Original Research Article

The Egyptian Journal of Immunology

The Egyptian Journal of Immunology
Volume 30 (4), 2023: 01–10.
www.Ejimmunology.org

TNF-α versus IL-6 Genes Expression levels in Active Rheumatoid Arthritis: Clinical and Laboratory Determinants

Enas I. Abdelhady[1], Hanaa I. Abd El-Hady[2], Shahenda G. Badran[2], and Mona Rabie[1]

[1]Department of Rheumatology & Rehabilitation, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
[2]Department Medical Microbiology & Immunology, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Corresponding author: Hanaa I. Abd El-Hady, Department of Medical Microbiology & Immunology, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
Email: hanaa4islam@yahoo.com.

Abstract

This study intended to compare the expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) genes in active rheumatoid arthritis (RA) patients who were receiving conventional synthetic disease-modifying drugs (csDMARDs) and to find the clinical and laboratory determinants affecting TNF-α and IL-6 genes expression levels among active RA patients. This was a cross sectional study that included 108 active RA patients who were receiving csDMARDs. A detailed history was reviewed for all patients in addition to a complete physical examination and assessment of the 28-joint disease activity score (DAS28). Some laboratory measures were recorded as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and serum rheumatoid factor (RF). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure expression levels of TNF- α and IL-6 genes. In active RA patients, TNF-α and IL-6 genes expression levels were significantly correlated to each other (p<0.001, r=0.788). Also, both had positive correlations with the age and DAS28 among RA patients (p<0.001). IL-6 and TNF-α expression levels were significantly higher in RA patients with high DAS28 scores (p<0.001). Most RA patients (81.5%) had relatively higher IL-6 gene expression levels than TNF-α. RA patients with relatively high IL-6 expression levels were younger in age and had shorter disease duration and less DAS28 than RA patients with relatively high TNF-α gene expression levels. In addition, they had higher CRP and RF levels. Young age was detected as a significant predictor for relatively higher IL-6 gene expression levels than TNF-α. In conclusion, most active RA patients had higher IL-6 gene expression levels than TNF-α. Young age could be considered a significant predictor for relatively high IL-6 gene expression levels among active RA patients.

Keywords: TNF-α gene expression, IL-6 gene expression, Rheumatoid arthritis (RA)

Date received: 23 March 2023; **accepted:** 12 July 2023

Introduction

Rheumatoid arthritis (RA) is an autoimmune condition that gradually damages the joints. It is characterized by a gradual, symmetrical inflammation of the affected joints, leading to cartilage degradation, bone erosion, and disability.[1] RA disease course varies from person to person, although it frequently leads to significant morbidity, decreased physical activity, and a poor quality of life. Studying RA pathogenesis has made significant contributions to identifying the key steps in the development

of the disease, the inflammatory process as well as the disease progression and severity.[2]

The inflammatory process involves both innate and adaptive immune responses, and cytokines are crucial for inflammation and the development of disease.[3] One of these cytokines is the tumor necrosis factor-alpha (TNF-α). Dysregulated TNF-α gene expression and signaling can cause chronic inflammation, which can result in autoimmune disorders and tissue damage.[4] TNF-α serum levels are increased in RA patients, and this elevation has been connected to the inflammation of joints and degeneration. It is believed to be one of the key mediating factors of this disease and one of the major mediators of its pathology.[5]

Interleukin 6 (IL-6) is a different cytokine involved in the pathophysiology of RA. It is a pleiotropic cytokine that plays various roles in adaptive immunity and possesses pro- and anti-inflammatory properties. IL-6 levels in synovial fluid and serum are elevated in RA patients, and this rise is strongly correlated to joint damage and disease severity.[6]

TNF-α and IL-6 both play important roles in the generation of pro-inflammatory cytokines, the development of inflammation, and disease activity in RA patients. For the treatment of RA, different conventional synthetic disease-modifying drugs (csDMARDs) are available, unfortunately, not all patients respond well to these drugs. Therapies targeting cytokines, including TNF and IL-6, have been proven to modify the disease course in these patients.[7]

