The Importance of The Inflammatory Cytokines IL21, TNF-A, IL6, And IL17A to The Flow Activity and Progression of Ankylosing Spondylitis Patients Treated With Anti-TNF Therapy

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Abstract—**Introduction: Ankylosing spondylitis (AS) is a prevalent inflammatory rheumatic condition that impacts the axial skeleton, resulting in distinctive inflammatory back pain, which can lead to structural and functional limitations and a reduction in quality of life. This study sought to determine the role of the inflammatory cytokines IL21, TNF-α, IL6 and IL17A in the flow activity and progression of ankylosing spondylitis in patients being treated with anti-TNF drugs.**
Method: A prospective case-control study was conducted at Basrah Teaching Hospital in southern Iraq. Eighty-one AS patients were divided into two groups: 67 were treated with anti-TNF therapy, 14 were newly diagnosed patients as positive controls, and 65 were healthy individuals. Disease activity was assessed using the AS Bath Disease Activity Index (BASDAI). Conventional radiography and MRI are used to measure the severity of the disease. IL-6, IL-17A, IL-21, and TNF-α were extracted from serum samples using ELISA.
Result: IL-21 had the highest difference between groups ($p = 0.001$), but IL6 had the highest combined significant difference ($p = 0.001$), with the most remarkable difference found between positive control and healthy individuals ($p = 0.001$). The only difference was between the positive control and healthy groups ($p = 0.006$) for both IL17A and TNF-α. There was a moderately positive correlation between serum levels of IL-21 and IL6 and BASDAI. There was a moderately negative relationship (-0.634) between the type of treatment and serum IL-21 levels and a moderately positive relationship (0.64) between sacroiliitis and IL21.
Conclusion: For the flow of AS activity and progression, the level of IL21 in the serum is more important than the level of IL6 in the serum, but there was no statistically significant difference in IL17A and TNF-α levels.

Keywords**;** Proinflammatory cytokine, Ankylosing spondylitis, BASDAI, Sacroiliitis

I. INTRODUCTION

Ankylosing spondylitis (AS) is an immune-mediated arthropathy prototype for a broader category of spondyloarthropathies (SpA). This category includes enteric arthritis, reactive arthritis, and psoriatic arthritis. Ankylosing spondylitis primarily impacts the axial skeleton, specifically the sacroiliac joints, spine, and surrounding soft tissues, including tendons and ligaments (1).

The potential origin of IL-17 induction in AS could be attributed to microbial dysbiosis due to the gut epithelium's ability to secrete IL-23. Patients with AS exhibit a significant increase in the production of IL-23 in the terminal ileum (2).

The cytokines IL-21 and IL-23 are essential factors in the development and expansion of the Th17 cell subset. IL-21 induces the differentiation of human naïve $CD4^+$ T cells into Th17 cells when combined with TGF-β, whereas IL-21 and IL-23 both function by maintaining and expanding Th17 cells (3, 4). IL-21 is produced by activated $CD4^+$ T cells and is reported to be produced in high amounts by the Th17 subset, although this association has recently been questioned (3, 4). The IL-21 receptor (IL-21R) is constitutively expressed in resting B, NK, and T cells and is potently upregulated in T cells upon T-cell receptor activation (5). IL-21 stimulates the proliferation and activation of NK, T, and B cells and can produce various proinflammatory cytokines, including IL-21 itself (6, 7). It plays an important role in chronic inflammatory diseases such as SpA because it can trigger the growth of Th17 cells.

