Correlation between Relative Expression of IL 17 and PERP in Rheumatoid Arthritis Patients and Disease Activity

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder. Decreased apoptosis is considered an important leading cause of autoimmune diseases. As IL17 and PERP can affect apoptosis process, they may contribute the pathogenesis and activity of RA. Objectives of this study were to investigate the possible correlation of IL 17 and PERP levels with RA pathogenesis and activity. Peripheral blood mononuclear cells (PBMCs) were isolated from fifty RA patients and fifty healthy subjects, RNA was extracted and subjected to real time PCR to detect the relative expression of IL17 and PERP. Results were correlated with RA disease activity parameters. Increased IL17 and decreased PERP mRNA expression levels were detected in patients as compared to the healthy controls (*P*<0.001) and they were positively and inversely correlated with disease activity score for 28 joints (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and rheumatoid factor (RF). A significant negative correlation between PERP and IL-17 mRNA expression levels was found (*P* <0.001). In conclusion, increased level of IL 17 and decreased level of PERP may constitute two major factors in the pathogenesis and activity of RA.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that primarily affects the synovial tissues of multiple joints with articular cartilage and bone destruction [1]. Improper immune regulation is involved in pathogenesis of RA through attacking the joints by self immune system due to lymphocytes, monocytes and other immune cells activation and recruitment [2].

Interleukin-17 (IL-17) is a pro-inflammatory cytokine, secreted by active Th17 cells. It exerts various biological functions in a wide range of inflammatory, infectious and autoimmune diseases. Playing the key role in pathogenesis of RA, it enhances chronic inflammation, cartilage damage, and bone erosion [3].

Pathogenesis of autoimmune diseases such as RA, systemic lupus, and multiple sclerosis may be attributed to low expression of p53 in peripheral blood mononuclear cells (PBMCs) of those patients which leads to inability to remove potentially pathogenic cells [4].

P53 effector related to PMP22 (PERP), a tetrapan protein, is a p53 transcriptional target proapoptotic gene with increased expression level during apoptosis rather than cell cycle arrest. It is one of peripheral myelin protein 22/growth arrest specific 3 (PMP-22/gas3) family members [5].

Autoimmune diseases such as RA may be attributed to defects in apoptotic cascades, as in normal inflammatory responses, initiation of appropriate apoptotic cascades is necessary for keeping lymphocyte homeostasis and improving inflammation to prevent normal tissues damage [6, 7].

The aim of this study was to investigate the possible correlation between IL 17 and PERP expression levels in RA patients and disease activity parameters, including DAS28, CRP, RF, antinuclear antibody

(ANA), anti-cyclic citrullinated peptide (anti-CCP), and ESR.

Patients and Methods

A case-control study was performed in Medical Microbiology and Immunology Department and Rheumatology & Rehabilitation Department, Faculty of Medicine, Zagazig University Hospitals in the period between September 2017 and December 2018.

Patients

The study was carried out on 100 subjects; they included two groups matched for age and sex as follows:

- Group I

It included fifty RA patients who fulfilled the 2010 American College of Rheumatology (ACR) / European League against Rheumatism (EULAR) classification criteria for rheumatoid arthritis [8]; they were selected from Rheumatology & Rehabilitation department, Zagazig University Hospitals. Their ages ranged from 22 - 70 year and the duration of the RA disease was between 5 months and 14 years.

- Exclusion criteria

Patients receiving corticosteroids or vitamin D, as well as patients with renal insufficiency or other coexisting rheumatic disease, were excluded.

- Group II

Fifty apparently healthy individuals free from any systemic diseases were considered as control group. Their ages ranged from 25- 71year.They had no family history of RA and never had any signs or symptoms of RA, other arthritis or joint diseases.

Written informed consent from each participant (patients and controls) was obtained prior to enrollment and the study was approved by the Institutional Review Board (IRB), Faculty of Medicine, Zagazig University.

All RA patients were subjected to full history taking including patient demographics, disease characteristics and disease duration. Clinical locomotor examination was performed to all patients to determine the total count of tender joints and swollen joints. Patients were also evaluated on the basis of the visual analogue scale. The disease activity score for 28 joints (DAS28), RF, CRP, ESR, anti-CCP and ANA levels were reported for all patients.

Assessment of RA disease activity

Disease activity was evaluated by DAS28 [9]. RA patients can be categorized into 4 levels; a DAS28 score greater than 5.1 implies highly active disease, a DAS28 score less than 5.1 and greater than 3.2 implies moderately active disease, a DAS28 score less than 3.2 implies low active disease, and a DAS28 score less than 2.6 implies remissions [10].