Some patients respond well to anti-TNF therapy and others show failure. The RA patient receiving csDMARDs may benefit from initial gene expression profiling to determine which cytokine is more predominant in their disease.[8] The bioactivity of the cytokine depends on multiple interacting variables which include gene expression, mRNA translation and post-transcriptional interactions at the receptor level, the signal transduction, or the cytokine level, so the gene expression is the initial determinant of certain bioactivity pathway.[9]
The link between variations in gene expression and clinical characteristics of patients will aid in tailored therapy for specific patients and enhance the field of personalized biological therapy. Therefore, the goal of the current study was to evaluate the peripheral blood monocyte gene expression of TNF-α and IL-6 in active RA patients, with a focus on disease-related clinical markers. We designed our study to analyze the expression levels of TNF-α and IL-6 genes in these patients taking into consideration the grade of disease activity and finding out the determinants of relatively high TNF-α or IL-6 gene expression levels.

Subjects and Methods

Patient selection

A total of 108 patients who were receiving csDMARDs participated in this study. Patients were recruited from the Rheumatology and Rehabilitation Department, Faculty of Medicine, Zagazig University Hospitals from September 2021 to March 2022. Laboratory work was carried out at the Medical Microbiology and Immunology Department and Scientific and Medical Research Center, Faculty of Medicine, Zagazig University.

Inclusion criteria

RA patients were diagnosed according to the 2010 American College of Rheumatology/ European League against Rheumatism classification criteria for the diagnosis of RA. [10] All patients had active disease with the 28-joint disease activity score (DAS28) ≥ 2.6 [11] despite they were receiving csDMARDs regularly for more than six months. They did not receive biologic DMARDs before (i.e., biologic-naïve patients).

Exclusion criteria

Patients who had severe comorbidities (e.g., cancer, severe cardiovascular, lung and renal dysfunction) were excluded from the study. Also, patients who had remission of disease or who were non-compliant with treatment were excluded from the study.

Ethical consideration

The protocol of the study was reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University (approval No. 8031-19-9-2021). Patients were included in the study after

providing written informed consent indicating their acceptance to participate.

Assessment of disease activity

All patients were subjected to history taking and detailed musculoskeletal examination. For every patient, DAS28 was calculated using the swollen and tender joints counts and the patient global assessment.[12] In addition, laboratory parameters such as erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP), and serum Rheumatoid Factor (RF) were collected from the patients' medical records. Based on DAS28 grades, patients were divided into categories as follows: low (2.6 ≥DAS28<3.2), moderate (3.2 ≥DAS28<5.1), and high activity (DAS28≥5.1). TNF-α and IL-6 genes expression were assayed by quantitative real-time polymerase chain reaction (qRT-PCR) method.

Separation of peripheral blood mononuclear cells (PBMC)

PBMCs were separated from other components of the blood, using the Ficoll-Paque (Lymphoflot, Biotest, Dreieich, Germany) density gradient centrifugation method. For this a venous blood sample (5 ml) was collected, from each study subject, in heparin containing tube then diluted with equal volume of phosphate buffer saline (PBS). Ficoll previously loaded into conical tubes was gently overlayed with the blood/PBS mixture. The samples were centrifuged at 400 x g for 30 minutes. Using a micropipette, the separated lymphocyte layer was gently aspirated and transferred into a new tube. For washing the cells, 3 volumes of PBS were further added to the lymphocyte layer and carefully homogenized, then the mixture was centrifuged at 100 x g for 10 min and the supernatant was discarded. PBMCs were washed twice with PBS followed by centrifugation. Finally, the cell pellet was collected for assessment of gene expression.

Assay of TNF-α and IL-6 genes expression

-RNA extraction from the PBMCs

Total RNA was extracted from the PBMCs using a commercial extraction kit (Fast HQ RNA Extraction Kit, iNtRON Biotechnology, Korea) according to the manufacturer's instructions. RNA yield and purity were confirmed by measuring optical density at 260 and 280 nm wavelengths using a spectrophotometer (Quantus^{Tm} Fluorometer, Promega, USA).

-Reverse transcription and complementary DNA (cDNA) synthesis

The extracted RNA was used for cDNA synthesis using commercial kits (TOPscript™ cDNA Synthesis Kit, enzynomics Inc., Korea), according to the manufacturer's instruction. Briefly, in a 25 µl reaction tube, 1 µg of total RNA from each sample was added to the reaction mixture containing 1 µl Random hexamer Primer, 2 µl RT Buffer, 2 µl of the dNTP's mixture, 0.5 µl RNase inhibitor,1 µl of reverse transcriptase, and the volume was completed to 20 µl with RNase-free water. The reaction tubes were incubated at 25°C for 15 minutes, 50°C for 60 minutes followed by 5 minutes of incubation at 95°C to denature the reverse transcriptase. The synthesized cDNA was stored at -20°C until used in real-time PCR assays.

