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A novel signature of interleukins 36α, 37, 38, 39 and 40 in ankylosing spondylitis

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ABSTRACT

The current study examined five cytokines, three belong to interleukin (IL)-1 family (IL-36α, IL-37 and IL-38), one belongs to IL-12 family (IL-39) and one has not been assigned to a family (IL-40), in the serum of 110 male patients with ankylosing spondylitis (AS) and 103 male controls. Studies regarding these cytokines in AS are very limited. Therefore, the significance of IL-36α, IL-37, IL-38, IL-39 and IL-40 as biomarkers of AS was evaluated. Cytokine levels were measured using enzyme-linked immunosorbent assay kits. Results revealed that serum levels (median and interquartile range) of IL-36α (90.7; 53.7–166.2 *vs* 39.7; 31.3–59.2 pg/mL; probability [*p*] < 0.001), IL-37 (161.3; 62.8–236.6 *vs* 58.4; 46.8–80.7 ng/mL; $p < 0.001$), IL-38 (135.8; 78.2–213.5 *vs* 65.8; 51.1–87.1 pg/mL; $p < 0.001$), IL-39 (57.7; 34.1–92.3 *vs* 29.1; 19.3–58.6 ng/L; $p < 0.001$) and IL-40 (3.89; 2.99–6.19 *vs* 2.10; 1.75–2.68 ng/L; $p < 0.001$) were significantly higher in AS patients than in controls. Besides, they were of value in distinguishing between AS patients and controls as evidenced by the receiver operating characteristic curve analysis: area under the curve = 0.797 (IL-36α), 0.75 (IL-37), 0.797 (IL-38), 0.728 (IL-39) and 0.886 (IL-40). Some of these cytokines were significantly correlated, but no correlation with AS activity was found. In conclusion, the levels of IL-36α, IL-37, IL-38, IL-39 and IL-40 were up-regulated in the serum of AS patients regardless of age, age at disease onset, disease duration, disease activity or HLA-B27.

1. Introduction

Ankylosing spondylitis (AS), also known as radiographic axial spondyloarthritis (axSpA), is a common chronic inflammatory autoimmune rheumatic disorder that primarily affects the spinal vertebrae and sacroiliac joints in about 1 % of the general population [1]. The disease etiology remains largely idiopathic, but a significant association between AS incidence and prevalence of human leukocyte antigen (HLA)-B27 (an immunogenetic marker) has been shown in different ethnic groups [2]. AS etiopathogenesis also includes chronic enthesitis associated with abnormal functions of certain immune cells such as T lymphocytes and macrophages [3]. In addition, it has been indicated that cytokines, which are crucial in mediating interactions and communications between these cells, play a key role in initiation and regulation of inflammatory processes at enthesitis sites [4]. In this context, the inflammatory process in AS has been found to be associated with high levels of circulating pro-inflammatory cytokines [5]. Besides, it has been disclosed that anti-inflammatory cytokines play key roles in maintaining immune homeostasis in AS [6–8].

A unique family of cytokines is the interleukin (IL)-1 family, in that it includes both pro-inflammatory (IL-1α, IL-1β, IL-18, IL-33, and IL-36) and anti-inflammatory (IL-37 and IL-38) cytokines [9]. Among the recently identified IL-1 family members that have not been well studied in AS are IL-36, IL-37 and IL-38. IL-36 is a pro-inflammatory cytokine and recent studies highlighted that IL-36 expression and function may be associated with an increased risk of inflammatory and autoimmune disorders [10]. Furthermore, it has been hypothesized that IL-36 may be involved in the pathogenesis of spondyloarthritides [11]. IL-37 is functionally characterized by its anti-inflammatory effects and its ability to suppress inflammation and production of pro-inflammatory cytokines

Abbreviations: AS, Ankylosing spondylitis; AUC, Area under the curve; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; CI, Confidence interval; ESR, Erythrocyte sedimentation rate.; HC, Healthy controls; IBD, Inflammatory bowel disease; IL, Interleukin; *p*, Probability; *pc*, Corrected probability; PBMC, Peripheral blood monocular cell; RA, Rheumatoid arthritis; r_s, Correlation coefficient; SLE, Systemic lupus erythematosus; TGF, Transforming growth factor; TNF, Tumor necrosis factor; WBC, White blood cell count.

