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Evaluating the Serum Level of IL-40 in a Sample of Iraqi Men with Ankylosing Spondylitis

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Abstract

Ankylosing spondylitis (AS) is a form of arthritis that predominantly affects the spine and is characterized by immune and skeletal disorders causing deterioration in structural integrity, functional capability, and overall quality of life. This study aimed to estimate IL-40 serum level in Iraqi men who had AS. Blood samples were obtained from 100 patients (with AS) and 100 men (controls group). The enzyme-linked immune sorbent assay was used to measure the IL-40 level in blood samples. The investigation's findings showed that the typical patient's age was 40.56 ± 10.19 years old. While the control group's average age was 38.76 ± 10.52 years. The results found that the duration of the disease was< 5 years in 44% of cases, 5-10 years in 50% and >10 years in 6% of cases. Patients without HLA-B27 had higher IL-40 levels, on average than those with HLA-B27 (p= 0.0001). Analysis of the receiver operating characteristic (ROC) curve suggested that a serum IL-40 level of 14.18 pg/mL (AUC = 0.93; p= 0.0001) was optimal which was considered the point at which a person was either affected or not. The differences in the interleukin level in serum between patients and controls were included. The results for levels of IL-40 appeared higher significant differences in patients as contrasted to healthy controls (19.10 ± 5.07 vs 9.13 ± 4.03 pg/ml; p<0.001). Also, the results showed that IL-40 was not substantially correlated with BASFI, BASDAI, Erythrocyte sedimentation, white blood cell count, disease duration, hemoglobin, urea, C-reactive protein or creatinine. While age had a significant positive association with IL-40 serum levels. In conclusion, IL-40 levels in the serum of AS patients were significantly higher than those of healthy controls which indicated that it could play an important pathogenetic role in AS.

Keywords: Ankylosing spondylitis, disease duration, HLA-B27, IL-40, Rheumatic immune diseases.

تقدير مستوى الانترلوكين 40 في عينة من الرجال العراقيين المصابين بالتهاب الفقار اللاصق

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الخلاصة

التهاب الفقار اللاصق (AS) هو شكل من أشكال التهاب المفاصل الذي يؤثر في الغالب على العمود الفقري ويتميز باضطرابات في الجهاز المناعي والهيكل العظمي. مما يسبب تدهور في السلامة الهيكلية والقدرة الوظيفية ونوعية الحياة بشكل عام. الهدف من هذه الدراسة هو تقدير مستوى IL−40 في المصل لدى الرجال

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العراقيين المصابين بالتهاب الفقار اللاصق (AS). تم الحصول على عينات الدم من 100مريض (مع AS) و 100 رجل (مجموعة السيطرة) .و تم استخدام طريقة الاليزا لقياس مستوى IL−40 في عينات الدم. أظهرت نتائج البحث أن عمر المريض النموذجي كان 40.56 ± 10.19 سنة. بينما كان متوسط عمر مجموعة السيطرة 38.76 ± 10.52 سنة. كانت مدة المرض أقل من 5 سنوات في 44٪ من الحالات ، و 5−10 سنوات في 50٪ ، و> 10 سنوات في 6٪ من الحالات. كان لدى المرضى الذين ليس لديهم HLA−B27 مستويات أعلى من IL−40 ، في المتوسط من أولئك الذين لديهم HLA−B27 (p = 0.0001). يشير تحليل منحنى خاصية تشغيل المستقبل (ROC) إلى أن مستوى IL−40 في الدم يبلغ 14.18 بيكوغرام / مل (المنطقة الواقعة تحت المنحنى = 0.93 ؛ p = 0.0001) هو الأمثل. والتي تعتبر النقطة التي يتأثر فيها الشخص أم لا. تم تضمين الفروق في مستوى الانترلوكين في مصل الدم بين المرضى والضوابط الصحية. أظهرت نتائج مستوى المصل من IL−40 اختلافات معنوية أعلى في المرضى على عكس الضوابط الصحية (19.10 ± 5.07 مقابل 9.13 ± 4.03 بيكوغرام / مل ؛ p <0.001). لم يكن IL−40 مرتبطًا بشكل كبير بـ BASFI أو BASDAI أو سرعة ترسيب كريات الدم الحمراء أو كريات الدم البيض أو مدة المرض أو هيموغلوبين أو اليوريا أو البروتين التفاعلي أو الكرياتين. في حين أن العمر كان له علاقة إيجابية كبيرة مع مستويات IL−40 في الدم. وفي الختام ، فإن مرضى AS لديهم مستوى IL−40 في مصلهم أعلى بكثير من مستواه في مجموعة السيطرة ، مما يشير إلى أن الانترلوكين 40 قد يلعب دورًا هامًا في التسبب في الإصابة بالتهاب الفقار اللاصق.

