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THE CORRELATION STUDY AMONG SERUM CONCENTRATIONS OF PRO-INFLAMMATORY CYTOKINES AND HEMATOLOGICAL PARAMETERS IN RHEUMATOID ARTHRITIS PATIENTS IN NAJAF GOVERNORATE

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Abstract- This study was performed to determine whether the serum concentra-tions of pro-inflammatory cytokines: interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, and interleukin (IL)-8 are elevated in patients with rheuma-toid arthritis (RA) and to investigate the relationship between these cytokines levels and hematological parameters in RA patients. Serum samples were obtained from 75-patientswith RA who had visited the Division of Rheumatology at Najaf Teaching Hospital in 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). Complete blood count (CBC) were performed using automated hematology analyzer Mythic™. Serum concentrations of IL-6, (TNF-α), (IL)-1α, and (IL)-8 were measured using an enzyme-linked im-munosorbent assay (ELISA). Serum concentrations of IL-6, (TNF-α), (IL)-1α, and (IL)-8 were significantly elevated (P<0.0001) in patients with RA compared to those of healthy controls. Although there was no significant rela-tionship between pro-inflammatory cytokines levels and RBC, Hb, HCT and red cell indices, the pro-inflammatory levels of pa-tients with RA showed a significant correlation with total leukocyte count (TLC), differential leukocytes and platelets. It has been concluded that the serum concentrations of IL-6, (TNF-α), (IL)-1α, and (IL)-8 were sig-nificantly elevated in patients with RA and strongly correlated with hematological alterations. These findings suggest a possible role for these cytokines in the pathogenesis of RA.

Keywords- *serum, cytokines, rheumatoid, arthritis*

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 environ-mental factors [1].

Various pro-inflammatory cytokines, such asIL-6, tumor ne-crotic factor (TNF)-α, interleukin (IL)-1α, and IL-8, are increased in the synovial tissue or sy-novial fluid of patients with RA [2,3]. Increased levels of pro-inflammatory 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]. In particular, IL-6 is a cytokine with various functions. When IL-6 is ac-tivated, acute inflammatory responses such as fever or ane-mia are induced. IL-6 promotes the proliferation of B cells and thus is involved in the production of the rheumatoid factor [6,7]. Tumor Necrosis Factor (TNF) is a multifunctional cytokine with potent pro-inflammatory 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 [8].

Pro-inflammatory 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 [9,10].

The pro-inflammatory cytokines IL-1, TNFα, and IL-6 in particular, were responsible for induction of several plasma proteins such as C-reactive protein and ferritin synthesis in a hepatic cell line [11,12].

The current study was sought to examine whether se-rum levels of IL-6, TNFα, IL-1, and IL-8 are increased in pa-tients with RA and whether the increased levels are signifi-cantly correlated with hematological parameters. The serum concentrations of these cytokines were compared in pa-tients with RA and those in normal controls and then inves-tigated the correlation between serum levels of pro-inflammatory cytokines and the hematological alterations of RA.

Materials and Methods

Subjects and clinical assessment. This study was conducted in 70 patients who had visited the Division of Rheumatology at Najaf teaching hospital in Najaf city between February and August 2011, and who fulfilled the American College of Rheumatology (ACR) 2010 revised criteria for the diagnosis of RA [13]. Twenty five age- and sex-matched healthy adults without any evi-dence of chronic inflammatory disease served as the con-trols. 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, 1ml for evaluation of ESR, and1ml for serological tests, 10µl were collected in anticoagulant for hematological assessments, whereas serum samples were collected in glass tubes without anticoagulant, stored for one hour at room temperature, centrifuged (2.500 r.p.m. for 10 minutes at 4°C) and then a liquoted in plastic tubes before being stored at -20°C until analysis.

Study Design

The group of patients was classified according to the duration of RA disease as follows [14]:

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

Erythrocyte Sedimentation Rate (ESR)

The International Committee on Standardization in Hematology (ICSH) recommends the use of the Westergren method [15,16].

Procedure

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

C - R Protein (Eactive CRP) Serology Test

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

Procedure

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

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

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

Hematological Investigation

The hematological parameters were performed on EDTA blood using Mythic™ 18 (RINGELSAN CO., Turk) in Hematology Laboratory of Najaf teaching hospital in Najaf city. Mythic 18 is a fully automated hematology analyzer performing complete blood count (CBC) on EDTA anti-coagulated 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.

Measurement of IL-6, TNFα, IL-1α, and IL-8

Serum samples were collected intravenously and stored in -20°C after centrifugation until analysis. The serum concentration of IL-6 was measured using an Assay Maxenzyme-linked immune sorbent assay (ELISA) kit (Assaypro, USA) at Virology Laboratory of Najaf teaching hospital in Najaf city.

Fifty microliters each of serum sample and assay diluents were placed in each well of a 96-well plate coated with a monoclonal mouse IgG against IL-6. This mixture was in-cubated for two hours at room temperature, and each well was aspirated and washed five times with wash buffer. Subsequently, 50 µl of BiotinylatedIL-6 Antibody was added to each well and incubated for two hours. Again, each well was washed five times with wash buffer. Following this, 50µlof 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 (H2O2) and tetramethylbenzidine, was added for a 20-minute reaction under dark conditions. The reaction was quenched by the addition of 50 µl stop solu-tion (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 IL-6 was determined based on a standard concentration curve. The correlation coefficient (r) of the standard concentration curve was 0.990.

The TNFα, IL-1α, and IL-8kits were purchased from Assaypro, USA, and serum concentrations of each cytokines determined using similar methods. The respective correlation coefficients (r) were 0.993,0.990, and 0.998 for TNFα, IL-1α, and IL-8, respectively.