TNF-α and IL-6 gene expression by quantitative real-time PCR assay

The obtained cDNA was used for TNF- and IL-6 geneS expression assay. For these commercially available kits (TOPreal™ qPCR 2X PreMIX, enzynomics Inc., Korea) were used. The reaction tube contained 1 µl of cDNA, 10 µl SYBR Green with low ROX, 1 µl of each of the forward and reverse primers and the volume was completed to 20 µl using Nuclease-free water. The reaction tubes were loaded in a real-time thermocycler (Agilent technologies Stratagene Mx3005p, Germany). The steps included an initial denaturation step at 95°C for 10 minutes followed by 40 cycles each of a denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds.

Primer pairs used in the reaction for β-actin (Human housekeeping gene) were forward: 5'-ACGGGGTCACCCACACTGTGC-3' and reverse: 5'-CTAGAAGCATTTGCGGTGGACGATG-3'. For the TNF-α gene, the primers were forward: 5'-CCTGCCCCAATCCCTTTATT-3' and reverse: 5'-CCCTAAGCCCCCAATTCTCT-3'. For the IL-6 gene, they were forward: 5'-GGTACATCCTCGACGGC

ATCT-3′ and reverse: 5′-GTGCCTCTTTGCTGCTTT CAC-3′.

Gene Expression Calculation

Cycle threshold (Ct) which is the cycle number at which the fluorescence generated during a reaction crosses the fluorescence threshold, was recorded using the real-time thermocycler for each gene. TNF-α and IL-6 genes expression values were calculated as their relative expression compared to β-actin human housekeeping gene expression for each sample. Firstly, sample ΔCt was calculated as the difference between the detected cycle threshold for each target gene and housekeeping gene in the same sample, then sample ΔΔCt was calculated as the difference between sample ΔCt and average control ΔCt, finally each target gene expression level was calculated using $2^{-\Delta\Delta Ct}$ formula for each sample which represents each target gene expression folds of housekeeping gene expression.[5] Relatively high gene expression was quantitatively assessed by comparing the TNF-α gene expression level of each patient with his/her IL-6 gene expression level, the gene expressing a higher level was considered relatively higher than the other gene (for example: when the TNF-α gene expression level of a patient was 0.7 fold and his/her IL-6 gene expression level was 1.3 fold, this patient was considered to have relatively high IL-6 gene expression).

Statistical Methods

For statistical analysis, we used the Statistical Package for the Social Sciences (SPSS) version 26.0 for Windows (SPSS Inc., Chicago, IL, USA). Qualitative data are presented as frequencies and percentages. For quantitative variables mean, standard deviation (SD) and median interquartile range (IQR) were calculated. Independent samples Student's t-test was used to compare two groups of normally distributed variables while Mann Whitney U test was used for non-normally distributed variables. When there were more than two groups, the Kruskal-Wallis test was used for non-normally distributed variables and the One-way ANOVA test for variables with a normally distributed distribution. When appropriate, the Chi-square test or Fisher's exact test was used to compare the percentages of categorical variables. The association between the various study variables was evaluated using Spearman's rank correlation coefficient for non-parametric variables and Pearson correlation for parametric variables; the (+) and (-) signs denote direct and inverse correlation, respectively; values close to 1 denote a strong correlation; while values close to 0 denote a weak correlation. To identify the factors that predict the predominance of IL-6, multivariate logistic regression was used. A *p*-value less than 0.05 was considered significant.

Results

Clinical and laboratory characteristics of RA patients

This study included 108 active RA patients, attended Zagazig University Hospital. The age of patients ranged from 30-67 years and the RA disease duration ranged from 1-17 years. CRP was positive in 66.7% of RA patients and RF positive in 70.4% of RA patients. This study included 22.2% of patients with low active disease, 55.6% of patients with moderately active disease and 22.2% with high disease activity. All patients had active disease despite being on regular csDMARDs and most patients were receiving a combination therapy as shown in Table 1.

