | 224.3±7.70 | $498.1\pm69.2_{a}^{***}$ | $346.5\pm29.1_{b}^{*}$ | $465.4\pm31.8_{a,b}^{***}$ | $401.3\pm29_{a,b}^{**}$ | 347.7 |

- **Data are expressed as means ± standard error (SE).**
- **The asterisks indicate significant difference based on Tukey's multiple comparison test.**
- **The same letters indicate non significant difference based on Tukey's multiple comparison test.**
- **ns: not significant.**

**Serum concentration of TNF-α**

**The standard curve was used to estimate the relationship between the optical density (O.D) of TNF-α and standards (pg/ml) and the results were calculated using GraphPad Prism version 5.04 Software (GraphPad Software Inc., San Diego, CA, USA) to determine each point (Figure 1).**

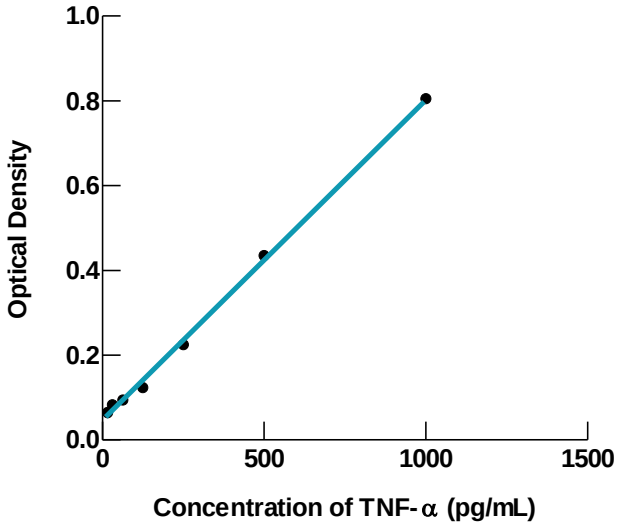


**Fig 1: TNF-α standard curve**

**Serum level of TNF-α of RA patients showed significant increase (P<0.0001) in the average values comparatively to the healthy group, in the long duration of the disease was much higher (Figure 2).**

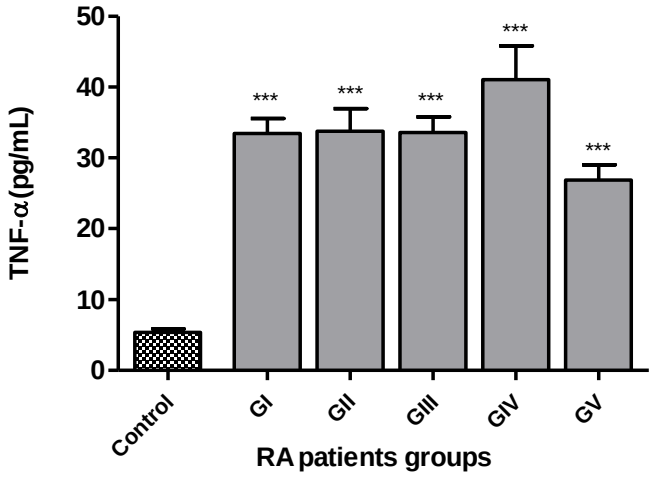


**Fig 2: Serum levels of TNF-α in healthy group and in the five groups of patients suffering from rheumatoid arthritis.**

- **Data are expressed as means ± standard error (SE).**
- **The asterisks indicate significant difference based on Tukey's multiple comparison test.**
- **P value <0.0001**
- **P value summary *****

**Relationship of TNF-α level to Hematological parameters**

**At baseline serum concentration of TNF-α correlated positively and significantly with TLC and GRA% (r = 0.3005, *p* = 0.0115 and r = 0.2589, *p* = 0.0305, respectively) (Fig. 4-A and D) and correlated positively but not significantly with MON% and PLT (r = 0.1751, *p* = 0.1472 and r = 0.2152, *p* = 0.0735, respectively) (Fig. 4 C and E). TNF-α showed a negative non-significant correlation with LYM% (r = -0.2242, *p* = 0.0620) (Fig. 4-B).**

**On the other hand there were no significant correlations between TNF-α and RBC, Hb, HCT and MCV (Fig. 3-A, B, C and D), respectively. TNF-α was significantly negatively correlated with MCH and MCHC (r = -0.2601, *p* = 0.0297and r = -0.3339, *p* = 0.0047, respectively) (Fig. 3-E and F).**