Detection of relative expression of IL 17 and PERP

- PBMCs isolation

PBMCs were isolated from peripheral blood of all participants by standard density-gradient centrifugation. Using Ficoll-Paque Plus (GE Healthcare, USA).

- RNA extraction and reverse transcription

RNA was extracted from PBMCs using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were subjected to RNA quantitation and purity assessment using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). Readings ranging from 1.8-2.1 indicated accepted purity. IScriptcDNA synthesis kit (Bio-Rad, Hercules, CA, USA), was used to make reverse transcription of RNA to cDNA according to the manufacturer's protocol. Briefly, A 20 μL reaction mixture was used including 4 μL 5×iScript reaction mix, 1 μL iScript reverse transcriptase, 1μg total RNA and finally the reaction mixture was completed with nuclease-free water to a final volume 20 μL. The reaction conditions were demonstrated in table ii.

- Quantitative real -time PCR

It was done to detect the relative expression of IL 17 and PERP using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a housekeeping gene. The primers used were supplied by Intron Biotechonolgy, Korea and their sequences were demonstrated in table i. Stratagene MX3005P thermal cycler (Agilent technologies,Germany) was used to perform RT-PCR using Real MOD^{TM} Grean Real-time Master mix kit (Intron Biotechonolgy, Korea). A total volume of 20 µl mixture of 10µl 2X Syber green master mix, 2µl of cDNA, 20 µM of each primer was used. Cycling conditions were demonstrated in table ii. Each sample was run in duplicate [11].

Expression of mRNA was normalized to the expression levels of a housekeeping gene, GAPDH (internal control). Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method [13]. The mean value of cycle threshold (C_T) readings for RA patients was used as calibrator for patients and the mean value of (C_T) readings for control groups was used as calibrator for controls. $\Delta\ C_{T\ calibrator}$ was estimated for each calibrator, then the following equations were estimated for patients and controls:

$\Delta\ C_{T=}$ mean $C_{T\ target\ gene}$ - mean $C_{T\ GADPH\ gene}$
$\Delta\ \Delta C_{T\ =} \Delta\ C_{T\ -} \Delta\ C_{T\ calibrator}$
$FC= 2^{-\Delta\Delta Ct}$

Statistical Analysis

All data were collected, tabulated and statistically analyzed using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA, 2011). Quantitative variables were described using their means, standard deviations and medians. The strength of the relationship between two sets of data was assessed using Spearman's rank correlation coefficient. Mann Whitney test was used to compare quantitative data as appropriate and one-way ANOVA for multigroup comparisons. *P* values for calculated test statistics were obtained. A *P* value <0.05 was considered statistically significant.

Table i. primer sequences used in RT-PCR [12].

Gene	Sequence
PERP	5 AGAGCCTCATGGAGTACGC-3
	5-CCTCACTTGCCGAAACAGC-3
IL 17	5-CTACAACCGATCCACCTCAC-3
	5-TGTGGTAGTCCACGTTCCCAT-3
GADPH	5-AGAAGGCTGGGGCTCATTTG-3
	5AGGGGCCATCCACAGTCTTC-3.

Table ii. Cycling conditions of RT-PCR [11].

RT		
Condition	Temperature	Time
First stage	25◦C	5 min
Second stage	42◦C	30 min
Third stage	85◦C	5 min
PCR		
Initial denaturation	95◦C	30 sec
40 cycle of 2 stages :		
First stage	95◦C	5 sec
Second stage	60◦C	30 sec
Dissociation step of 2 stages:		
First stage	95◦C	15 sec
Second stage	Begins at 60◦C and ends at 90◦C	1 min

Result

Fifty RA patients as well as fifty apparently healthy subjects matched in age and sex were enrolled in this study. Disease duration, ESR, CRP and DAS28 were reported for all patients. ANA, RF and Anti CCP were reported for RA patient group only (Table 1).

Relative expression of PERP was significantly higher in healthy subjects in comparison to RA patients while that of IL 17 was higher in RA patients than in healthy control with a high significant difference (Table 2).

Table 1. Demographic characteristics of RA patient (N=50) versus control group (N=50).