1. Introduction

Ankylosing spondylitis (AS) is a member of the group of rheumatic diseases known as axial spondyloarthropathies (axSpA) [1] and is characterized by progressive inflammatory responses mediated by the immune system, predominantly affecting the axial skeleton [2]. AS enthesitis increases spinal ankylosis and the formation of new bone. Osteoporosis results from the loss of bone trabeculae in the vertebral body of AS patients which decreases local bone mineral density [3, 4], and can have significant effects on health-related quality of life (HRQL) [5], impacting patients' physical function and mental health [6], and resulting in a substantial loss of work productivity [7]. Although men are more likely to be diagnosed with such a condition than women (3:1), recent research has suggested that gender may not be a predictor of AS [8]. The cause of autoimmunity in AS and the molecular pathogenesis involved have not been well established. However, it has been strongly hypothesized that interactions between genetic, immunological and infectious factors play an important role [9].

Human leukocyte antigen (HLA-B27) has been the primary genetic and immunological factor associated with AS disease [10, 11]. HLA-B27 is not always detectable in patients with AS-like symptoms [12]. Many inflammatory disorders have been linked to cytokines which are crucial mediators of inflammation. It has been observed that the inflammatory and proliferative cascades of AS are largely mediated by inflammatory mediators such as tumor necrosis factor - α (TNF)- α, interleukin (IL)-6, and IL-17 cytokines [13, 14, 15]. This role is supported by the fact that disease symptoms and activity could be affected by blocking the cytokines such as anti-IL-17A [16]. IL-12 and IL-23 are indispensable for the differentiation of Th1 and Th17 cells respectively [17]. It seems that Th1 and Th17 cells are both involved in the development of the disease. Acute inflammation in AS is thought to be driven by Th17 cells, while the inflammatory process is thought to be maintained by Th1 cell [2]. In 2017, Catalan-Dibene *et al*. were the first to describe the new IL-40. Because of its unusual structure, IL-40 was considered to be one of the few "orphan" cytokines [18]. The cytokine IL-40 has been associated with immune response mechanisms and B cell homeostasis [19].

C17orf 99 (chromosome 17 open reading frame 99) encodes IL-40 in humans [19]. This gene produces a short-secreted protein (27 KDa) consisting of 265 amino acids, including a signal peptide of 20 amino acids. By demonstrating down-regulation in response to anti-inflammatory treatment in a human cell model of lung inflammation, a recent study has hypothesized that this gene may function as a pro-inflammatory cytokine [20]. In light of this

finding, it is plausible that it contributes to the pathophysiology of a variety of human disorders [18]. New studies have demonstrated that IL-40 levels in rheumatoid arthritis (RA) are increased and linked to disease activity, autoantibodies, and neutrophile extracellular traps externalization (NETosis) [21]. Additionally, it has been shown that the C17orf99 protein is one of four autoantigens that distinguished autoimmune hepatitis patients from healthy controls [22]. Despite this, the prior information has not precisely identified the role of IL-40 in AS. The purpose of this study was to examine IL-40 levels in AS patients as a potential biomarker for disease activity and to learn more about the role of IL-40 in pathophysiology of this disease.

2. Methods

2.1 Study Subject

Blood samples from 100 males with AS who were admitted to the rheumatology department of the Baghdad Teaching Hospital and 100 males serving as healthy controls (blood donors) were collected at the National Blood Bank (Baghdad) between October 2022 and February 2023. Both the modified New York criteria for AS and the Assessment Spondylarthritis International Society (ASAS) criteria for axSpA were met by all patients in this study [23]. All individuals provided written informed consent before participation in the research. The local Ethics Commission (Ref: CSEC/0423/0033) approved and recorded in writing each participant's informed assent. Under the supervision of medical professionals at the City of Medical, Baghdad Hospital, a group from the University of Baghdad conducted research in Baghdad, Iraq. Patients' data included their age, HLA-B27 phenotypes, white blood cell count (WBC), medications, hemoglobin (Hb), disease duration, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index score (BASFI) were used to measure disease activity and physical function respectively, and to determine functional status [24, 25]. Patients in both cases were divided into two groups: < 4.0 (low) and ≥ 4.0 (high). Scores above the cut-off value indicated more functional impairments or active disease.