TNF-α can target a wide range of cell types [8, 9], but the specific cell types targeted in AS are unknown. They release TNF to alert other immune system cells as part of an inflammatory response. [10]

Infliximab is a recombinant chimeric monoclonal antibody that contains a murine variable region and a human IgG1 constant region. It is specific to all forms of TNF-α in human and effectively blocks the binding of TNF-α to its soluble and transmembrane receptors [11]. Etanercept is a fusion protein comprising two identical extracellular regions of TNFR2 consisting of (p75 TNF-α receptors) attached to the Fc portion of human IgG1 [12]. biosimilars that are not generic products but closely resemble the originators have received FDA approval. These have only slight differences in the clinically

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inactive ingredients in molecular structure, which are about the same in terms of purity, safety, and efficacy [13].
TNF blockers improve the signs and symptoms of AS, but they do not stop new bone from growing. Anti-TNF therapy, such as Th17 cellular plasticity, reduces Th17 cell proliferation in patients with various types of inflammatory arthritis, including AS. The decrease in Th17 cells is not apparent. Nevertheless, it could be that inflammatory cells from infected joints move around because of changes in chemokine gradients or the expression of adhesion molecules in the synovium (14, 15). This study aimed to evaluate the role of the inflammatory cytokines IL-21, TNF-α, IL6, and IL-17A as indicators to follow the activity and progression in patients with AS who received anti-TNF therapy.

II.Patients and methods

Prior to data collection, each participant signed a permission form after explaining the objective of the study and ensuring that the data was protected, and the current study protocol was reviewed and approved by the Ministry of Higher Education and Scientific Research, University of Basrah, College of Medicine 030403/034/2022, and Basrah Health Department at the Ministry of Health. They were given permission to start work on the research project.. The study reviewed 81 AS patients who were divided into two groups: 67 treated with anti-TNF therapy and 14 newly diagnosed patients who were not given treatment (biologically naïve) as positive controls. All patients were assessed based on the criteria of the Assessment of SpondyloArthritis International Society (ASAS) (16). Anti-TNF drugs (infliximab, etanercept, remsima, and amjevita) were given to the patients for at least three months before the samples were taken. In addition, 65 healthy individuals were enrolled in the study as a healthy control group, and disease activity was assessed using the AS Bath Disease Activity Index (BASDAI) (17). BASDAI scores <4 indicate inactive disease, while BASDAI scores ≥4 indicate active disease. Information about treatment was collected at the time of the clinical material sampling. Conventional radiography and sacroiliac joint MRI were used with the following sequences: semi-coronal T1-weighted spin echo (T1), semi-coronal T1 fat-saturated (T1FS), and semi-axial short tau inversion recovery (STIR).

A total of 5 mL of blood samples was taken from the established AS patient and positive and healthy control groups. The blood samples were placed in a gel tube and centrifuged at 724 x g for 10 min (Eppendorf 5415C; Eppendorf, Hamburg, Germany) to perform an enzyme-linked immunosorbent assay (ELISA). The samples were either tested immediately or put in a freezer at -20 °C until they were needed again.

IL-6, IL-17A, IL-21, and TNF-α were extracted from serum samples using an ELISA (Biotek Instruments ELx800 Absorbance Microplate Reader, 400 to 750 nm wavelength, 96-Well amplitude) following the manufacturer's instructions (Beijing Solarbio Science & Technology, China). The levels of cytokines in the blood were checked in triplicate to improve accuracy.

III.Statistical analysis

All analyses were performed using SPSS software v21.0 (IBM, Chicago, USA). The Shapiro–Wilk test was used to determine the normality of the variables. The differences between parameters that were not normally distributed were analyzed with the Kruskal-Wallis test alternative to the one-way ANOVA test and Friedman's test alternative to the two-way ANOVA test and were defined as the mean rank. A categorical variable was compared using chi-square and Fisher exact test analysis. The power of the correlation among the variants was realized with the Spearman correlation test, as the distributions were nonparametric. In the correlation analysis, a rho (correlation coefficient) value between 0.00 and 0.49 was accepted as a weak correlation, 0.50 to 0.69 as a medium correlation, and ≥ 0.70 as a strong correlation. A p-value of ≤ 0.05 was considered significant.

IV.Result

The ages of the participants ranged from 20 to 63 years, and there was no apparent difference in age between the established AS patients and the positive and healthy control groups. There were no differences between the groups in terms of urban or rural residence (p=0.126). The participants' sociodemographic characteristics are listed in Table 1.
There was a significantly higher difference in inflammatory markers (ESR and CRP) between patients with AS and positive controls than in healthy individuals (p=0.001). The complete blood count showed a significant difference between the three groups with regard to lymphocyte, neutrophil, and platelet counts (p< 0.001).
Radiographic changes in the sacroiliac joint showed more progression and destruction in patients with established AS, with 22/67 (32.8%) having grade 4 joint damage compared to 2/12 (14.4%) biologically naïve patients, a significant difference (p=0.001). There were no significant differences in hip involvement between the two groups (p=0.744).