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and chemokines [12]. In AS, two previous studies demonstrated that IL-37 was up-regulated in the serum of patients [8,13]. IL-38 is a recently discovered member of the IL-1 family with anti-inflammatory functions [12,14]. Increasing evidence indicates that IL-38 is a vital cytokine in the pathogenesis of several inflammatory autoimmune diseases, such as rheumatoid arthritis (RA), psoriatic arthritis and systemic lupus erythematosus (SLE) [15]. In AS, although systemic levels of IL-38 have not been determined, an association of *IL38* gene polymorphism with AS risk has been proposed [16].

IL-39 and IL-40 are newly discovered cytokines that have been proposed to play a role in the pathogenesis of autoimmune and inflammatory diseases but have not been explored in AS [12]. IL-39 is the most recently identified member of the IL-12 cytokine family, and in lupus-like mice, it showed pro-inflammatory effects [17,18]. IL-40, a 27-kDa secreted protein, is considered an orphan cytokine because no structural homology has been identified with any of the recognized cytokine families [12]. Regarding inflammation, the coding gene (Chromosome 17 Open Reading Frame 99; *C17orf99*) was down-regulated in IL-38-treated macrophages [19]. In addition, a study showed that C17orf99 protein was among four autoantigens that could distinguish patients with autoimmune hepatitis from healthy individuals [20].

The current study sought to assess the potential of IL-36α, IL-37, IL-38, IL-39 and IL-40 as biomarkers of AS in the serum of male patients. Besides, their correlation with AS activity and other patient characteristics was also evaluated. To the researchers' best knowledge, this study is the first to explore IL-38, IL-39 and IL-40 in AS.

2. Materials and methods

2.1. Patients and controls

A study was conducted on 110 male patients with AS (mean age ± standard deviation (SD) = 38.9 ± 10.2 year) and 103 healthy male controls (mean age ± SD = 37.9 ± 10.2 year). Patients were referred to the Rheumatology Outpatient Clinic at Baghdad Teaching Hospital during the period from November 2021 to February 2022. Diagnosis of AS was according to the modified New York Classification Criteria [21]. Female patients and those with other rheumatological diseases were excluded. Data for patients included age, age at onset, duration of disease, medication, HLA-B27, white blood cell count (WBC), erythrocyte sedimentation rate (ESR), blood urea nitrogen and serum creatinine. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index (BASFI) were used to evaluate disease activity and functional impairment, respectively. In both cases, patients were categorized into two groups; < 4.0 (low) and ≥ 4.0 (high) [22]. The control sample included blood donors who were healthy and did not suffer from inflammatory or autoimmune diseases. The Ethics Committee at the College of Science (University of Baghdad) approved the study (Reference: CSEC/0921/0079), and written consent was provided by all participants.

2.2. Immunoassay of cytokines

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum levels of IL-36α, IL-37, IL-38, IL-39 and IL-40 (Catalogue Number: MBS755062, MBS165041, MBS269990, MBS167915 and E0147Hu, respectively). They were products of MyBioSource (USA), and instructions of manufacture were followed. The sensitivity of kits (minimum detectable human cytokine level) were 1 pg/mL (IL-36α), 4.6 ng/L (IL-37), 5 pg/mL (IL-38), 1.1 ng/L (IL-39) and 0.2 ng/L (IL-40).

2.3. Statistical analysis

Normality tests (Kolmogorov-Smirnov and Shapiro-Wilk tests) revealed that IL-36α, IL-37, IL-38, IL-39 and IL-40 levels were distributed in a non-parametric manner and were therefore given as median and interquartile range (IQR). Significant differences between medians were assessed using Mann-Whitney *U* test (comparing two groups) or Kruskal-Wallis test (comparing more than two groups). Receiver operating characteristic (ROC) curve analysis was performed to calculate area under the curve (AUC) and 95 % confidence interval (CI). Spearman's rank order correlation analysis was applied to determine correlation coefficient (r_s). A probability value (p) ≤ 0.05 was taken statistically significant. The *p*-value was corrected using Bonferroni correction. GraphPad Prism version 8.0.0 (San Diego, CA, USA) and IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) were used to perform statistical analysis. G*power software (version 3.1.9.7) was used to estimate power of sample size [23].