2.2 Interleukin-40 Immunoassay

Three milliliters of peripheral blood samples were drawn and deposited in a gel tube. The blood was then coagulated at room temperature for one hour, centrifuged at 2000g for ten minutes, and the serum was then frozen at -20°C. A sandwich enzyme-linked immune sorbent assay (ELISA) kit (Cat. No.: E4654Hu) was used to determine the serum level of IL-40. The kit was a product of MyBioSource, and the instructions of the manufacturer were followed. Colour development was monitored with an ELISA microplate reader at 450 nm.

2.3 Statistical Analysis

Both GraphPad Prism version 8.0.0 and SPSS version 23.0 were used to analyze the collected data. Number and percentage, mean, and standard deviation (SD) were used to determine the results. The differences between the two groups were calculated, and the Pearson Chi-square test was utilized to assess the significant difference. The one-way analysis of variance (ANOVA) was used to determine significant differences. A receiver operating characteristic (ROC) curve analysis was carried out to determine the area under the curve (AUC), the 95% confidence interval (CI), the sensitivity, and the specificity. The relationships between serum IL-40 levels and various AS characteristics (age, BASDAI, BASFI, disease duration, WBC, urea, creatine, ESR, and CRP) were assessed using the Pearson correlation coefficient (rs) test. *P*-values less than ≤ 0.05 were regarded as significant in statistical analyses.

3. Results and Discussion

Table 1 shows the baseline characteristics of the patients and controls. The results showed that the mean age of patients with AS (40.56 ± 10.19 years) was the non-statistical difference

(p=0.221) with control groups (38.76 ± 10.52 years). Patients were divided into three disease duration groups based on the AS duration which was 5.38 ± 4.15 years: <5 years in (2.34 ± 1.07), 5-10 years in (6.80 ± 1.64), and >10 years in (17.50 ± 4.50) of cases. In 44% of cases the duration of the disease was< 5 years, 5-10 years in 50% and >10 years in 6% of cases. The results revealed that 73% of patients had negative HLA-B27, while 27% of them had positive HLA-B27. Also, BASDAI and BASFI scores were used to calculate disease activity. Over 50% of the patients had a score of less than 4.0 in both situations (61% and 53% respectively). Hb and WBC for AS patients were significantly higher compared with controls (p = 0.0008; p = 0.014 respectively). Urea and creatinine did not show a significant difference compared with control (p = 0.092; p = 0.299 respectively). While ESR and CRP levels in patients with AS were higher (25.37 ± 19.23 mm/h and 16.09 ± 7.77mg/l respectively) compared to the controls (18.27 ± 7.44mm/h and 11.13 ± 6.12 mg/l respectively). Almost 89.00% people were on anti-TNF therapy and only 11.00% of cases were newly diagnosed. Serum IL-40 protein levels were substantially higher in AS patients compared with controls (19.10 ± 5.07 *vs* 9.13 ± 4.03 pg/ml; p<0.0001) (Table 1, Figure 1).

Table 1: Baseline characteristics of the AS patients compared to controls.

Characteristics	AS Patients (n =100) Mean ±SD	Controls (n = 100) Mean ±SD	*p*-value
Age (Year)	40.56 ± 10.19	38.76 ± 10.52	0.221 NS
Total Disease Duration (Year) **< 5** **5-10** **>10**	5.48 ± 4.10 2.34 ± 1.07 6.80 ± 1.64 17.50 ± 4.50	---	---
HLA-B27 Positive **HLA-B27 Negative**	27 (27.00%) 73 (73.00%)	---	---
BASDAI	3.45 ± 2.03	---	---
BASFI	3.75 ± 2.61	---	---
HB (g/dl)	13.45 ± 2.06	12.56 ± 1.59	0.0008 **
WBC (10^9\L)	8.33 ± 2.06	7.67 ± 1.65	0.014 *
Urea (mg/dl)	24.68 ± 9.48	22.42 ± 9.35	0.092 NS
Creatinine (mg/dl)	1.11 ± 0.55	1.04 ± 0.28	0.299 NS
ESR (mm/hour)	25.37 ± 19.23	18.27 ± 7.44	0.0007**
CRP (mg/L)	16.09 ± 7.77	11.13 ± 6.12	<0.0001**
Drugs anti-TNF **Drugs Newly Diagnosed Cases**	89 (89.00%) 11 (11.00%)	---	---
IL-40 pg/ml	19.10 ± 5.07	9.13 ± 4.03	<0.0001**