Items	Patients (N=50)		Control (N=50)		*P* value
Age					
Range	(22-70)		(25-71)		NS
Mean±SD	36.8± 8.9		37.02±10.3		
Sex	No	%	No	%	
Male	7	14.0	10	20.0	NS
Female	43	86.0	40	80.0	
Disease duration (months)					
Range	(5 months- 14 years)				
Mean±SD	50.9± 41.05				
Median	12				
ESR					
Range(mm\hr)	(1.3-89)				
Mean±SD	45.57±27.68				
Median	50				
CRP					
Range(mg\l)	(0.15-88)				
Mean±SD	42.36±25.37				
Median	50				
DAS28					
Range	(1-6.8)				
Mean±SD	3.22±1.64				
Median	4.6				
RF					
Range(IU\ml)	(0-2000)				
Mean±SD	1162±442.58				
Median	1150				
ANA					
Range(U\ml)	(0-900)				
Mean±SD	210±171.73				
Median	200				
AntiCCP					
Range(EU/mL)	(30-500)				
Mean±SD	102±105.25				
Median	100				

**P* >0.05 is non significant (NS). ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; DAS28: Disease activity score; ANA: RF: Rheumatoid factor; Antinuclear antibody; AntiCCP: Cyclic citrullinated peptide antibody.

Table 2. Relative expression of PERP and IL-17 in patients versus control by Mann whitney test.

Items	Patients (n=50)	Control(n=50)	*P* value
PERP			
Range	(0.1-1.4)	(0.7-3.6)	<0.001*
mean±SD	0.65± 0.33	1.96±0.96	
Median	0.4	1.75	
IL-17			
Range	0.1-2.9)(	0-2)(	<0.001*
mean±SD	1.59±0.94	0.72±0.61	
Median	1.6	0.55	

P <0.05 is significant

Furthermore, a statically significant difference was found in the relative expression of IL 17 among patient groups with different DAS28 scores (Table 3).

Similarly, a statically significant difference regarding the relative expression of PERP among patient groups according to DAS28 was also detected (Table 4).

Table 3. Relative expression of IL 17 in patient groups according to DAS28.

DAS28	IL17 (mean±SD)	Median	Range	P value	LSD
Remission (N=5)	0.16±0.04	0.1	(0.1-0.3)	<0.001*	0.16**1 0.00**2 0.00**3 0.00**4 0.00**5 0.00**6
Low activity (N=10)	0.44±0.05	0.4	(0.3-0.8)		
Moderate activity (N=12)	1.28±0.08	1.2	(0.9-1.7)		
High activity (N=23)	2.43±0.01	2.6	(1.1-2.9)		

P <0.05 is significant. **1*P* value between remission and low activity; **2*P* value between remission and moderate activity; **3*P*value between remission and high activity; **4*P*value between moderate and low activity; **5*P* value between low and high activity; **6*P* value between moderate and high activity. LSD: least significant difference.

Table 4. Relative expression of PERP in patient groups according to DAS28.

DAS28	PERP (mean±SD)	Median	Range	*P* value	LSD
Remission (N=5)	1.1±0.14	1.2	(0.6-1.4)	<0.001*	0.005**1 0.00**2 0.00**3 0.02**4 0.00**5 0.001**6
Low activity (N=10)	0.74±0.08	0.8	(0.3-1)		
Moderate activity (N=12)	0.52±0.08	0.5	(0.2-0.9)		
High activity (N=23)	0.23±0.34	0.2	(0.1-0.6)		