TABLE 1 COMPARISON OF SOCIO-DEMOGRAPHIC CHARACTERISTICS AMONG ANKYLOSING SPONDYLITIS PATIENTS, NAÏVE PATIENTS, AND HEALTHY CONTROLS

Characteristic	Patients with AS No. (%)	Positive control (AS-Naïve patients) No. (%)	Healthy control N0. (%)	P value
Age group				
20-29	10(14.9)	6(42.9)	18(27.3)	0.065
30-39	20(29.9)	4(28.6)	26(39.4)	
40-49	29(43.3)	2(14.3)	17(26.8)	
>50	8(11.9)	2(14.3)	5(7.6)	
Gender				
Male	60(89.6)	12(85.7)	58(87.9)	0.807
Female	7(10.4)	2(14.3)	8(12.1)	
Occupation				
Employee	13(19.4)	2(14.3)	47(71.2)	<0.001
Free job	14(20.9)	3(21.4)	9(13.6)	
Unemployed	26(38.8)	6(42.9)	8(12.1)	
Retired	14(20.9)	3(21.4)	2(3)	
Residency				
Central	50(74.6)	8(57.1)	39(59.1)	0.126
Peripheral	17(25.4)	6(42.9)	27(40.9)	

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BMI				
Under weight	8(11.9)	0(0)	6(9.1)	0.017
Normal	34(50.7)	10(71.4)	51(77.3)	
Grade1 overweight	16(23.9)	4(28.4)	9(13.6)	
Grade2 overweight	5(7.5)	0(0)	0(0)	
Grade3 overweight	4(6)	0(0)	0(0)	
Smoking				
Active	31(46.3)	5(35.7)	8(12.1)	<0.001
Passive	1(1.5)	2(14.3)	13(19.7)	
Non-smoking	35(52.2)	7(50)	45(68.2)	
Education				
Illiterate	16(23.9)	4(28.6)	3(4.5)	0.001
Primary	21(31.3)	0(0)	7(10.6)	
Intermediate	9(13.4)	1(7.1)	20(30.3)	
Secondary	3(4.8)	3(21.4)	15(22.7)	
University & more	18(26.9)	6(42.9)	21(31)	

There was a highly significant difference between the three groups of participants in terms of proinflammatory markers related to propagated local and systemic inflammation and activity and joint destruction in AS. IL-6 showed the highest combined significant difference (p=0.001), and within the group, the highest difference was found between positive controls and healthy individuals (p=0.001). IL-17A had the highest combined significant difference (p=0.007), and within the group, the only difference was found between the positive and healthy groups (p=0.006). Finally, IL-21 had the highest difference between groups (p=0.001) due to the wide variation of mean rank measures between the positive controls and patients with AS and healthy individuals. There was a

significant difference in TNF-α cytokine levels between the groups (p=0.001), and there was also a significant difference between patients with AS and positive controls compared with healthy individuals (p=0.001). However, the only difference was found between the positive and healthy groups (p=0.006) figure 1.

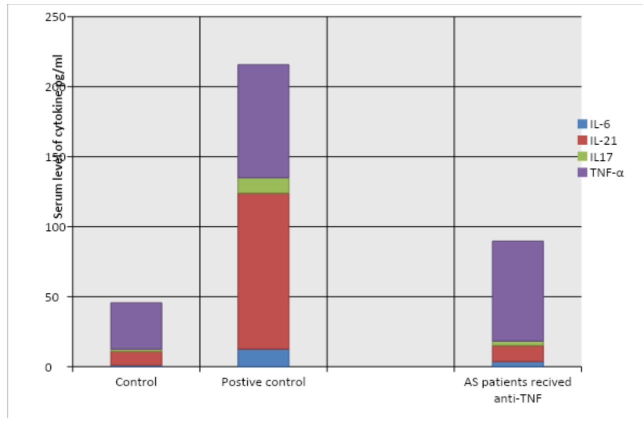

Figure1. Shows the levels of proinflammatory cytokine levels in three groups that were evaluated in this study