Non-Significant: NS. Significantly (p≤0.01).AS: Ankylosing spondylitis; HLA: Human leukocyte
antigen; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; ESR: Erythrocyte sedimentation
rate; HB: Hemoglobin; WBC: White blood cell; CRP: C-Reactive Protein.
**Highly Significant (P≤0.01); * Significant (P≤0.05).

This study also found that IL-40 serum levels were higher in AS patients with age over 50 years (Figure 2). IL-40 levels were significantly higher in HLA-B27-negative individuals than in HLA-B27-positive patients (18.99 ± 4.93 *vs* 15.17 ± 4.51 pg/ml; p= 0.0001) (Figure 3).

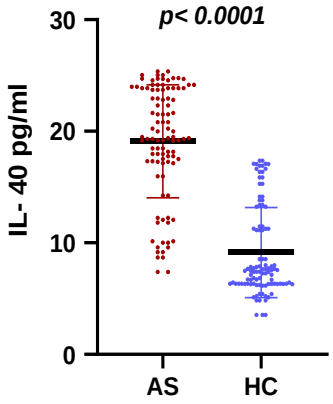


Figure 1: Interleukin-40 (IL-40) serum concentrations in ankylosing spondylitis (AS) patients and healthy controls (HC).

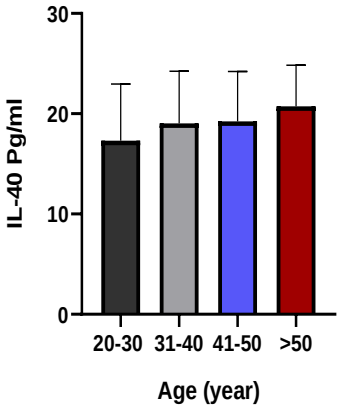


Figure 2: Interleukin-40 (IL-40) serum concentrations in ankylosing spondylitis (AS) patients and age (20-30, 31-40, 41-50, >50).