Expression levels of IL-6 and TNF-α genes

The expression of TNF-α and IL-6 genes are shown in Figure 1. The mean levels of gene expression of both IL-6 and TNF-α genes among patients are demonstrated in Table 1.

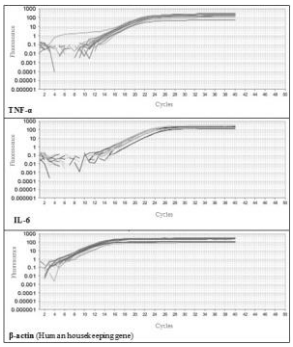

Figure 1. Amplification curves for TNF-α, IL-6 and β-actin genes.

Table 1. Clinical and laboratory characteristics of the 108 RA patients.

Characteristic	RA patients	
Sex	Male	12 (11.1%)
	Female	96 (88.9%)
Age (years)	Mean ±SD	42.5±9.56
	Range	(30-67)
Disease duration (years)	Mean ±SD	6.07±4.57
	Median (IQR)	5 (2-10)
	Range	(1-17)
TJC	Range	(1-28)
	Median (IQR)	5 (3-18)
SJC	Range	(0-10)
	Median (IQR)	1 (0-3)
PGA	Range	(10-80)
	Median (IQR)	40 (20-50)
DAS28	Mean ±SD	4.53±1.13
	low	24 (22.2%)
	Moderate	60 (55.6%)
	High	24 (22.2%)
Treatment	Corticosteroids	60 (55.6%)
	csDMARD monotherapy*	20 (18.5%)
	csDMARD combination therapy**	72 (66.7%)
	Triple csDMARD therapy***	16 (14.8%)
ESR (mm)	Range	(3-61)
	Median (IQR)	24 (15-28)
CRP (mg/L)	Range	(1-68)
	Median (IQR)	7 (2.7-16)
	Positivity	72 (66.7%)
RF (IU/ml)	Median (IQR)	25 (10-64)
	Positivity	76 (70.4%)
IL-6 gene expression level (folds)	Mean±SD	3.47±5.6
	Median (IQR)	2.2 (1.1-5.6)
TNF-α gene expression level (folds)	Mean±SD	1.6±2.49
	Median (IQR)	1.0 (0.7-1.9)

RA: rheumatoid arthritis, SD: standard deviation, IQR: interquartile range, TJC: tender joint count, SJC: swollen joint count, PGA: a patient global assessment, DAS28: disease activity score 28, csDMARD: conventional synthetic disease modifying antirheumatic drug, ESR: erythrocyte sedimentation rate, CRP: C reactive protein, RF: rheumatoid factor, IL-6: interleukin 6, TNF-α: tumor necrosis factor. *csDMARD monotherapy: methotrexate, leflunomide, sulfasalazine or hydroxychloroquine.
**csDMARD combination therapy: a combination of any two csDMARDs.
***Triple csDMARD therapy: a combination of any three csDMARDs. "folds" of housekeeping gene expression.
Data are presented as frequency (%), median (IQR) or mean±SD.

Correlation between IL-6 and TNF-α Gene Expression levels and clinical and laboratory variables

We investigated the relationship of the expression levels of the studied genes with the clinical and laboratory variables and found significant positive correlations with age and DAS28 for both genes (p<0.001 for each correlation) as shown in Table 2. On comparing RA patients of different DAS28 grades, there were significant differences regarding the IL-6 and TNF-α genes expression levels (p<0.001 for both). The highest IL-6 and TNF-α genes expression levels were among RA patients with high activity compared to low and moderate activity as presented in Table 3.

Table 2. Correlation between IL-6 and TNF-α Genes Expression levels and clinical and laboratory variables.

Variable	IL-6 gene expression level		TNF-α gene expression level	
	r	*p* value*	r	*p* value*
Age (years)	0.510	<0.001	0.522	<0.001
RF (IU/ml)	0.031	NS	0.192	NS
CRP (mg/L)	0.156	NS	0.092	NS
ESR (mm)	0.063	NS	0.119	NS
DAS28	0.482	<0.001	0.465	<0.001

IL-6: interleukin 6, TNF-α: tumor necrosis factor, RF: rheumatoid factor, CRP: C reactive protein, ESR: erythrocyte sedimentation rate, DAS28: disease activity score 28.
*Pearson correlation is used for parametric variables (age and DAS28) while Spearman correlation was used for non-parametric variables. *P* > 0.05 is not significant (NS).