Statistical Analysis

Data analyses were performed with GraphPad Prism version 5.04 Software (GraphPad Software Inc., San Diego, CA, USA). All of the descriptive vari-ables were expressed as the mean ± standard error (SE). The correlations between the concentrations of IL-6and hematological parameters were tested using Pearson's correla-tion test. The group analyses were per-formed 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 dis-ease 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%	94.70%	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%	90.90%	80%

Hematological Parameters

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

- 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.

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)	Group V (n=10)	P value
RBC(×1012/L)	5.67±0.12	4.19±0.175a***	4.49±0.13a***	4.43±0.10a***	4.21±0.17a***	4.57±0.08a***	<0.0001***
Hb(g/dL)	15.49±0.21	11.05±0.40a***	11.27±0.29a***	10.81±0.40a***	11.08±0.44a***	11.52±0.27a***	<0.0001***
HCT %	46.67±0.75	33.71±0.84a***	33.39±0.75a***	32.75±1.13a***	33.54±1.16a***	33.96±0.57a***	<0.0001***
MCV(fL)	91.51±1.10	76.38±2.48a***	74.57±1.56a***	75.64±1.79a***	76.85±2.56a***	71.53±0.99a***	<0.0001***
MCH(pg)	31.36±0.53	25.89±0.87a***	24.91±0.63a***	24.61±0.78a***	25.81±1.04a***	25.79±0.97a***	<0.0001***
MCHC(g/dL)	35.29±0.17	33.71±0.38ans	34.14±0.45ans	33.34±0.67a**	33.06±0.30a*	33.7±0.30ans	0.0042**
TLC(×109/L)	7.00±0.25	9.51±1.15ans	7.97±0.61ans	8.66±0.63ans	8.05±0.54ans	8.6±1.09ans	0.1190ns
LYM%	33.06±1.01	26.33±2.38a, bns	30.74±2.96ans	28.58±1.92a,bns	28.68±0.84a,bns	20.72±2.02b**	0.0047**
MON%	3.95±0.21	12.61±3.13a**	11.84±1.53a**	9.42±1.37ans	16.51±2.78a***	12.07±2.35a*	<0.0001***
GRA%	59.83±1.37	64.73±3.95a,bns	66.09±2.21a,bns	63.57±2.55ans	65.8±0.78a,bns	74.43±2.28b***	0.0027**
PLT(×109/L)	224.3±7.70	498.1±69.2a***	346.5±29.1b*	465.4±31.8a,b***	401.3±29a,b**	347.7±18.93a,bns	<0.0001***

Standard Curves of Cytokines

The serum concentrations of each cytokine were determined based on astandard concentration curve. The standard curve was used to estimate the relationship between the optical density (O.D) of each cytokine and standards (pg/ml) and the results were interpolated using Graph Pad Prism version 5.04 Software (Graph Pad Software Inc., San Diego, CA, USA) to determine each point [Fig-1] to [Fig-4]. The correlation coefficients (r) of the standard concentration curve were0.98730.9992, 0.9998 and 0.9986 for IL-6, TNF-α, IL-1αandIL-8, respectively.

Serum Concentrations of Cytokines

The statistical analysis revealed a significant increase (P<0.0001) in IL-6, TNFα, IL-1α, and IL-8 with all groups of patients with RA compared to the healthy control group [Fig-5] to [Fig-8].

- 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 ***
- Data are expressed as means ± standard error (SE).
- The asterisks indicate significant difference compared to control based on Tukey's multiple
- comparison test.
- The same letters indicate non-significant difference between groups based on Tukey's multiple comparison test.
- P value summary ***

Relationship of Cytokines Levels to Hematological Parameters

Relationship of IL-6 levels to hematological parameters

Serum concentration of IL-6 correlated positively and significantly with TLC (r = 0.3169, p = 0.0075) and GRA% (r = 0.2635, p = 0.0276) [Fig-9] A and D, respectively and correlated positively but not significantly with MON% (r = 0.2118, p = 0.0784) and PLT (r = 0.2071, p = 0.0854) [Fig-9] C and E respectively. IL-6 showed a negative non-significant correlation with LYM% (r = -0.2044, p = 0.0896) [Fig-9] B. On the other hand there were inverse correlations between IL-6, RBC, and Hb [Fig-10] A and B, respectively. IL-6 was significantly negatively correlated with MCH (r = -0.2583, p = 0.0309), and MCHC (r = -0.3310, p = 0.0051) [Fig-10] E and F, respectively.

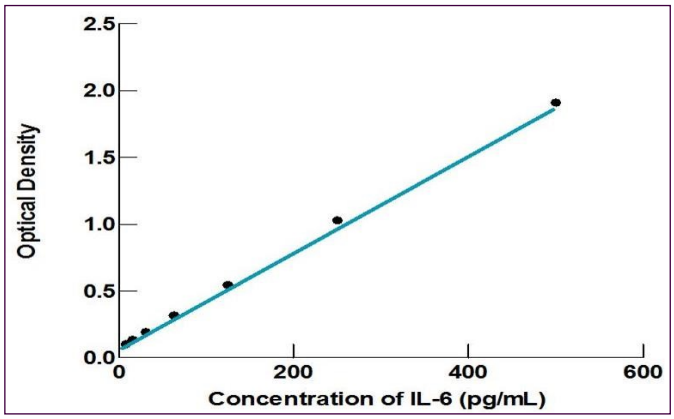

Fig. 1- Interleukin-6 (IL-6) standard curve.

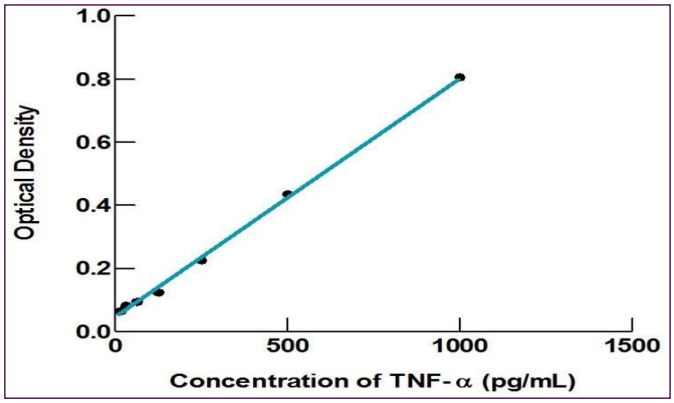

Fig. 2- Tumor necrosis alpha (TNF-α) standard curve.