3. Results

3.1. Power of sample size

Numbers of AS patients and controls were computed using G*power software to estimate power of sample size. At a two-tailed α error probability of 0.05 and an effect size d of 0.5, the power of sample size (1-β error probability) was 0.95. The acceptable power of sample size is considered to be 0.8.

3.2. Baseline characteristics of AS patients

Mean age of AS patients was 38.9 ± 10.2 years and most of them were<45 years old (75.5 %). The age at onset of AS patients was 32.6 ± 9.6 years, but>50 % of patients were in the range of 25–39 years (53.6 %). AS duration was 6.1 ± 6.8 year, and patients were classified into four disease duration groups: ≤ 1(19.1 %), 2–5 (41.8 %), 6–10 (25.5 %) and > 10 (13.6 %) years. Disease activity was determined using BASDAI and BASFI scores. In both cases, <50 % of patients had a score of ≥ 4.0 (36.4 and 44.5 %, respectively). Most patients were on anti-TNF medication (91.8 %), while newly diagnosed cases accounted for only 8.2 %. HLA-B27 data were available for 36 patients, of whom 58.3 % were positive for the marker. WBC, blood urea nitrogen and serum creatinine were within the reference range, while ESR was above 15 mm/h (18.9 ± 18.1 mm/h) (Table 1).

3.3. Serum cytokine levels

Serum levels (median and interquartile range) of IL-36α (90.7; 53.7–166.2 *vs* 39.7; 31.3–59.2 pg/mL; $p < 0.001$), IL-37 (161.3; 62.8–236.6 *vs* 58.4; 46.8–80.7 ng/mL; $p < 0.001$), IL-38 (135.8; 78.2–213.5 *vs* 65.8; 51.1–87.1 pg/mL; $p < 0.001$), IL-39 (57.7; 34.1–92.3 *vs* 29.1; 19.3–58.6 ng/L; $p < 0.001$) and IL-40 (3.89; 2.99–6.19 *vs* 2.10; 1.75–2.68 ng/L; $p < 0.001$) were significantly higher in AS patients than in controls (Fig. 1). ROC curve analysis revealed the discrimination potential of these cytokines between AS patients and HC in the following descending order based on the calculated AUC: IL-40 (AUC = 0.886; 95 % CI = 0.842–0.929), IL-36α (AUC = 0.797; 95 % CI = 0.738–0.857), IL-38 (AUC = 0.797; 95 % CI = 0.737–0.856), IL-37 (AUC = 0.75; 95 % CI = 0.678–0.822) and IL-39 (AUC = 0.728; 95 % CI = 0.661–0.795) (Fig. 2).

3.4. Serum cytokine levels classified by characteristics of patients

Serum IL-36α, IL-37, IL-38, IL-39 and IL-40 levels were stratified according to the characteristic groups of AS patients (age, age at onset, disease duration, BASDAI, BASFI, medication and HLA-B27) in order to evaluate the effects of these characteristics on the cytokine levels. In fact, no significant difference was found in each stratum. The age at onset and BASFI groups were an exception, where the *p*-value was significant ($p = 0.05$ and 0.018, respectively), but lost significance when the Bonferroni correction was applied ($pc = 0.25$ and 0.09, respectively)

Table 1
Characteristics of ankylosing spondylitis patients.