A comparison was made between patients with AS who received different types of anti-TNF therapy originators and biosimilar drugs and positive controls treated with NSAIDs to observe the effect of treatment type on serum levels of proinflammatory cytokines. Serum IL6 levels were significantly lower in patients with AS treated with different anti-TNF drugs than in patients treated with NSAIDs (p=0.024). However, serum IL-17A levels did not differ between the two groups of patients (p=0.146). There was a significant decrease in IL21 levels between groups (p=0.001), especially when comparing amjevita to NSAID or etanercept to NSAID. Serum TNF-α levels did not differ significantly between the two groups (p=0.483). **Table 2** shows the levels of inflammatory cytokines in patients treated with anti-TNF drugs and biologically naïve AS patients.

TABLE 2 COMPARISON OF SERUM CYTOKINE LEVELS AMONG GROUPS OF AS PATIENTS AND POSITIVE CONTROLS BY TYPE OF TREATMENT RECEIVED

Cytokine	Patients with AS	Positive control	P- value				
		Infiximab N (%) 13(19.4)	Remsima N (%) 4(6)	Etanercept N (%) 44(65.7)	Amjevita N (%) 6(9)	NSAID N (%) 14(100)	
IL-6	Median (Min.-Mx) Between group (combined) Within drug Inflxi.-NSAID Rems.-NSAID Etaner.-NSAID Amj.-NSAID	0.74 (0-37.2)	0.84 (0-3.392)	2.37 (0-22.98)	0.69 (0-97.03)	12.56 (0-4)4.7)	0.024 0.039 0.008 0.022 0.012
IL-17A	Median (Min-Max)	5.42 (0-35.8)	1.69 (0-14.8)	2.09 (0-146.8)	2.7 (0-58.7)	11.05 (3.7-14.5)	0.146
IL21	Median (Min-Max) Between group (combined) Within drug Amj. -NSAID Etaner. -NSAID	64.391 (3.9-124)	27.2 (0-113.04)	10.6 (0-272.5)	5.3 (3.07-16)	111.4 (11.3-290)	<0.001 0.003 <0.001
TNF-α	Median (Min-Mix)	65.8 (289-35.3)	49 (143-25.9)	72.2 (0-266.5)	37.8 (0-180)	80.7 (190.1-24)	0.483

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There was a positive medium correlation between serum levels of IL-21 and IL6 and BASDAI. There was a negative correlation between enthesitis and IL-6 levels and a negative correlation between the duration of treatment and levels of both IL-17A and IL-21. There was also a medium-negative correlation between treatment type and serum IL-21 levels, with a weaker negative correlation between IL-6 and IL-17A levels. There was a moderate positive correlation between the serum level of IL21 and sacroiliitis. All of these correlations are shown in Table 3

TABLE 3 CORRELATION BETWEEN MULTIPLE CLINICAL PARAMETERS AND A CYTOKINE PROFILE

Clinical parameters	Cytokine							
	IL6	IL17A	IL21	TNF-α				
	P	r	P	r	P	r	P	r
Disease onset	0.232	-0.134	0.853	0.021	0.875	-0.018	0.708	-0.042
Duration of diagnosis	0.226	-0.136	0.383	-0.096	*0.018	-0.262	0.686	-0.045
Family history	0.625	0.066	0.898	-0.015	0.376	-0.1	0.266	0.126
BMI	0.599	0.044	*0.05	0.158	0.477	0.059	0.751	-0.026
Smoking	0.849	-0.016	*0.05	-0.18	0.634	0.062	0.363	-0.076
BASDAI	*0.01	0.266	0.641	0.069	*0.001	0.455	0.840	0.023
Hip arthritis	0.920	-0.011	0.696	0.044	0.495	0.077	0.499	-0.076
Enthesitis	*0.05	-0.201	0.314	0.113	0.318	-0.112	0.615	0.073
Uveitis	0.838	-0.023	0.483	0.078	0.385	-0.098	0.395	0.096
Sacroilitis	0.099	-0.185	0.225	0.136	0.671	0.64	0.663	0.067
Duration of treatment	0.132	-0.169	*0.05	-0.196	*0.01	-0.283	0.404	-0.094
Type of treatment	*0.043	-0.226	*0.027	-0.246	*0.001	-0.634	0.234	-0.134