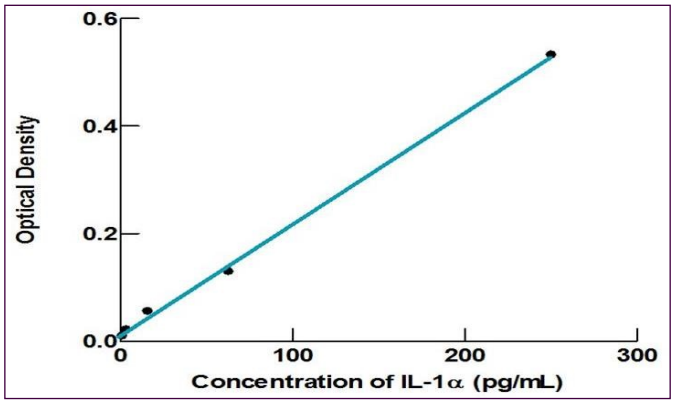

Fig. 3- Interleukin-1 (IL-1α) standard curve.

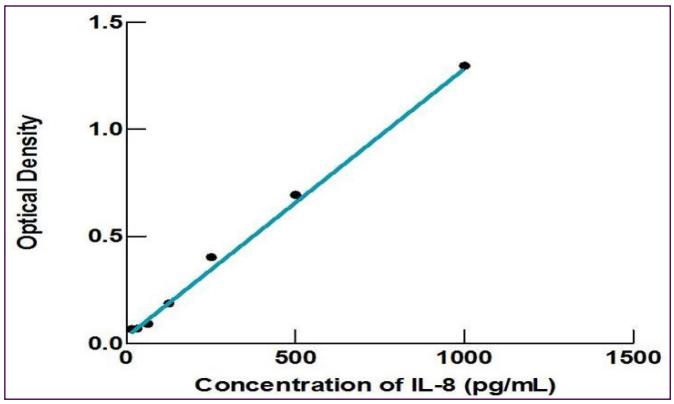

Fig. 4- Interleukin-8 (IL-8) standard curve.

Relationship of TNF-α level to hematological parameters

Serum concentration of TNF-α correlated positively and significantly with TLC (r=0.3005, p=0.0115) and GRA% (r=0.2589, p=0.0305) [Fig-11] A and D, respectively and correlated positively but not significantly with MON% (r=0.1751, p=0.1472) and PLT (r=0.2152, p=0.0735) [Fig-11] C and E, respectively. TNF-α showed a negative non-significant correlation with LYM% (r= -0.2242, p=0.0620) [Fig-11] B. On the other hand there were inverse correlations between TNF-α, HCT and MCV [Fig-12] C and D, respectively). TNF-α was significantly negatively correlated with MCH (r= -0.2601, p=0.0297), and MCHC (r= -0.3339, p= 0.0047) [Fig-12] E and F, respectively.

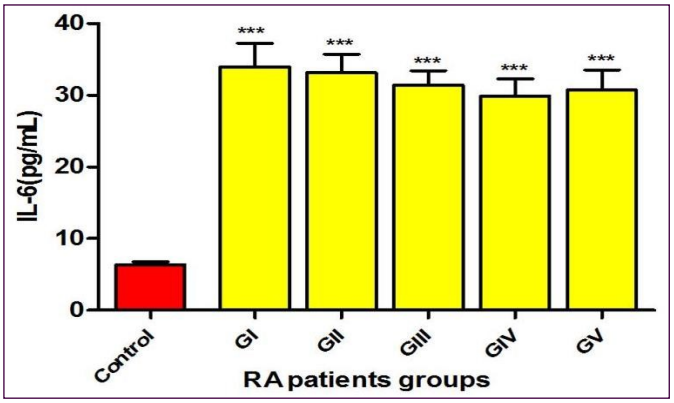

Fig. 5- IL-6 in healthy control group and in the groups of patients suffering from RA

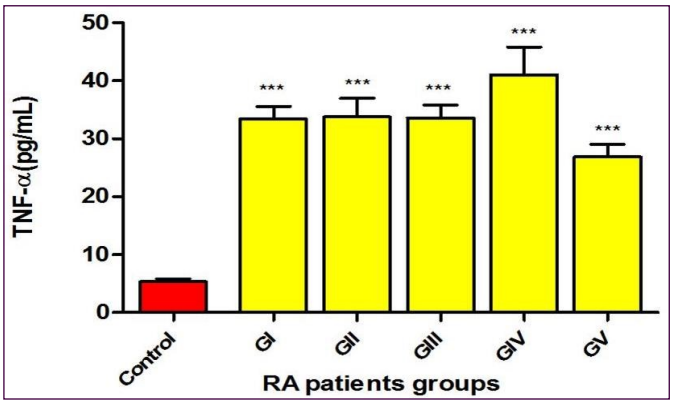

Fig. 6- IL-1α in healthy control group and in the groups of patients suffering from RA

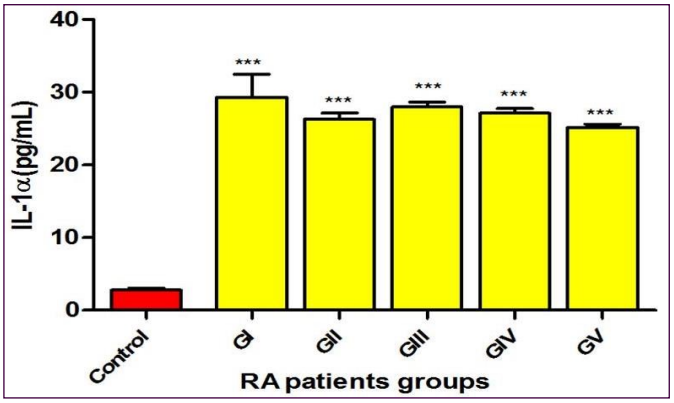

Fig. 7- TNF-α in healthy control group and in the groups of patients suffering from RA