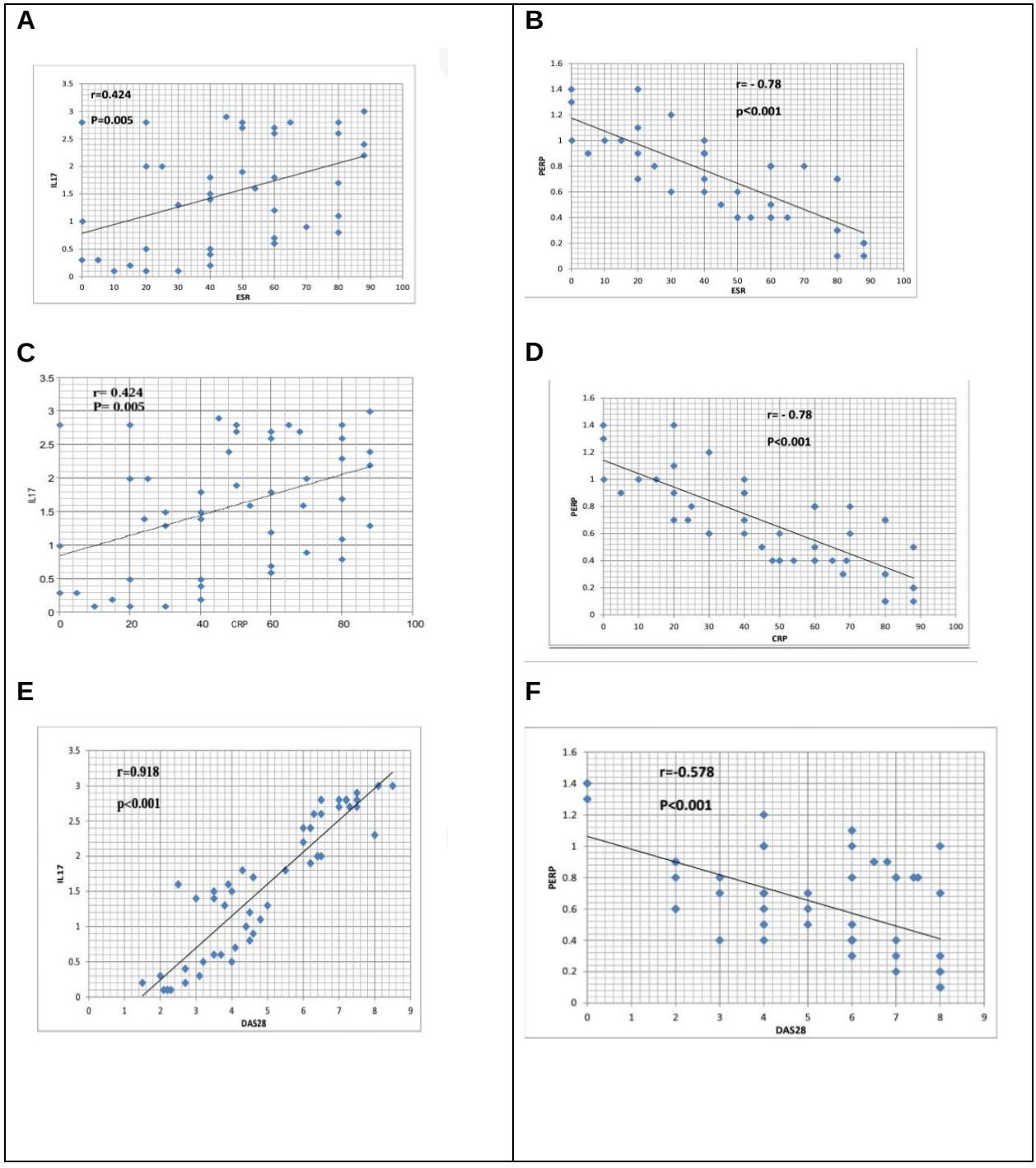
P <0.05 is significant. **1*P* value between remission and low activity; **2*P* value between remission and moderate activity; **3*P*value between remission and high activity; **4*P*value between moderate and low activity; **5*P* value between low and high activity; **6*P* value between moderate and high activity. LSD: least significant difference.

The correlations between relative expression of IL 17 and PERP with ESR, CRP, DAS28, RF, ANA and Anti CCP were demonstrated in Fig 1(A to L)

The expression levels of IL 17 and PERP in patients with RA were positively and inversely correlated with ESR, CRP, DAS28, RF, respectively (A - H), but did

not have correlations with ANA nor with Anti-CCP levels (I – L).

Correlation between PERP and IL-17 mRNA expression levels was demonstrated in figure 2. There was a highly significant negative correlation between PERP and IL-17 mRNA expression levels