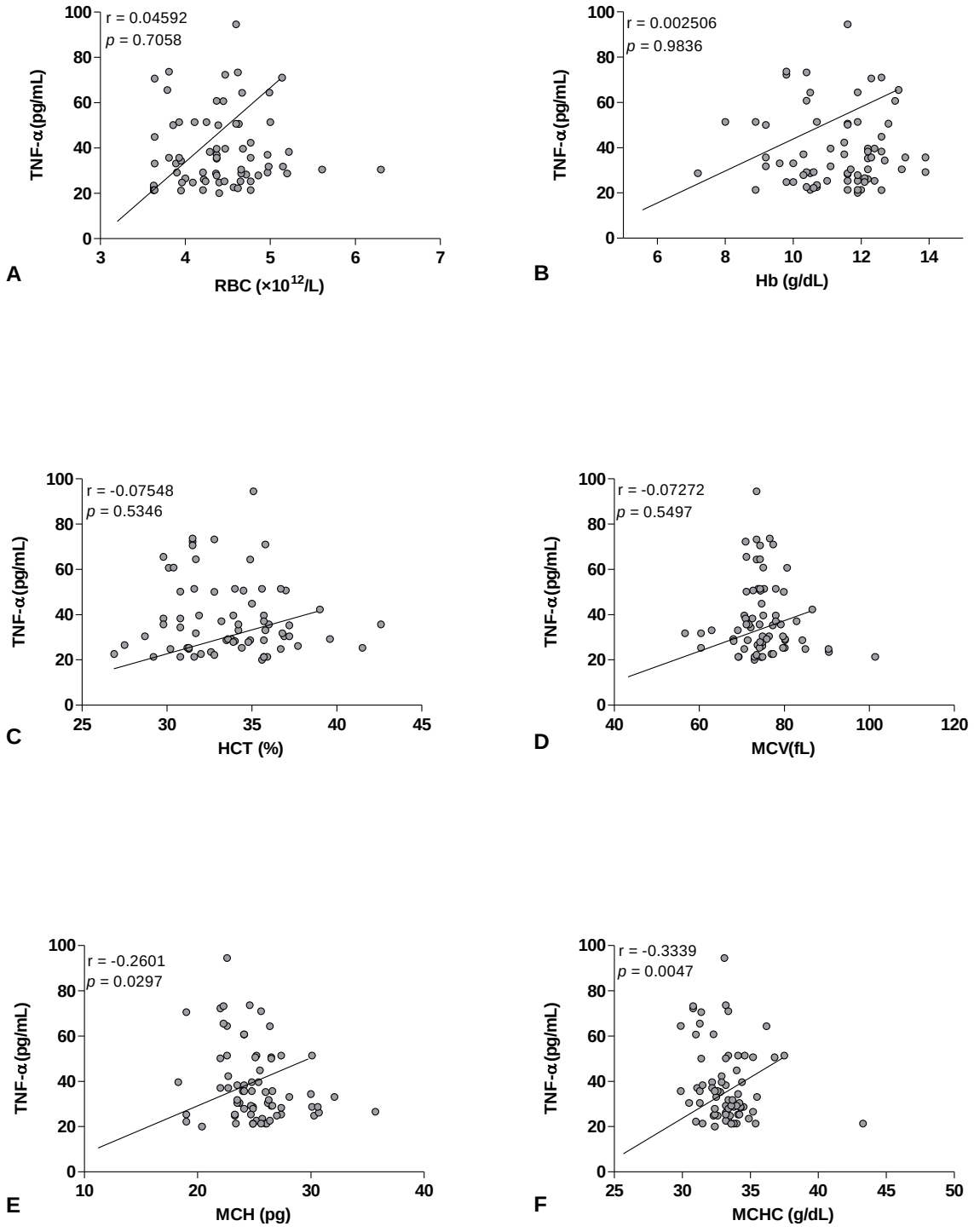


**Fig.3: The correlation among serum concentrations of TNF-α, RBC, Hb, HCT, and red cell indices in rheumatoid arthritis patients.**