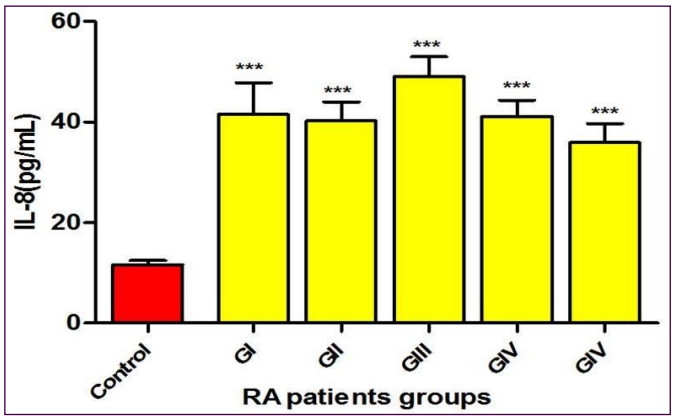

Fig. 8- IL-8 in healthy control group and in the groups of patients suffering from RA

Relationship of IL-1α levels to hematological parameters

Serum concentration of IL-1α correlated positively and significantly with GRA% and PLT (r = 0.2459, p = 0.0402 and r = 0.2458, p = 0.0402, respectively) [Fig-13] D and E and correlated positively but not significantly with TLC and MON% (r = 0.2096, p = 0.0817 and r = 0.1710, p = 0.1570, respectively) [Fig-13] A and C. IL-1α showed a negative non-significant correlation with LYM% (r = -0.1387, p = 0.2523) [Fig-13] B. On the other hand there were inverse correlations between IL-1α, Hb, HCT, and red cell indices [Fig-14] B, C, E and F, respectively).

Relationship of IL-8 levels to hematological parameters

Serum concentration of IL-8 correlated positively and significantly with TLC and GRA% (r = 0.3114, p = 0.0087 and r = 0.2540, p = 0.0338, respectively) [Fig-15] A and D and correlated positively but not significantly with MON% and PLT (r = 0.2121, p = 0.0780 and r = 0.1916, p = 0.1122, respectively) [Fig-15] C and E. IL-8 showed a negative non-significant correlation with LYM% (r = -0.2291, p = 0.0564) [Fig-15] B. RBC, Hb, HCT, and MCV did not significantly correlated with IL-8 [Fig-16] A, B, C and D, respectively, whereas MCH and MCHC significantly negatively correlated with IL-8 (r = -0.3085, p = 0.0094 and r = -0.3664, p = 0.0018, respectively) [Fig-16] E and F.