Table 3. Comparison between clinical and laboratory variables of RA patients of different disease activity grades.

Variable	Low No. (24)	Moderate No. (60)	High No. (24)	*p*-value
Age (years) Mean ± SD	41.8±8.78	40.3±4.88	48.6±15.31	NS**
Disease duration (years) Median (IQR)	1.5 (1-3)	5 (3-10)	9 (2-15)	<0.0001*
ESR (mm) Median (IQR)	17 (15-19)	26 (21-29)	22.5 (16-32)	0.004*
CRP (mg/L) Median (IQR)	3.5 (1-8)	8.8 (2.7-16)	11.5 (6.5-21)	0.001*
RF (IU/ml) Median (IQR)	27 (16-32)	32 (10-98)	13 (8-57)	NS*
TNF-α gene expression levels (folds) Median (IQR)	0.65 (0.5-0.9)	0.9 (0.7-1.9)	1.55 (1.2-1.9)	<0.001*
IL-6 gene expression levels (folds) Median (IQR)	1 (0.9-1.9)	2.2 (1.1-5.2)	9.9 (5.6-14.2)	<0.001*

No: number, SD: standard deviation, IQR: interquartile range, ESR: erythrocyte sedimentation rate, CRP: C reactive protein, RF: rheumatoid factor, TNF-α: tumor necrosis factor, IL-6: interleukin 6.
* Kruskal Wallis test is used to compare three groups of non-parametric data.
**ANOVA is used to compare three groups of parametric data. *P* > 0.05 is not significant (NS).
"folds" of housekeeping gene expression

Correlation between IL-6 and TNF-α gene expression levels among csDMARDs non-responder RA patients

There was a significant correlation between the gene expression levels of TNF-α and IL-6 among RA patients (*p* <0.001, r= 0.788) (Figure 2). When we compared TNF-α and IL-6 gene expression levels in each studied individual, most RA patients (81.5%) had relatively higher IL-6 gene expression levels than TNF-α, while 18.5% of RA patients had relatively high TNF-α gene expression levels as shown in Table 4.

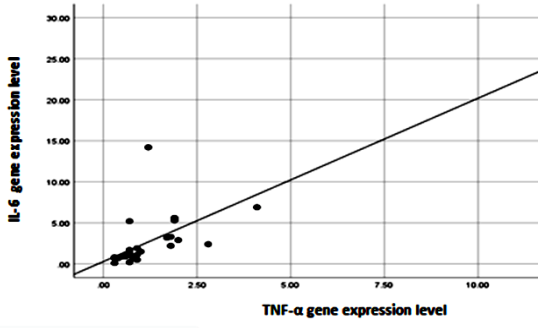

Figure 2. Correlation between TNF-α and IL-6 gene expression levels among active RA patients. Spearman correlation.

Table 4. Comparison between RA patients with relatively high TNF-α versus RA patients with relatively high IL-6 gene expression levels.

Variable	Relative gene expression		*p*-value
	Relatively high TNF-α gene expression No. (20)	Relatively high IL-6 gene expression No. (88)	
Age (years) (Mean ± SD)	49.0±13.28	41.04±7.86	<0.001**
Disease duration (years) Median (IQR)	8 (5-8)	3.5 (2-10)	0.005*
DAS28 Median (IQR)	5.04 (4.78-5.86)	4.55 (3.15-4.87)	0.004*
CRP (mg/L) Median (IQR)	2.07 (2-6.09)	8.4 (4-16.0)	0.013*
ESR (mm) Median (IQR)	24 (24-29)	21 (15-27)	NS*
RF (IU/ml) Median (IQR)	10 (10-20.2)	30.4 (10-64.1)	0.031*

TNF-α: tumor necrosis factor, IL-6: interleukin 6, No: number, SD: standard deviation, IQR: interquartile range, DAS28: disease activity score 28, CRP: C reactive protein, ESR: erythrocyte sedimentation rate, RF: rheumatoid factor.
* Mann Whitney test is used to compare two groups of non-parametric data.
** T test is used to compare two groups of parametric data. *P* > 0.05 is not significant (NS).