Significantly p value , r correlation coefficient

In the pairwise analysis of immunological profiles in patients with AS, positive controls, and healthy controls except for IL-6 and IL-17A, there was a significant difference between the cytokines (P = 0.312), as shown in Table 4.

TABLE 4 PAIRWISE ANALYSIS OF IMMUNOLOGICAL PROFILES IN PATIENTS WITH AS, POSITIVE CONTROLS, AND HEALTHY CONTROLS

Pairwise cytokine analysis	P. value	Adj. P. value
IL-6 - IL-17A	0.052	0.312
IL-6 - IL-21	0.001	0.001
IL-6 - TNF-α	0.001	0.001
IL-17A - IL-21	0.001	0.001
IL-17A - TNF-α	0.001	0.001
IL-21 - TNF-α	0.001	0.001

V. DISCUSSION

Ankylosing spondylitis is a chronic inflammatory disorder affecting skeletal and extraskeletal tissues. AS is more common among young men in the workforce and places a high health burden on the community.

This study found that the immunological profiles of IL-6, IL-17A, IL-21, and TNF-α differed significantly between established AS patients, biologically naïve AS patients, and healthy controls. However, the serum levels of the investigated cytokines were found to differ significantly between the biologically naïve AS and the other two groups. The most apparent cytokine change was in IL-21 levels. This indicated its significance in disease activity and progression. A previous study showed that most IL-21 was produced by activated CD4+ T cells, while IL-21R was expressed in human B cells, NK cells, activated T cells, and some nonhematopoietic cells (18, 19). The initial functions of IL-21 are to induce B and T cell proliferation and to generate highly lytic NK cells (19). Studies on the expression of IL-21 in inflammatory bowel disease (IBD) revealed increased cytokine production in the inflamed gut of patients with Crohn's disease and patients with ulcerative colitis in comparison to the inflamed and non-inflamed guts (20). $CD4^{+}$ T cells and T follicular helper cells produce IL-21 in the IBD mucosa, and the vast majority of these cells also express IFN- and, to a lesser extent, IL-17A or IL-4 (21). The current study is the first to confirm the role of IL-21 in the pathogenesis of AS directly or indirectly through the significant relationship of IL-21 with IBD associated with AS. It also demonstrated that a decrease in IL-21 levels, rather than a decrease in TNF-α level, is the cause of the disease's decline in disease activity.

The present study observed a significant decrease in IL-17A in established AS patients treated with anti-TNF therapy. This is consistent with another study that showed that anti-TNF drugs increase the number of regulatory T cells (Tregs) via the inhibition of IL-6 compared to untreated patients (22).

No decrease in serum TNF-α level was found in this study. However, patients receiving anti-TNF therapy had significantly higher TNF-α levels during treatment (p=0.736), consistent with a previous study by Martin Schulz et al. [23]. In contrast, the amount of TNF-α measured in the infliximab-treated patients remained unchanged. This cannot rule out the possibility that autoantibodies to infliximab could also influence the quantification of TNF. Perhaps no change in TNF-α is caused by cigarette smoking in established AS, which is characteristic of most AS patients treated with anti-TNF drugs. Cigarette smoking triggers local immunity via the induction of pattern recognition receptors (TLR4) and the MyD88 signaling pathway, resulting in the generation of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, and chronic inflammation [24]. Chronic inflammation distorts the balance of the T cell population toward the Th1 and Th17 subsets, which causes more IFN-γ, IL-10, IL-17, and IL-21 to be produced.