Characteristic [a]		AS patients; n = 110
Age; year		38.9 ± 10.2
Age group; year	< 45	83 (75.5)
	≥ 45	27 (24.5)
Onset age; year		32.6 ± 9.6
Onset age group; year	17–24	27 (24.6)
	25–39	59 (53.6)
	≥ 40	24 (21.8)
Disease duration; year		6.1 ± 6.8
Disease duration group; year	≤ 1	21 (19.1)
	2–5	46 (41.8)
	6–10	28 (25.5)
	> 10	15 (13.6)
BASDAI		3.3 ± 2.3
BASDAI group	< 4.0	70 (63.6)
	≥ 4.0	40 (36.4)
BASFI		4.0 ± 2.9
BASFI group	< 4.0	61 (55.5)
	≥ 4.0	49 (44.5)
Medication	Anti-TNF	101 (91.8)
	Newly diagnosed cases	9 (8.2)
HLA-B27; n = 36	Positive	21 (58.3)
	Negative	15 (41.7)
WBC; × 10^9/L		8.1 ± 2.1
ESR; mm/hour		18.9 ± 18.1
Blood urea nitrogen; mg/dL		27.8 ± 11.6
Serum creatinine; mg/dL		0.97 ± 0.75

[a] Data are given as mean ± standard deviation or number followed by percentage in parenthesis. AS: Ankylosing spondylitis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; TNF: Tumor necrosis factor; WBC: White blood cell count; ESR: Erythrocyte sedimentation rate.

(Table 2).

3.5. Spearman's rank order correlation analysis

Spearman rank order correlation analysis was performed to understand the relationship between IL-36α, IL-37, IL-38 and IL-39 and some AS variables (age, age at onset, disease duration, BASDAI, BASFI, WBC and ESR). In addition, the understanding was expanded to evaluate the relationship between the five cytokines that may be affected by AS. None of the AS variables showed a significant correlation with any of the five cytokines (Table 3). On the contrary, five significant positive and negative correlations were found between IL and 36α, IL-37, IL-38, IL-39 and IL-40 among the ten possible combinations. Positive correlations included IL-36α with IL-37 ($r_s = 0.285$; $p = 0.003$), IL-37 with IL-38 ($r_s = 0.318$; $p = 0.001$) and IL-39 with IL-40 ($r_s = 0.326$; $p = 0.001$). Negative correlations included IL-36α with IL-40 ($r_s = -0.434$; $p < 0.001$) and IL-37 with IL-40 ($r_s = -0.443$; $p < 0.001$) (Fig. 3).

4. Discussion

It is generally proposed that the chronic inflammatory response has a causative role in AS. Mounting evidence indicates that several cytokine pathways are associated with the disease pathogenesis including those involved in pro-inflammatory and anti-inflammatory interactions [4,5,24]. The first focus of the current study was on three members of the IL-1cytokine family (IL-36α, IL-37 and IL-38) and the literature indicates a paucity of information regarding their role in AS pathogenesis. IL-36α is associated with pro-inflammatory effects, while IL-37 and IL-38 have anti-inflammatory functions [12]. Therefore, their role in AS pathogenesis can be expected. Indeed, the levels of the three cytokines were significantly elevated in the serum of AS patients and ROC curve analysis suggested their performance as diagnostic tests, particularly IL-36α and IL-38, as the AUC approached 0.8.

Among the three IL-36 agonists (IL-36α, IL-36β and IL-36γ), IL-36α was investigated and the present study results showed significantly elevated serum levels in AS patients compared to controls. Consistent with our observation, all members of IL-36 have recently been suggested to be associated with the pathogenesis of AS and other inflammatory diseases [11]. Up-regulation of IL-36α signaling has been positively linked to the pathogenies of psoriasis, arthritis, SLE and inflammatory bowel disease (IBD) [25]. Experimental evidence has demonstrated that IL-36α and through its pro-inflammatory functions can influence other pro-inflammatory cytokines and chemokines, which in turn may be involved in the inflammatory process of AS. It has been shown that stimulation of normal human keratinocytes with IL-36α was associated with increased production of chemokines, including CXCL1, CXCL8, CCL3, CCL5 and CCL20 [26]. Further, when dendritic cells were stimulated with IL-36α, they showed a strong expression of pro-inflammatory cytokines, including IL-1β, IL-6 and tumor necrosis factor (TNF)-α [27]. IL-36α was also found to promote the infiltration of inflammatory monocytes, macrophages and neutrophils *in vivo* after administration of recombinant IL-36α. In addition, IL-36α was significantly effective in driving and enhancing T helper (h)1 responses [28]. These findings may indicate functional roles for IL-36α in regulating both innate and adaptive pro-inflammatory responses in AS.