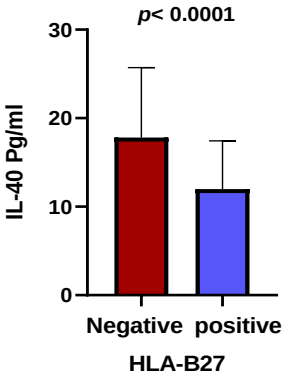


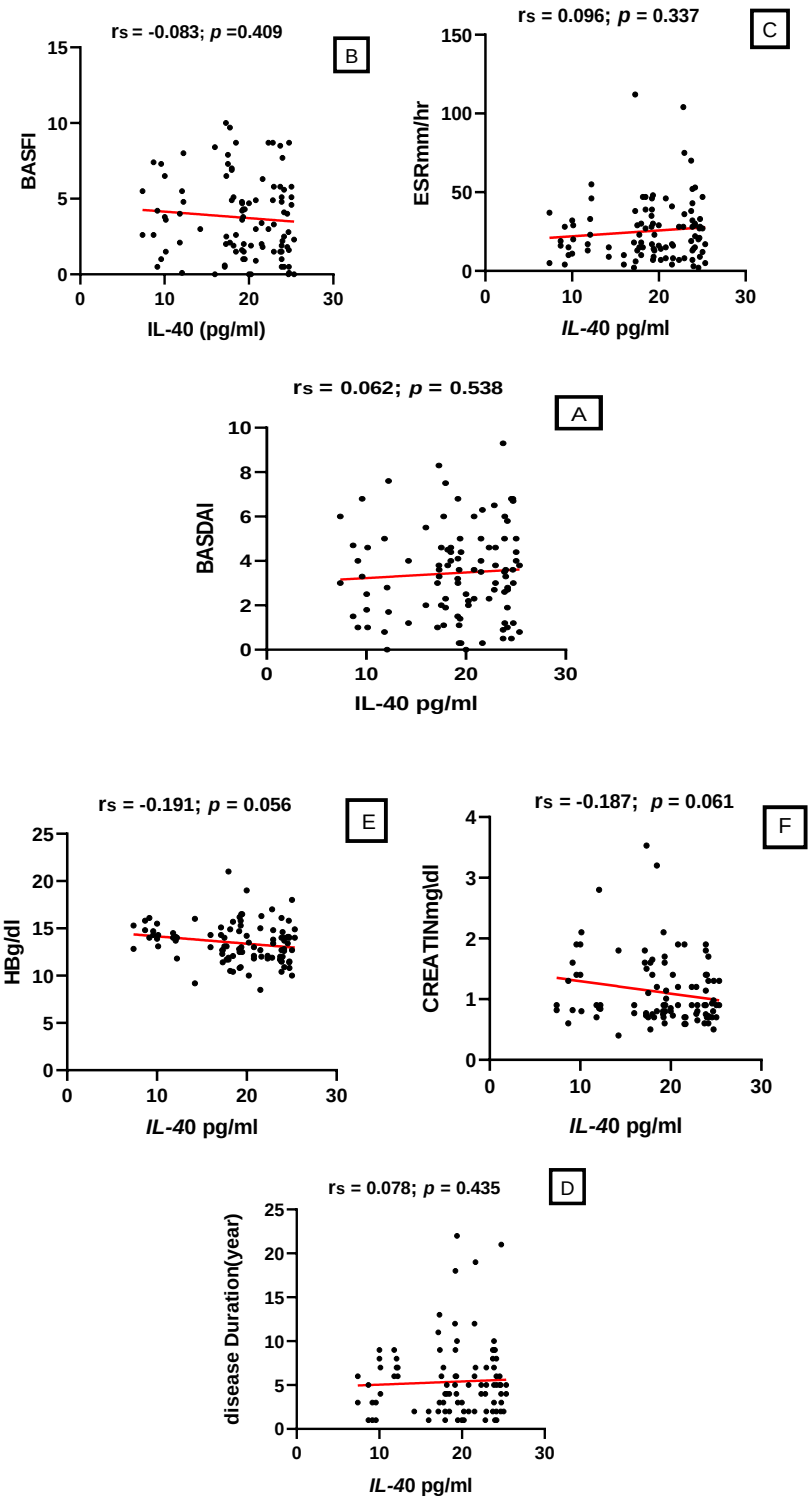
Figure 3: Interleukin-40 (IL-40) serum concentrations in ankylosing spondylitis (AS) patients with positive and negative HLA-B27.

3.1 Pearson Correlation between the AS Patients' Baseline Characteristics and IL-40 Serum Levels.

Figure 4 shows the correlation calculated by Pearson between IL-40 serum levels and some baseline features of AS patients. The results showed that there was a significant negative correlation between IL-40 levels and the BASFI (Figure 4-B), Hb (Figure 4-E), creatin (Figure 4-F), urea (Figure 4-G), as well as WBC (Figure 4-H), (r = -0.083; *p* = 0.409: r = -0.191; *p* = 0.056: r = -0.187; *p* = 0.061: r = -0.006; *p* = 0.950: r = -0.044; *p* = 0.658 respectively). IL-40 levels were not substantially associated with other parameters: BASDAI (Figure 4-A), ESR (Figure 4-C), disease duration (Figure 4-D), and CRP (Figure 4-Z), (r = 0.062; *p* = 0.538: r = 0.096; *p* = 0.337: r = 0.078; *p* = 0.435: r = 0.032; *p* = 0.748 respectively). While age (Figure 4-K) had a significant positive association with IL-40 serum levels (r = 0.208; p = 0.037) when $p \leq 0.05$ was considered to be a statistically significant value (Table 2).

Table 2: Pearson correlation coefficient between interleukins-40 and parameters in AS patients.

Variable	Pearson Coefficient (r) *(p-value)*
A- BASDAI	r = 0.062; (0.538) NS
B- BASFI	r = -0.083; (0.409) NS
C- ESR mm/hr	r = 0.096; (0.337) NS
D- Disease duration (year)	r = 0.078; (0.435) NS
E- HB g/dl	r = -0.191; (0.056) NS
F- Creatin mg/dl	r = -0.187; (0.061) NS
G- Urea mg/dl	r = -0.006; (0.950) NS
H- WBC 10^9\L	r = -0.044; (0.658) NS
K- Age (year)	r = 0.208; (0.037) *
Z- CRP mg/l	r = 0.032; (0.748) NS


rs = -0.083; p =0.409
B
BASFI
IL-40 (pg/ml)
rs = 0.096; p = 0.337
C
ESRmm/hr
IL-40 pg/ml
rs = 0.062; p = 0.538
A
BASDAI
IL-40 pg/ml
rs = -0.191; p = 0.056
E
HBg/dl
IL-40 pg/ml
rs = -0.187; p = 0.061
F
CREATINmgldl
IL-40 pg/ml
rs = 0.078; p = 0.435
D
disease Duration(year)
IL-40 pg/ml