***Pearson's correlation analysis was performed.**

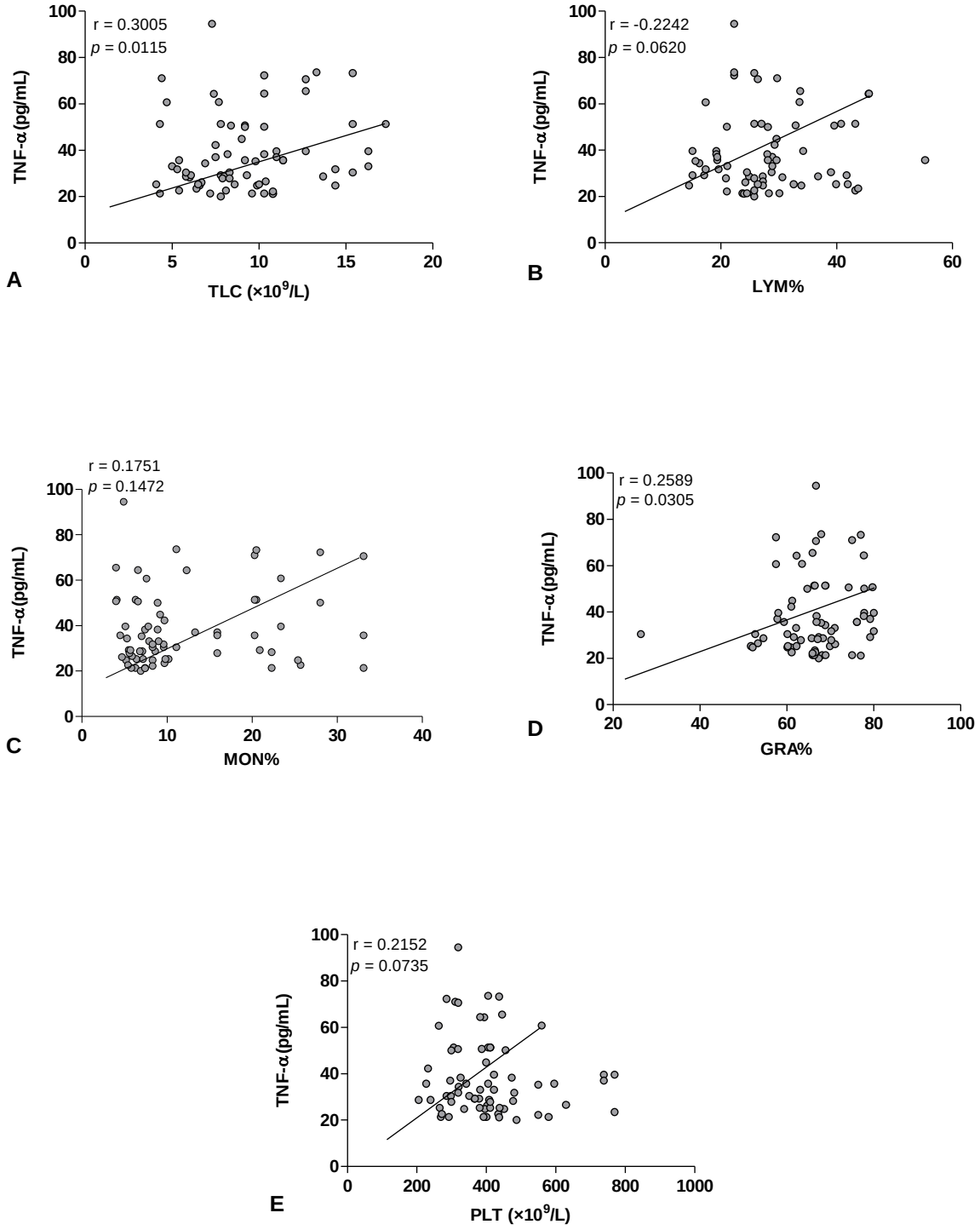


**Fig.4: The correlation among serum concentrations of TNF-α, TLC, differential leukocytes, and PLT in rheumatoid arthritis patients.**

***Pearson's correlation analysis was performed.**

## DISCUSSION

**The current study showed that serum concentrations of TNF-α, was significantly elevated in patients with RA compared to those in healthy controls. As seen in previous reports, this finding supports the hypothesis that TNF-α is involved in the pathogenesis of RA.[14-16]**

**Proinflammatory cytokines such as IL-1 and TNFα and chemokines are key mediators of the inflammation attracting inflammatory cells to the synovium which induces bone and joint destruction in RA.[17-20] In addition to these previous observations, the significant increase in the TNFα observed in the current study indicates that these cytokines might play a role in inducing inflammatory responses or mediating anti-inflammatory responses in the pathogenesis of RA.[21]**

**The present study also indicates significant decrease in RBC count, Hb, HCT and red cell indices (Table 2) in all groups of patients with rheumatoid arthritis. As seen in previous reports, this finding supports the hypothesis that the anemia is the most frequent extra-articular manifestation of the disease [22,23]. Anemia, defined by the World Health Organization [24] as a hemoglobin concentration below 12 g/dl in women and 13 g/dl in men, is common in people with arthritis. In RA, it is estimated that 30-60% of patients are anemic. One of the most frequent causes of anemia in RA patients is iron deficiency anemia (IDA). Anemia of chronic disease (ACD) which does not usually respond to iron supplementation is another major cause of anemia in patients with RA[25].**