Determinants of relatively high IL-6 gene expression levels among active RA patients

There were significant associations between the relative gene expression and some clinical and laboratory variables. Active RA patients with relatively high IL-6 gene expression levels had younger age, shorter disease duration and less activity than RA patients with relatively high TNF-α expression levels. On the other hand, high CRP and RF levels were associated with relatively higher IL-6 gene expression levels than TNF-α as shown in Table 4. Finally, regression analysis demonstrated that young age was a predictor factor for relatively high IL-6 gene expression levels among RA patients, as shown in Table 5.

Table 5. Logistic regression analysis of determinants of relatively high IL-6 gene expression levels.

Variable	Regression coefficient	SE	Wald Test	*p*-value*	OR (95%CI)
Age (years)	-0.072	0.028	6.59	0.010	0.93 (0.88-0.98)
Disease duration (years)	-0.058	0.061	0.89	NS	0.94 (0.84-1.06)
DAS28	0.031	0.026	1.36	NS	1.03 (0.98-1.09)
CRP (mg/L)	0.003	0.003	0.96	NS	1.003 (1-1.01)
RF (IU/ml)	-0.041	0.028	2.10	NS	0.96 (0.91-1.02)

SE: standard error, OR: odds ratio, CI: confidence interval, DAS28: disease activity score 28, CRP: C reactive protein, RF: rheumatoid factor. * Wald test is used in case of logistic regression *P* > 0.05 is not significant (NS).

Discussion

The present study intended to determine expression levels of TNF-α and IL-6 genes in RA patients and to correlate such levels with disease activity, and to identify the determinants of relatively high TNF-α or IL-6 gene expression levels. Most RA patients (81.5%) had relatively higher IL-6 gene expression levels than TNF-α. RA patients with relatively high IL-6 expression levels were younger in age and had shorter disease duration and less DAS28 than RA patients with relatively high TNF-α gene expression levels. Young age was detected as a significant predictor for relatively high IL-6 gene expression levels among RA patients.

In our work, the mean levels of TNF-α and IL-6 genes expression were 1.6±2.49 and 3.47±5.6, respectively. On the other hand, a study on etanercept-treated RA patients demonstrated a higher mean level of TNF-α gene expression of 5.759±1.834.[5] In a study of IL-6 gene expression in RA patients, Ad'hiah et al., 2018, reported a higher mean level of 9.08±0.97.[6]

In our study, there was a significant positive correlation between both genes' expression levels and DAS28 in RA patients. Also, we categorized these patients according to DAS28 grade and then compared their clinical and laboratory variables. Regarding the expression levels of the IL-6 and TNF- genes, there was a statistically significant difference between patients of different activity grades. IL-6 and TNF-α genes expression levels among RA patients with high activity had the highest levels compared to low and moderate activity. These findings agreed with those of Shafiaa et al., 2016, who stated that TNF-α gene expression was higher in RA patients with high DAS28 scores compared to RA patients with low and moderate DAS28 scores.[13] On the other hand, Mahmood et al., 2017, found that TNF-α gene expression among RA patients with moderate activity had the highest level but with no significant difference. They attributed this finding to that etanercept, as an anti-TNF drug, can normalize the TNF-α gene expression levels among etanercept-treated RA patients, therefore, no significant difference was found among patients of different activity grades.[5]

Regarding IL-6 gene expression levels and DAS28, a previous study noticed no significant relation between IL-6 gene expression levels in synovial fluid and DAS28 scoring in RA patients.[13] In addition, Ad'hiah et al., 2018, stated that there was no significant difference in IL-6 gene expression levels among RA patients of different disease activity grades.[6]
In the current study, there was a significant positive correlation between both genes' expression levels and age in RA patients. The relationship between IL-6 gene expression with age had been investigated by multiple studies. Some claim that IL-6 regulates age-related changes in macrophages resulting in immune cell function to decline with aging and this is supported by the observation of increased IL-6 gene expression in elderly.[14] However, Beharka et al., 2001, found no difference between healthy young and elderly human individuals in terms of the average circulating levels of IL-6.[15]

In this study, we observed a positive correlation between TNF-α and IL-6 genes expression levels (p <0.001). This result is supported by the previous note that the crosstalk between these two cytokines is not limited to the post-transcriptional or cytokine level but also extends to the gene expression levels.[16]

Gene expression profiling is recently used in research to develop biomarkers that predict response to biological therapeutic agents. It focuses on identifying differentially expressed genes that may predict and distinguish between responders and non-responders to anti-TNF therapy.[17] Therefore, in the current study, we compared the gene expression levels of TNF-α versus IL-6 in RA patients receiving csDMARDs and investigated the laboratory and clinical determinants of their differential expression.