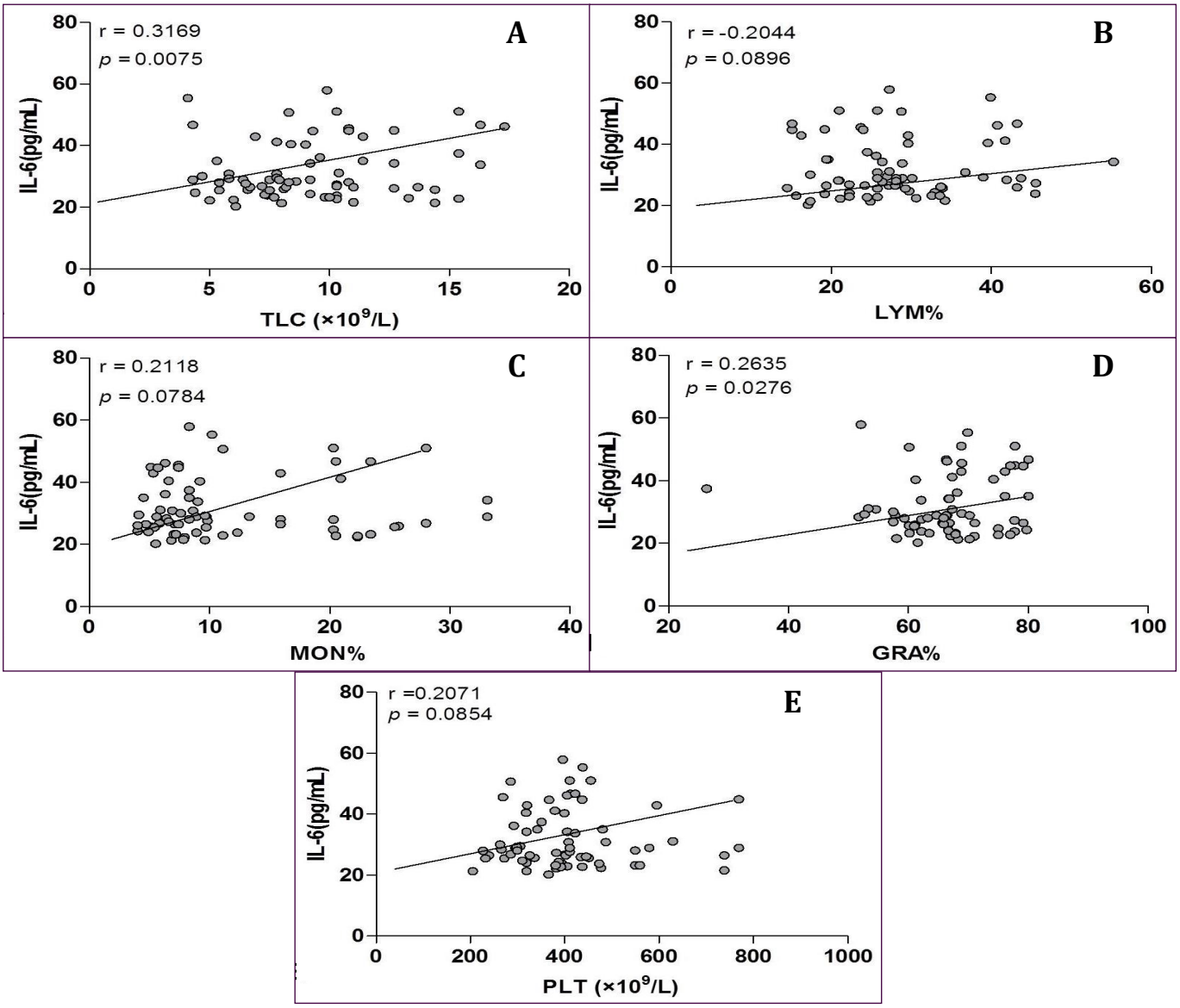

Fig. 9- The correlation among serum concentrations of IL-6, TLC, differential leukocytes, and PLT in rheumatoid arthritis patients.

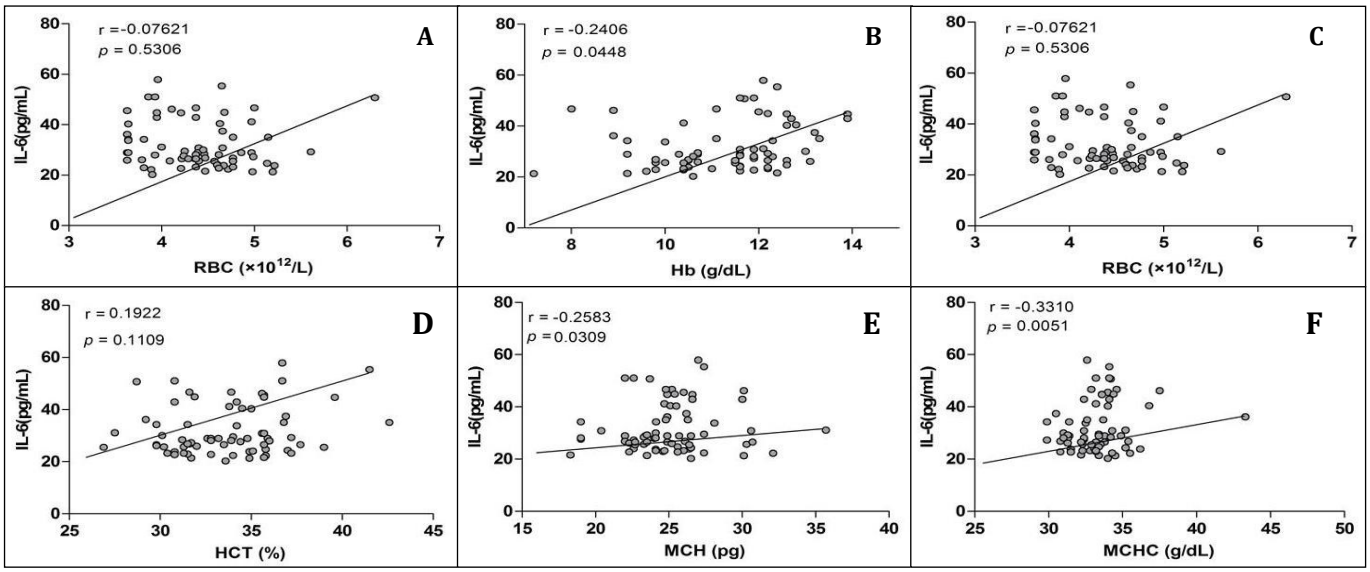

Fig. 10- The correlation among serum concentrations of IL-6, RBC, Hb, HCT, and red cell indices in rheumatoid arthritis patients.

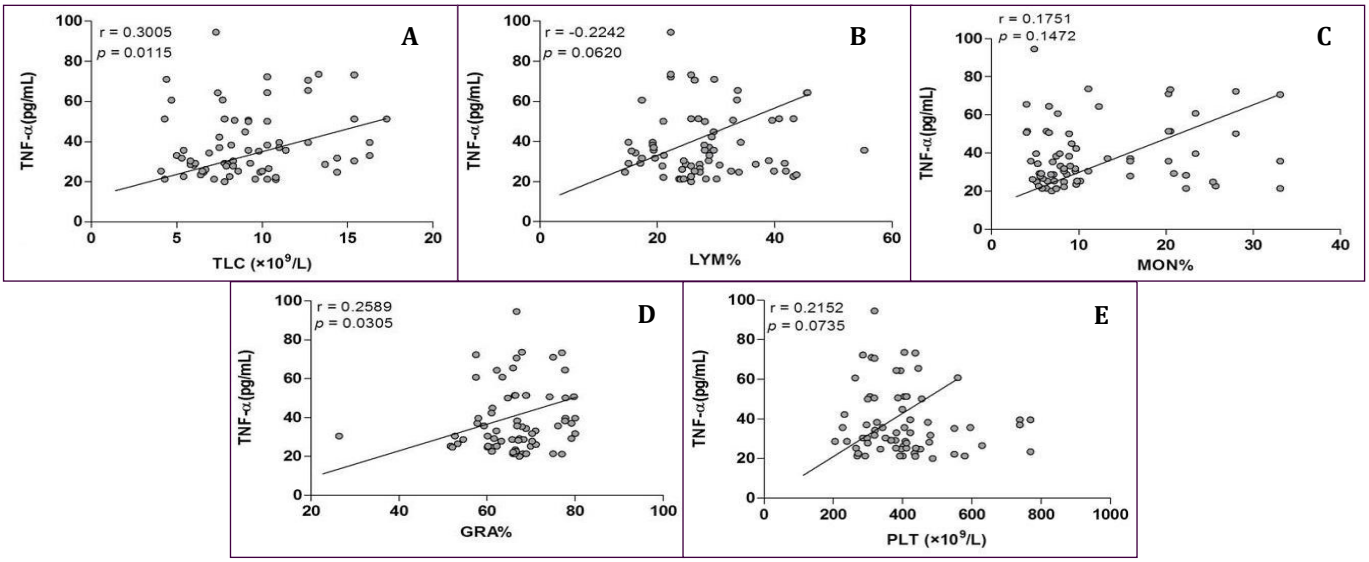

Fig. 11- The correlation among serum concentrations of TNF-α, TLC, differential leukocytes, and PLT in rheumatoid arthritis patients.