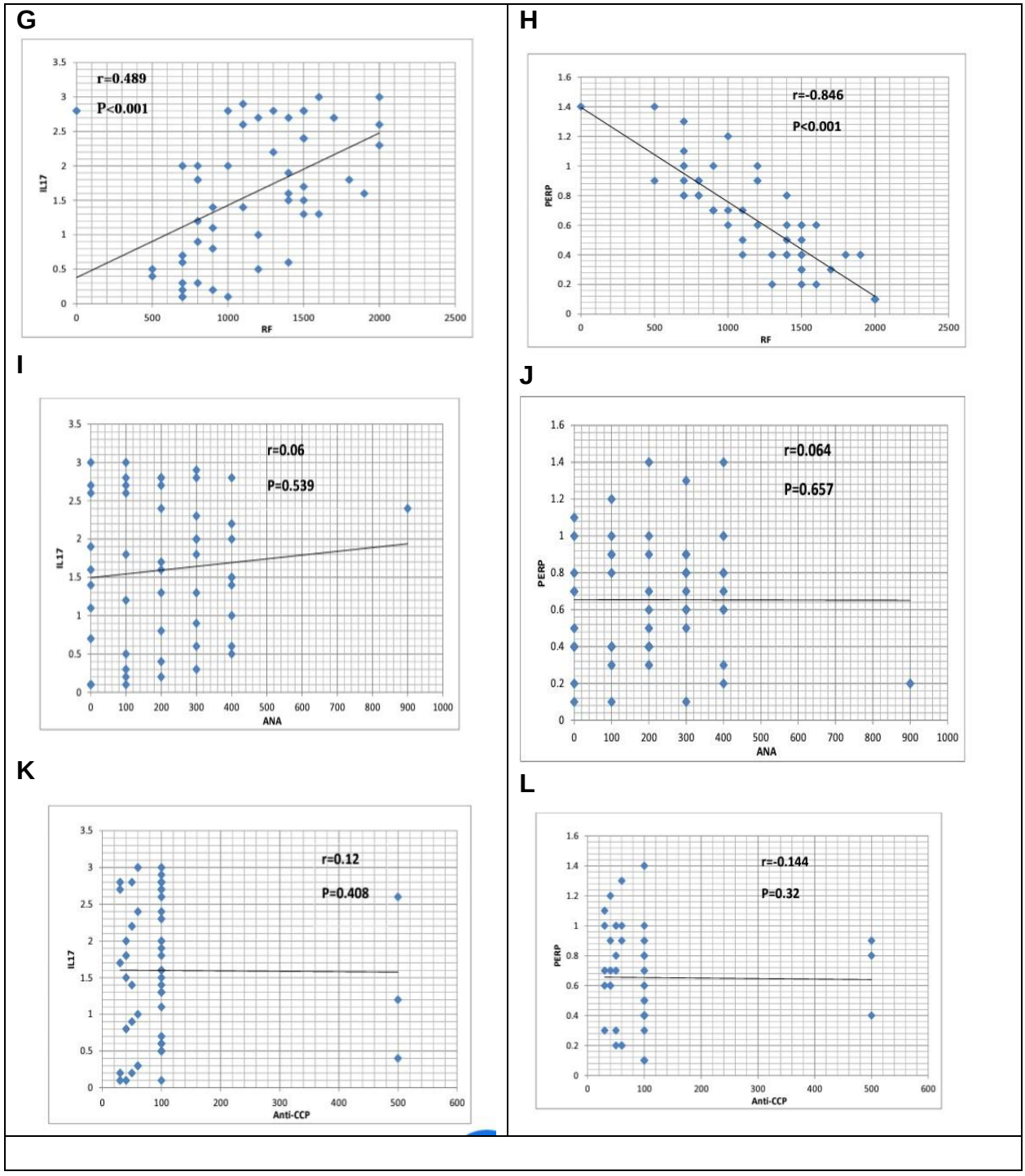


Figure 1. Correlations between relative expression of IL 17 and PERP with ESR (A, B), CRP (C, D), DAS28 (E, F), RF (G, H), ANA (I, J) and Anti CCP (K, L).

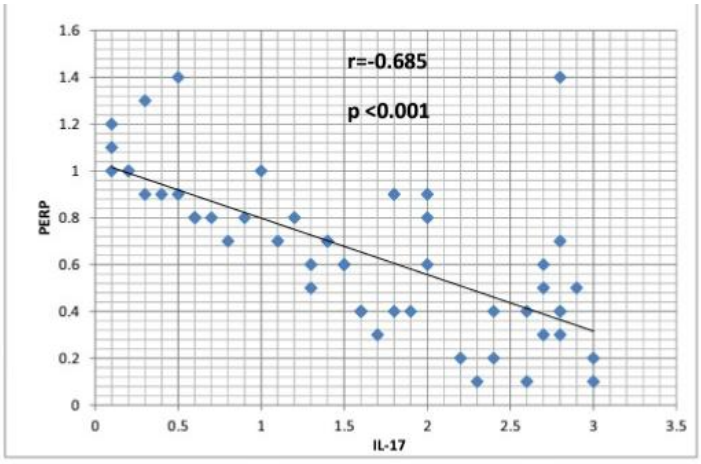


Figure 2. Correlation between PERP and IL-17 mRNA expression levels.