On comparing the relative expression of the IL-6 and TNF-α genes to the human housekeeping gene (β-actin) for each patient, we noticed that IL-6 gene expression levels were relatively higher than TNF-α gene expression levels in 81.5% of RA patients. This finding can be supported by the linkage of IL-6 to local inflammation and joint destruction in

RA patients and its overexpression in inflammatory conditions.[18] Moreover, most RA patients included in the current study (66.7%) had positive CRP which is a closely related indicator of differential IL-6 overactivity and/or overexpression rather than TNF-α.[19,20]

As regards the relation between the relative gene expression state and clinical and laboratory variables. There were significant relations between relative gene expression state and age, disease duration, DAS28, CRP and RF. RA patients with relatively high TNF-α expression levels had longer disease duration and more disease activity than patients with relatively high IL-6 expression levels. Furthermore, an Indian study reported that an increased TNF-α expression is linked to a more severe form of RA disease.[13]

In this study, RA patients with relatively high IL-6 expression levels had higher CRP and RF than patients with relatively high TNF-α expression levels. This result agreed with those reported by Ad'hiah et al., 2018, who noted that there was a significant relation between IL-6 gene expression in RA patients and CRP. [6] Several studies have found links between CRP and locally produced IL-6 in inflamed joints as CRP synthesis is typically IL-6 dependent.[19] On the other hand, Mahmood et al., 2017, found that there was no significant difference in TNF-α gene expression between CRP-positive and CRP-negative RA patients.[5] This is supported by the previous report of IL-6 as the main inducer of CRP production rather than TNF.[21]

In our study, RA patients with relatively greater TNF-α expression levels were significantly older compared to patients with relatively high IL-6 expression levels. The IL-6 predominance among the younger group might be attributed to the underlying characteristics of this group as not only the younger age was recorded among the patients with relatively high IL-6 expression levels, but also higher CRP was an eminent characteristic of this group. There were significant differences regarding the clinical and laboratory variables (age, disease duration, DAS28, CRP and RF) between RA patients with high IL-6 gene expression levels and who had high TNF-α expression levels. So, regression analysis was performed and revealed that young age was a significant predictor for high IL-6 gene expression versus TNF-α among RA patients.

It is necessary to show some limitations of the current study. This study did not include synovial tissue gene expression analysis. In addition, all patients were biologic-naïve, so the study lacked comparing the gene expression levels to biologic-treated patients as a step forward to a more personalized biological treatment.

In conclusion, most RA patients had higher IL-6 gene expression levels than TNF-α. There were significant relations between relative gene expression state and age, disease duration, DAS28, CRP, and RF. Young age could be a significant predictor for relatively high IL-6 gene expression levels among active RA patients.

Author Contributions

MR was responsible for the research conceptualization and proposal design. EIA. contributed to the data collection, formal analysis, and interpretation. HIA and SGB. were responsible for the gene expression assay steps and analysis. MR, HIA and SGB were responsible for writing and editing the original manuscript. EIA was responsible for the final editing and revision. All authors approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Zagazig University (approval No. 8031-19-9-2021).

Informed consent

A written informed consent was obtained from each participant before being included in the study.

References

1. Lin, YJ., Anzaghe, M., Schülke, S. (2020). Update on the Pathomechanism, Diagnosis, and Treatment Options for Rheumatoid Arthritis. *Cells*; 9(4): 880.

2. Daikh, DI. (2022). Rheumatoid arthritis: Evolving recognition of a common disease. *Best Practice and Research Clinical Rheumatology*; 36(1):101740.

3. Mateen, S., Zafar, A., Moin, S., et al. (2016). Understanding the role of cytokines in the pathogenesis of rheumatoid arthritis. *Clinica Chimica Acta*; 455:161–171.