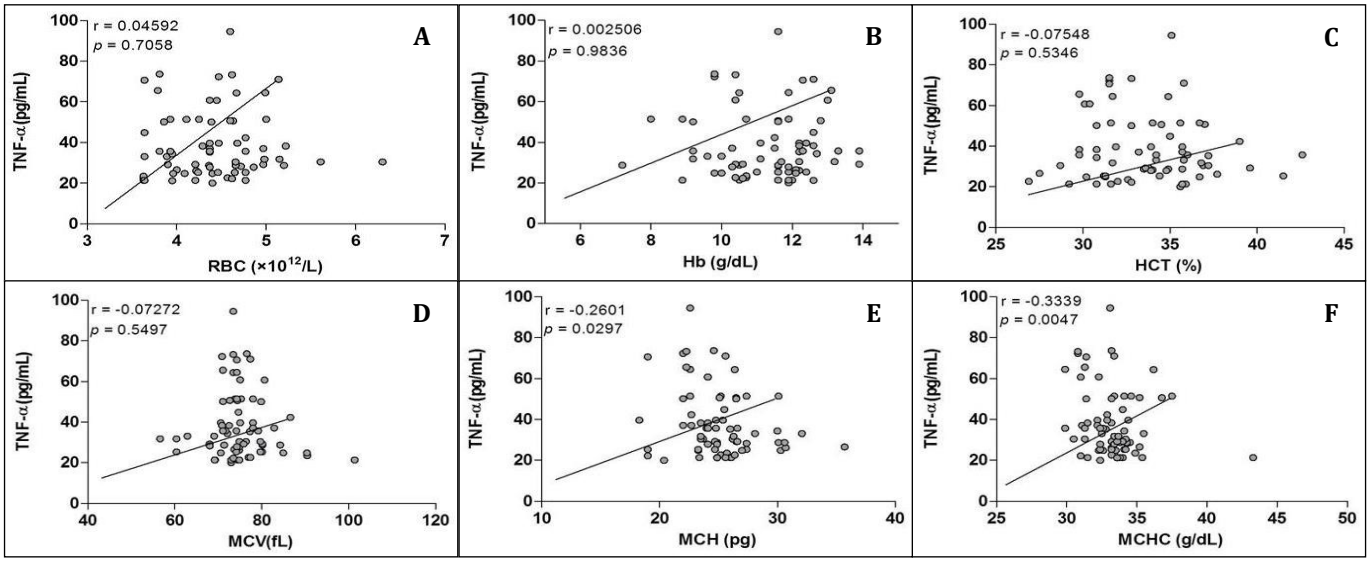

Fig. 12- The correlation among serum. concentrations of TNF-α, RBC, Hb, HCT, and red cell indices in rheumatoid arthritis patients.

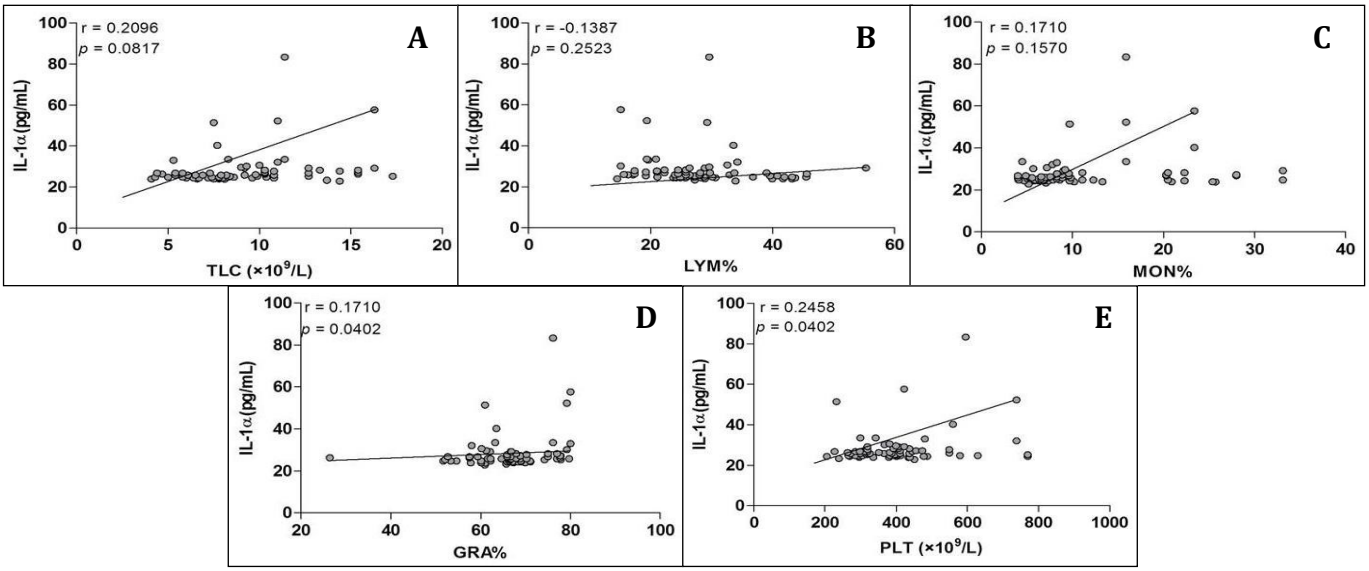

Fig. 13- The correlation among serum concentrations of IL-1α, TLC, differential leukocytes, and PLT in rheumatoid arthritis patients.