IL-6 is a multifunctional cytokine produced primarily by adipocytes and macrophages in fatty tissues, skeletal muscle, and the liver (25). High levels of IL-6 were found in sacroiliac biopsies of AS patients and may be partly responsible for the inflammatory response (26). According to the results obtained in this study, there were higher serum IL-6 levels in both established and biologically naïve AS patients than in healthy individuals. This aligns with Bal A et al. and Pedersen SJ et al. (26, 27). In this study, circulating levels of IL-6 were decreased as a result of anti-TNF therapy. There were significant

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differences in serum levels between established and biologically naïve AS patients. This finding is consistent with Rudwaleit et al. (28), who discovered a decrease in IL-6 levels in patients receiving anti-TNF treatment. In the current study, IL-6 could indicate the disease activity parameters in AS patients.

In the present study, serum levels of IL-6 and IL-21 varied based on the type of therapy received by established and biologically naïve AS patients. There was a significant decrease in IL-6 serum levels in patients taking infliximab, etanercept, remsima, and amjevita compared to patients taking NSAIDs. Anti-TNF therapy also reduced serum IL-21 levels, but only amjevita and etanercept were statistically significant. TNF-α (p=0.483) and IL-17A (p=0.146) serum levels did not change significantly more in established AS patients than in biologically naïve patients when NSAIDs were used. Perhaps the reason for this is that dose-dependent anti-TNF drugs can lower serum TNF-α and IL-17A levels, or it could be a side effect of developing immunogenicity to anti-TNF therapy. According to Martin Schulz et al., soluble TNF-R1 may be a good marker of inflammation in AS patients. In contrast, TNF-α, CRP, and IL-6 levels are often below the detection limits in commonly used assays. The fact that AS patients treated with infliximab and etanercept had lower levels of TNF-R1 in their blood supports the idea that soluble TNF-R1 is linked to TNF activity and inflammatory events in AS patients (29).

There was a significant difference and moderate negative correlation between proinflammatory cytokine levels and NSAIDs in biologically naïve AS patients and anti-TNF therapy in established AS patients, most notably IL-21 (p=0.001, r-0. 634) and less with IL-17A and IL-6 (r-0.246 and r-0.226, respectively) and TNF-α (r-0.134). In a study by Yong Yan et al., 148 patients with AS were selected to receive NSAID treatment. After treatment, IL-6, IL-17, and TNF-α levels were significantly reduced, whereas IL-10 levels increased in the positively effective, effective, and moderately effective groups and decreased in the completely effective and effective groups (30). These results contrast with those of Nuh Atas et al., who found no significant difference in TNF-α levels after six months of treatment between axial spondylitis (ax-SPA) patients and healthy controls (30.7 (12.8–35.6) vs. 18.1 (12.1–28.4) peg/ml, p=0.156). After six months of anti-TNF treatment, average IL-17, IL-22, and IL-33 serum levels increased significantly (31).

In the current study, there was a significant positive correlation between serum IL-21 and IL-6 levels and BASDAI (p=0.001, r=0.455 and p=0.01, r=0.266, respectively), but there was no correlation with the levels of IL-17A and TNF-α. This indicates the importance of IL-21 over IL-6 to the follow-up of the disease activities of established AS patients. Patients were treated with anti-TNF therapy to help them decide whether to switch or increase the drug dosage. Biologically naïve patients were used as a marker to help in deciding on early begging of receiving anti-TNF therapy.

In our study, there was a significantly positive correlation between sacroiliitis and the serum level of IL21, suggesting that this is related to the effect of the anti-TNF drug on decreased serum levels of IL21. IL21 is a pleiotropic cytokine that regulates innate and specific immunity-related activity. Indeed, it costimulates T and natural killer (NK) cell proliferation and function and regulates B cell survival, differentiation, and the role of dendritic cells.

VI. AUTHOR CONTRIBUTIONS

HAA, NHA, and FA collected the data. NHA and FA reagents and quality control were provided. HAA performed the experiments and analyzed the data. All of the authors contributed to the article and approved the final version. All authors read and approved the final manuscript.

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