IL-37 results of the current study were consistent with those reported by two previous studies. In the first, AS patients were found to have significantly higher serum IL-37 levels compared to controls. Further, peripheral blood monocular cells (PBMCs) of AS patients showed a significantly increased expression of IL-37. The study also confirmed the anti-inflammatory effects of IL-37, and *in vitro* analysis of PBMCs cultured with recombinant IL-37 showed a significant decrease in the production and gene expression of TNF-α, IL-6, IL-17 and IL-23 (pro-inflammatory cytokines) in AS patients [13]. In the second, serum IL-37 levels were significantly higher in AS patients than in controls, particularly in AS patients with osteoporosis. Similarly, IL-37 gene expression was significantly up-regulated in AS patients, and was higher in patients with active disease (25-fold) than in patients with inactive disease (12-fold) [8]. These data indicate that IL-37 is up-regulated in AS patients likely to down-regulate the disease-associated inflammatory response through its potentially suppressive effects on pro-inflammatory cytokines, which show up-regulated levels in AS. Inhibition of pro-inflammatory cytokines in AS patients, for instance IL-6, IL-17A and TNF-α, has been associated with a dramatic alleviation of axial and peripheral inflammation [4].

IL-38 is another cytokine of IL-1 family that showed up-regulated levels in the serum of AS patients. This cytokine has not been explored in AS, except for a proposed association of a genetic polymorphism of IL-38 with the disease risk [16]. But in other inflammatory autoimmune disorders, particularly arthritis, there has been accumulating evidence suggesting a role for IL-38 in the pathogenesis of these disorders. Recent findings have indicated that IL-38 shows up-regulated expressions in numerous inflammatory diseases, including RA, psoriatic arthritis, SLE, primary Sjogren's syndrome and IBD [12,14]. Functionally, IL-38 is broadly described as a cytokine with anti-inflammatory properties. IL-38 was found to inhibit fungal-induced Th17 responses, and *in vitro* treatment of PBMCs with IL-38 caused a decrease in IL-17A and IL-22 production. It has also been observed that recombinant IL-38 can inhibit *in vitro* the production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-17A. Pro-inflammatory effects have also been identified and this may depend on IL-38 concentration. For instance, at low concentrations of IL-38, IL-22 and IL-17A were suppressed *in vitro* but increased at high concentrations [15]. These findings suggest that IL-38 may represent a potential biomarker for predicting the development of inflammatory autoimmune diseases including AS. Indeed, the link between IL-38 and the pathogenesis of these diseases has recently been recognized and its functional involvement in the regulation of etiological mechanisms has been proposed [29].

IL-39 and IL-40 were the second focus of the current study. The first is not related to the IL-1 family of cytokines, while the second has not