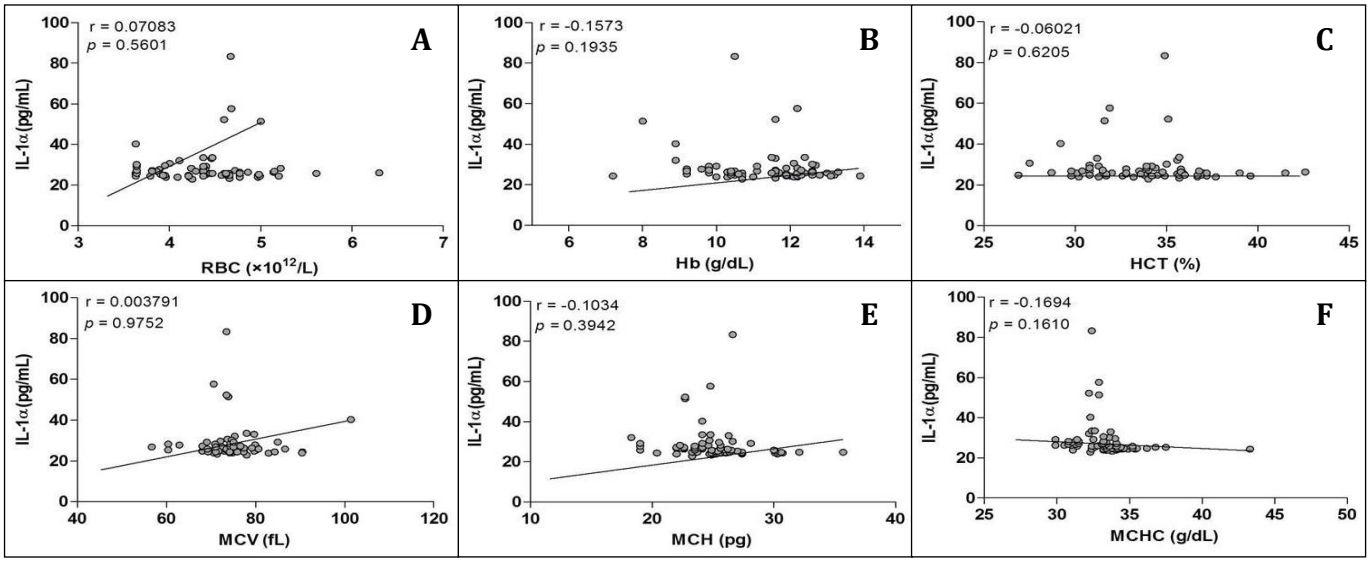

Fig. 14- The correlation among serum concentrations of IL-1α, RBC, Hb, HCT, and red cell indices in rheumatoid arthritis patients.

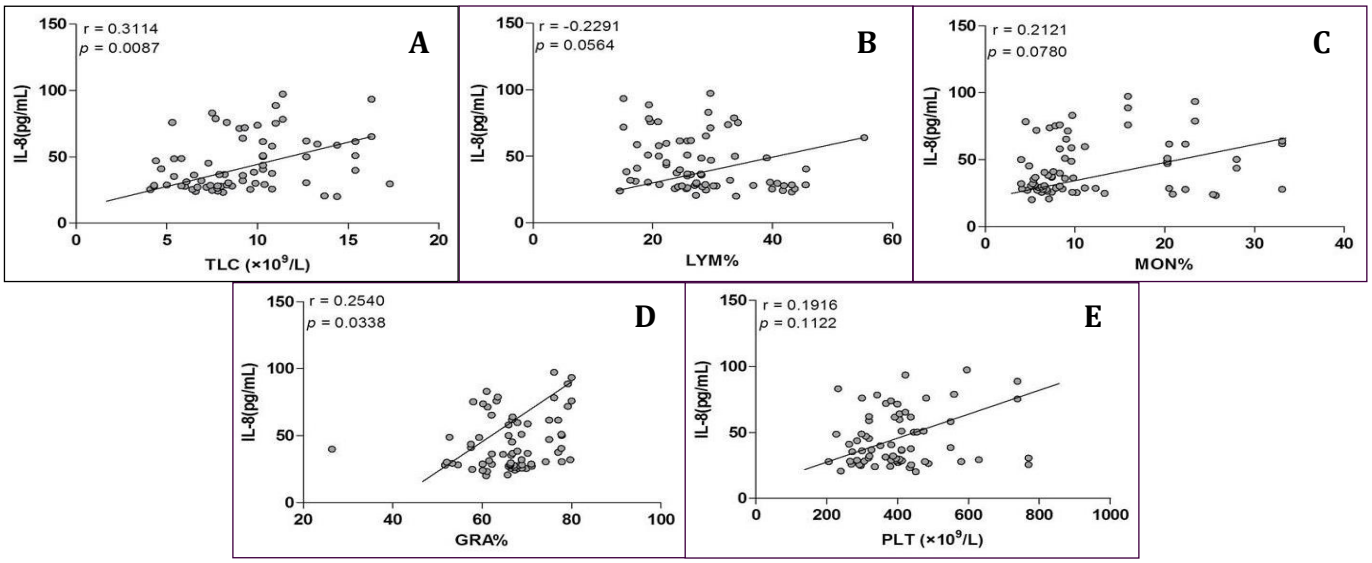

Fig. 15- The correlation among serum concentrations of IL-8, TLC, differential leukocytes, and PLT in rheumatoid arthritis patients.