4. Brzustewicz, E., & Bryl, E. (2015). The role of cytokines in the pathogenesis of rheumatoid arthritis – Practical and potential application of cytokines as biomarkers and targets of personalized therapy. *Cytokine*; 76(2):527–536.

5. Mahmood, AS., Al-Kazaz, A-KA., Ad'hiah, AH., et al. (2017). Gene Expression of Tumor Necrosis Factor-Alpha in Etanercept-Treated Rheumatoid Arthritis Patients. *J Biosciences and Medicines*; 05(09):1–9.

6. Ad'hiah, AH., Mahmood, AS., Al-kazaz, A-KA., et al. (2018). Gene expression and six single nucleotide polymorphisms of interleukin-6 in rheumatoid arthritis: A case-control study in Iraqi patients. *Alexandria J Med*; 54(4):639–645.

7. Aterido, A., Cañete, JD., Tornero, J., et al. (2019). A combined transcriptomic and genomic analysis identifies a gene signature associated with the response to anti-TNF therapy in rheumatoid arthritis. *Front Immunol*; 10(JUL): 1459.

8. Wysocki T & Paradowska-Gorycka A. (2022). Pharmacogenomics of Anti-TNF Treatment Response Marks a New Era of Tailored Rheumatoid Arthritis Therapy. *International journal of molecular sciences*; 23(4):2366.

9. Deon, D., Ahmed, S., Tai, K., et al. (2001). Cross-talk between IL-1 and IL-6 signaling pathways in rheumatoid arthritis synovial fibroblasts. *J Immunology*;167(9):5395–5403.

10. Aletaha, D., Neogi, T., Silman, AJ., et al. (2010). A joint working group from the ACR and the European League Against Rheumatism devel. *Arthritis Rheumatism*; 62(9):2569–2581.

11. Van Gestel, AM., Haagsma, CJ., van Riel, PL. (1998). Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. *Arthritis Rheum*; 41(10):1845-1850.

12. Prevoo, MLL., Van'T Hof, MA., Kuper, HH., et al. (1995). Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis and Rheumatism*; 38(1):44–48.

13. Shafiaa, S. & Shaha, ZA. (2016). TNF-A, IL-1β and IL-6 Cytokine Gene Expression in Synovial Fluid of Rheumatoid Arthritis and Osteoarthritis Patients and Their Relationship with Gene Polymorphisms. *Rheumatology Current Research*; 06(01):1–6.

14. Kovacs EJ, Gomez CR, Karavitis J, et al. (2010). Interleukin-6 contributes to age-related alteration of cytokine production by macrophages. *Mediators Inflamm*. 2010;2010.

15. Beharka AA, Meydani M, Wu D, et al. (2001). Interleukin-6 Production Does Not Increase With Age. *Journals Gerontol Ser A [*Internet]; 56(2):B81–8.

16. Valin, A., Ruano, Y., Del Rey, MJ., et al. (2016). Crosstalk Between IL-6 and TNF-Alpha Signaling Pathway in Rheumatoid Arthritis Synovial Fibroblasts [abstract]. *Arthritis and Rheumatology*; 68 (suppl 10).

17. Kim, TH., Choi, SJ., Lee, YH., et al. (2014). Gene expression profile predicting the response to anti-TNF treatment in patients with rheumatoid arthritis; analysis of GEO datasets. *Joint Bone Spine*; 81(4):325–330.

18. Lopez-Lasanta, M., Julià, A., Maymó, J., et al. (2015). Variation at interleukin-6 receptor gene is associated to joint damage in rheumatoid arthritis. *Arthritis Research and Therapy*; 17(1):1–8.

19. Baillet A, Gossec L, Paternotte S, et al., (2015). Evaluation of Serum Interleukin-6 Level as a Surrogate Marker of Synovial Inflammation and as a Factor of Structural Progression in Early Rheumatoid Arthritis: Results from a French National Multicenter Cohort. *Arthritis Care Res.*;67(7):905–12.

20. Tanaka, T., Narazaki, M., Kishimoto, T. (2014). Il-6 in inflammation, Immunity, And disease. *Cold Spring Harbor Perspectives in Biology*; 6(10) a016295.

21. Sproston, N. R., & Ashworth, J. J. (2018). Role of C-Reactive Protein at Sites of Inflammation and Infection. *Frontiers in immunology*, 9, 754.