**On the other hand, the current study showed that the TLC, MON% and GRA% and platelets were significantly elevated in patients with RA compared to those in healthy controls (Table 2). Cascão and his colleagues agreed with present findings in that the GRA% (especially neutrophils) and MON% play an important role in the onset of RA[26]. In fact, neutrophils are the most abundant leukocytes in the SF of patients with active RA, and in early RA, these cells show significantly lower levels of apoptosis. Additionally, there is a delay in the apoptosis of circulating neutrophils in VERA patients and that these cells heavily infiltrate the synovial tissue during RA onset [27]. Whereas , Haynes who studied the inflammatory cells in rheumatoid arthritis showed that the macrophages and lymphocytes have a well established role in the onset and progression of arthritis, but the role of neutrophils has been less clear [28].**

**The present results also revealed thrombocytosis associated with all groups of patients who suffering from rheumatoid arthritis. Gasparyan and his coworkers have shown in their RA study an increased number of platelets and platelet-derived proteins (growth factors) within the synovium and synovial fluid [29]. Chung et al., (2007) are also showed that circulating platelets are an abundant source of prothrombotic agents closely related to inflammatory markers, and play a crucial role in the initiation and propagation of vascular disease[30].**

**However, there is positive correlation was found between serum concentrations of TNF-α and TLC, GRA% MON% and platelets. TNF-α has pleiotropic activities in inflammatory response[20]. TNF-α might have dual roles in inflammation; pro-inflammatiory[31,32] or anti-inflammatiory[33]. Additionally, serum concentrations of TNFα was inversely correlated with hemoglobin, HCT, RBC, and red cell indices. Recent studies suggest that TNF-αcould specifically inhibit Erythropoietin (Epo) which responsible for stimulation of bone marrow to erythropoiesis, so suppress normal bone marrow in a dose-dependent manner[28].**

**In summary, it has been found the serum concentrations of TNF-α was significantly increased in patients with RA compared with those of normal controls. The cytokine levels**

**were significantly correlated with total leukocytes and differential leukocytes. But serum levels of TNF-α inversely correlated hemoglobin, hematocrit, erythrocytes and red cell indices. These findings suggest that TNF-α might be involved in the pathogenesis of RA and that levels of TNF-α might reflect the activity of the disease.**

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# دراسة العلاقة بين تركيز عامل التنخر الورمي نوع الفا (TNF-α) والمعايير الدموية لدى مرضى التهاب المفاصل الرثوي في محافظة النجف

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## الخلاصة

اجريت الدراسة الحالية لتحديد تركيز عامل التنخر الورمي نوع الفا (TNF-α) لدى مرضى التهاب المفاصل الرثوي في محافظة النجف الاشرف وكذلك التحري عن العلاقة بين مستوى تركيز TNF-α والمعايير الفسلجية للدم.

اجريت الدراسة الحالية على (70) مريض بالتهاب المفاصل الرثوي راجعوا الى مستشفى الصدر العام في محافظة النجف و (25) شخص طبيعي مقارب لهم بالعمر والجنس اعتبروا كمجموعة سيطرة، تم تحديد المعايير السرسرية لكل مريض والتي تضمنت معدل ترسيب كريات الدم الحمراء (ESR) وبروتين عالي التفاعل-C (CRP) والعامل الرثوي (RF). اما صور الدم الكاملة (CBC) فقد تم قياسها باستعمال جهاز™Mythic بينما تم قياس تراكيز عامل التنخر الورمي نوع الفا (TNF-α) باستعمال جهاز (ELISA).

اظهرت النتائج ارتفاعا معنويا في تراكيز عامل التنخر الورمي نوع الفا (TNF-α) مقارنه بالسيطرة . بالرغم من عدم وجود علاقة معنويه بين TNF-α وعدد كريات الدم الحمراء، خضاب الدم، حجم الكريات المرصوص ودلائل كريات الدم الحمراء، الا ان النتائج اظهرت علاقة موجبه معنوية مع خلايا الدم البيضاء والعدد التفريقي لها والصفيحات الدموية.

يستنتج من الدراسة الحالية ان عامل التنخر الورمي نوع الفا (TNF-α) قد ارتفع ارتفاعا معنويا لدى مرضى التهاب المفاصل الرثوي مقارنة بالأشخاص الاصحاء وهذا الارتفاع مرتبط ارتباطا قويا مع التغيرات في المعايير الدموية. وهذا الاستنتاج يدل على الدور الذي يلعبه عامل التنخر الورمي نوع الفا (TNF-α) في إمراضيه التهاب المفاصل الرثوي.