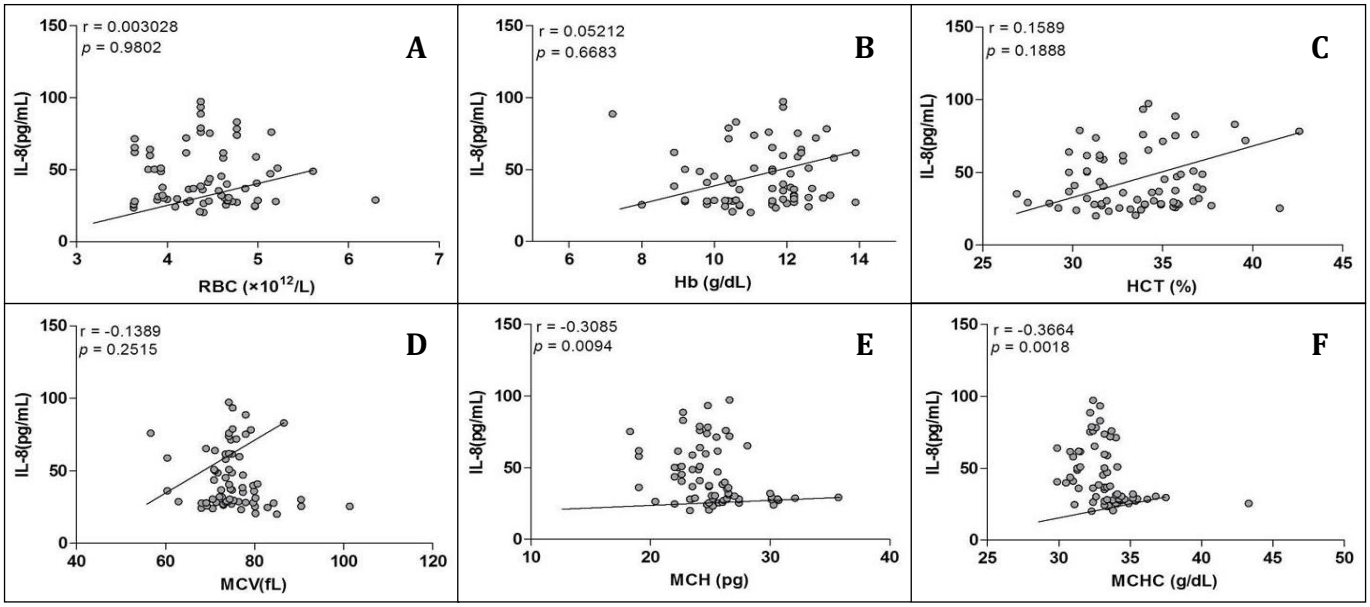

Fig. 16- The correlation among serum concentrations of IL-8, RBC, Hb, HCT, and red cell indices in rheumatoid arthritis patients.

Discussion

[Table-3] showed that serum concentrations of IL-6, TNFα, IL-1α and IL-8 were significantly elevated (P<0.0001) in patients with RA compared to those in healthy controls. As seen in previous reports, this finding supports the hypothesis that these cytokines are involved in the pathogenesis of RA [17-20].

IL-6 is a cytokine that causes an acute inflammatory re-sponse, and it is well-documented that IL-6 plays a crucial role in the pathogenesis of various inflammatory diseases including RA [21,22].

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 [23,24]. Pro-inflammatory cytokines such as IL-1 and TNF-α and chemokines such as IL-8 are key mediators of the inflammation attracting inflammatory cells to the synovium which induces bone and joint destruction in RA [25,26].

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 [27,28]. Anemia, defined by the World Health Organization [29] as a hemoglobin concentration below 12 g/dl in women and 13 g/dl in men, is common in people with arthritis. On the other hand, The current study showed [Table-2] that the TLC, MON% and GRA% and platelets were significantly elevated in patients with RA compared to those in healthy controls.

Cascão and his colleagues agreed with our findings in that the GRA% (especially neutrophils) and MON% play an important role in the onset of RA28. 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 [30]. 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 [31]. Our 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 [32]. Chung, et al. [33] 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. However, there is positive correlation was found be-tween serum concentrations of IL-6 and TLC, GRA% MON% and platelets. IL-6 has pleiotropic activities in in-flammatory response [17]. IL-6 might have dual roles in in-flammation; pro-inflammatory [34,35] or anti-inflammatory [36]. Additionally, serum concentrations of I that IL-6 could specifically inhibit Erythropoietin (Epo) which responsible for stimulation of bone marrow to erythropoiesis, so suppress normal bone marrow in a dose-dependent manner [22].

Table 3- Cytokines concentrations (pg/mL) in healthy control group and in the groups of patients suffering from rheumatoid arthritis.

Cytokine (pg/mL)	Healthy control (n=25)	RA patients (n=70) Duration of disease groups					
		Group I (n=10)	Group II (n=19)	Group III (n=20)	Group IV (n=11)	Group V (n=10)	P value
IL-6	6.35±0.40	33.99±3.26a***	33.14±2.56a***	31.41±1.98a***	29.89±2.41a***	30.78±2.76a***	<0.0001***
TNF-α	5.36±0.48	33.44±2.1a,b***	33.77±3.19a,b***	33.6±2.22a,b***	41.021±4.74a***	26.91±2.12b***	<0.0001***
IL-1α	2.82±0.25	29.31±3.16a***	26.31±0.82a***	28.01±0.65a***	27.11±0.69a***	25.18±0.43a***	<0.0001***
IL-8	11.61±0.87	41.51±6.30a***	40.26±3.77a***	49.09±3.92a***	41.10±3.23a***	35.93±3.80a***	<0.0001***

Conclusion

It has been concluded that the serum concentrations of pro-inflammatory cytokines IL-6, TNFα, IL-1α and IL-8 were significantly increased in patients with RA compared with those of normal controls. The cytokine levels were significantly correlated with hematological parameters. These findings suggest that these cytokines might be involved in the pathogenesis of RA and that levels of it might reflect the activity of the disease.

Conflicts of Interest: None Declared.

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