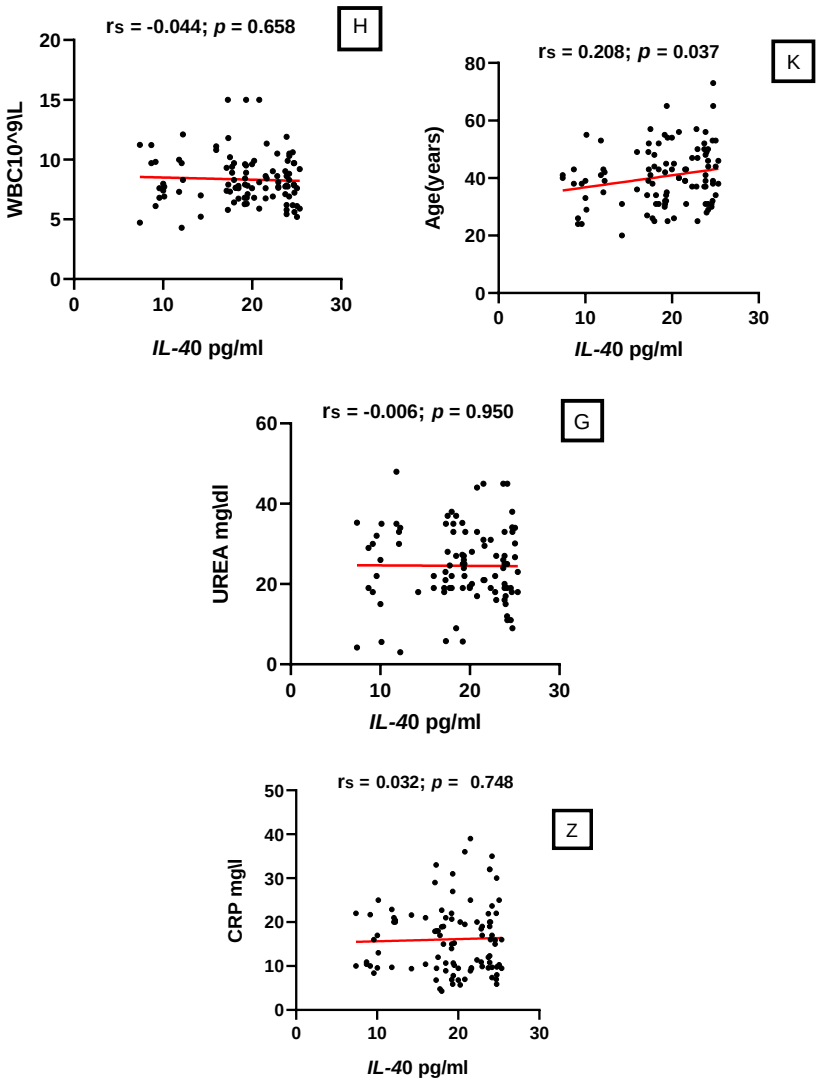


Figure. 4. Correlation between interleukin-40 (IL-40) level and Bath Ankylosing Spondylitis Disease Activity Index (A), Bath Ankylosing Spondylitis Functional Index (BASFI) (B), ESR (C), disease duration (D), HB (E), Creatin (F), Urea (G), WBC (H), Age (K), CRP (Z).

3.2 Receiver Operating cCaracteristic (ROC) of IL-40

According to the ROC curve analysis, IL-40 blood levels at an optimal cut-off point of 14.18 pg/mL may discriminate AS patients and controls meaningfully (AUC = 0.93; 95% CI = 0.90 to 0.96; *p* 0.0001), with a sensitivity and specificity of 84% and 82% respectively (Figure 5). The ability of a diagnostic marker to distinguish between patients who had the disease or condition being tested for and those who didn't was measured by the area under the curve (AUC). The AUC between 0.50 and 0.59 meant no discrimination; and that between 0.60 and 0.69 meant that there was moderate discrimination; and values between 0.70 and 0.79 were taken as acceptable; whereas those between 0.80 and 0.89 were considered to be outstanding; and 0.9 meant it was exceptional.