Discussion

Apoptosis in RA patients could be down regulated due to IL17 expression. On the other hand, Th1 and Th17 functions in those patients could be regulated by P53. Previous studies had detected P53 mutation in rheumatoid arthritis synovium [14, 15]. The low expression of p53 is implicated in pathogenesis of RA however, the role of PERP, as a transcriptional target of p53, in pathogenesis of RA and in regulating IL17 is still unclear.

In this case - control study, the role of IL17and PERP in RA disease activity could be clarified suggesting that PERP may be used as a target for management of RA.

A total of 50 patients with RA (Case group) and 50 apparently healthy individuals (Control group) were enrolled in our study. They were matched for age and sex. Data concerning CRP (42.36±25.37, Median= 50), ESR (45.57±27.68, Median=50), disease activity score (3.22±1.64, Median=4.6), RF (1162±442.58, Median=1150), ANA (210±171.73, Median=200), and AntiCCP (102±105.25, Median=100) were collected from patient records.

The expression levels of both IL-17 and PERP in the PBMCs were determined in the case group and compared with that of the healthy controls and then correlated with the disease activity parameters.

We found higher IL17 gene expression in the diseased group (1.59±0.94, Median=1.6) compared with the healthy group (0.72±0.61, Median=0.55) with a high statistical significant difference between both groups (P<0.001), a finding that suggests IL-17 to have an important role in the pathogenesis of RA. This comes in keeping with Kohno *et al.* [16] who showed that IL-17 gene expression in PBMCs of patients with RA was significantly higher than the healthy controls.

When we correlated the IL17 gene expression with disease activity parameters, we found significant positive correlations with all recorded parameters, another finding that further denotes the key role of IL-17 in the pathogenesis of RA. This comes in agreement with Kohno *et al.* [16] who declared that IL-17 expression in PBMC might be associated with the inflammatory process of RA. Also with Elhewala *et al.* [17] and Tofiq and Merza [18] who found

increased IL-17A levels in patients with disease activity, considering it an indicator for high disease activity and suggesting it as a treatment option for patients with poor prognosis.

Moreover, our results come in accordance with Kim *et al.* who found in their study that IL17 and Th17 cells essentially contributed in RA pathogenesis and declared that the disease activity was correlated with Th17 cells level in peripheral blood of RA patients [19].

Apoptosis is a multi-step cell death pathway that occurs in a variety of physiological conditions. The balance of apoptosis is an essential mechanism of physiological cell death, required for homeostasis of the body systems including the immune system.

Furthermore, defects in the apoptotic cascades either by elevation or failure might contribute to immunodeficiency or autoimmunity respectively. Several previous studies demonstrated impaired PBMCs apoptosis in RA patients [20].

In this study, we found that PERP mRNA expression levels in PBMCs from patients with RA (0.65± 0.33, Median=0.4) were significantly decreased compared with healthy controls (1.96±0.96, Median=1.75) (P<0.001) and they were inversely correlated with disease activity parameters (DAS28), CRP, ESR and RF. This comes in accordance with Du *et al.* [12] who declared the same results.

The results of previous studies support also our findings as they found that defects in downstream p53 target gene had a vital role in promoting systemic autoimmunity diseases. Maas *et al.* declared that p21which is a transcriptional target of p53 and downstream cyclin dependent kinase inhibitor was also down expressed in patients with RA [21].

Our findings have shown that there were statistically significant differences among disease activity groups regarding the relative expression of IL-17 that increased proportionally with activity of RA where the highest concentrations were in patients with severe disease activity (P< 0.05). This comes in agreement with Siloşi *et al.* [22].

On the other hand, our study has found decreased relative expression of PERP consistently with activity of RA, where the lowest concentrations were in patients with severe activity. Statistically significant differences were observed between mild, moderate, severe and remission groups (P< 0.05) this comes in agreement with Du *et al.* [12].

Another important correlation, in our study, was detected between the expression levels of PERP and IL-17 levels in PBMCs where they were inversely correlated in a significant way (P<0.001).This comes also in accordance with Du *et al.* [12] who suggested that PERP might be one of the regulators of IL-17 expression participating in the pathogenesis of RA.

On the other hand, our study found no correlations between the IL-17 and PERP expression levels with either anti-CCP or ANA (P >0.05).This comes in keeping with Eshed *et al.* and Kim and Kim [23, 24] who demonstrated that anti-CCP and ANA have no significant relationship with either disease activity or severity.

In conclusion, IL 17 and PERP can contribute to the pathogenesis and disease activity of rheumatoid arthritis and can be utilized as indicators for high disease activity and probably as a treatment option for patients with aggressive disease and/or poor prognosis.

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