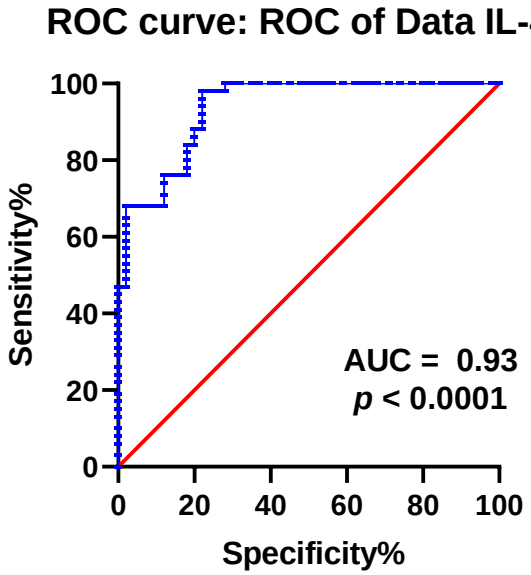


Figure 5: Receiver operating characteristic (ROC) curve of serum interleukin40 (IL-40) for differentiating ankylosing spondylitis (AS) patients from healthy controls.

There has been a little research that examined the correlation between AS and IL-40. The present study's findings revealed no distinct differences between the mean ages of control subjects and AS patients. These findings were in line with those of an earlier study [26] in which it was discovered that the mean age of patients was 38.0 ± 9.0 years which did not differ significantly from that of the control. According to the current investigation, neither the BASFI nor the BASDAI were responsible for the considerable increase in blood IL-40 levels. Regarding the autoimmune disease component, a study showed that type 3 immunity and the HLA-B27-related pathway improved in AS patients [27, 28]. In addition, HLA-B27-negative cases had higher IL-40 levels than HLA-27-positive patients. Moreover, a separate study [29] found that Syrian patients with AS had a lower incidence of HLA-B27 antigen appearance than patients from other populations. CRP and ESR are the two disease activity indices most commonly used in clinical practice and research. Inflammation in the lumbar and cervical regions are the initial manifestations of the disease [30], so these parameters are typically elevated in AS patients with lumbar and cervical pain. In 50% to 70% of individuals with active AS, an augmented acute phase response, including an elevated erythrocyte sedimentation rate (ESR) and high C-reactive protein (CRP), may be present. About 30% of patients with non-radiographic axSpA (nr-axSpA) reported such changes [31]. Deregulated inflammatory responses are a hallmark of AS, an autoimmune rheumatic disease [9]. IL-40 has been considered a possible RA biomarker. According to numerous studies, patient synovial tissue contains the highest concentration of IL-40. This has further been explained by the fact that RA patients had six-fold higher levels of IL-40 in synovial fluid compared to blood [21]. This study looked into IL-40's potential as a biomarker for AS patients, and the ROC analysis indicated that there could be a connection between the two. IL-40 is not only secreted by activated B cells but also by cells in the bone marrow and the mammary glands [32]. After being stimulated with anti-CD40, anti-IgM, and IL-4, human B cells express increased IL-40. The lack of IL-40 has also been linked to deregulated microbiota [33], and IL-40-deficient rats exhibited a systemic IgA deficit. This second finding had relevance for AS because some studies did suggest a function for the gastrointestinal flora in the development of AS [34]. One limitation of this current study was the small sample size which increased the possibility of discrimination.

Furthermore, no radiographic information was collected from AS patients. These findings also suggested that IL-40 could serve as a biomarker for other autoimmune inflammatory diseases such as systemic lupus erythematosus or Hashimoto's thyroiditis.

4. Conclusions

According to our findings, serum IL-40 levels in AS patients were significantly greater than in healthy controls. This value was not related to any of the AS variables tested (disease duration, BASDAI, BASFI, white blood cell count, erythrocyte sedimentation rate, urea, hemoglobin, C-reactive protein, or creatinine). However, serum levels of IL-40 tended to increase with age. According to these results, IL-40 levels in the serum may function as a potential biomarker to identify AS in Iraqi patients.

Interest Discrepancies

The authors claim that their publication of this work is free of any conflicts of interest.

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