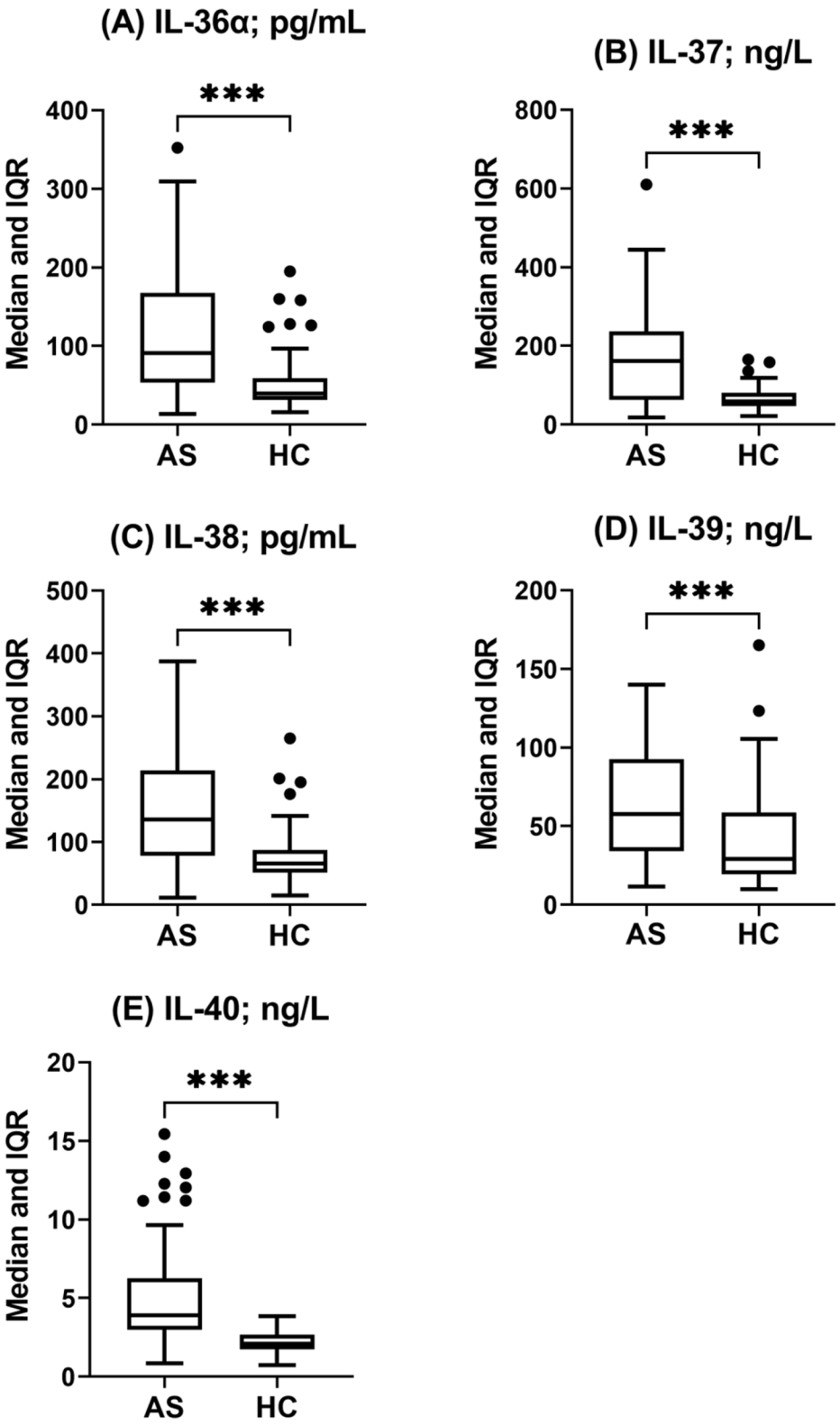

Fig. 1. Box and whisker plots (Tukey method) for IL-36α (A), IL-37 (B), IL-38 (C), IL-39 (D) and IL-40 (E) levels in serum of ankylosing spondylitis (AS) patients and healthy controls (HC). Horizontal lines within boxes represent median and whiskers represent interquartile range (IQR). Outliers are shown (black circles). Significant differences between medians were assessed using Mann-Whitney *U* test. Serum levels of the five cytokines were significantly higher in AS patients than in HC (***$p < 0.001$; Bonferroni correction was applied).

been assigned to any cytokine family. Both cytokines have not been explored in AS. IL-39 showed significant up-regulated levels in the serum of AS patients with an acceptable AUC; 0.728. IL-39 is a member of the IL-12 cytokine family, which comprises four additional cytokines (IL-12, IL-23, IL-27, IL-35) that are key immune regulators involved in the pathogenesis of a variety of inflammatory and autoimmune diseases [30]. Accumulating evidence indicates that IL-39 may have pro-inflammatory effects. A study showed that splenic B cells of lupus-like mice secreted a large amount of IL-39 when activated with lipopolysaccharides. Besides, IL-39 was linked to the induction of inflammatory responses and exacerbation of disease severity in these mice [18]. Autoimmune symptoms were also ameliorated in lupus-like mice after treatment with anti-IL-39 polyclonal antibodies. It was found that these antibodies were effective in reducing the numbers of inflammatory cells

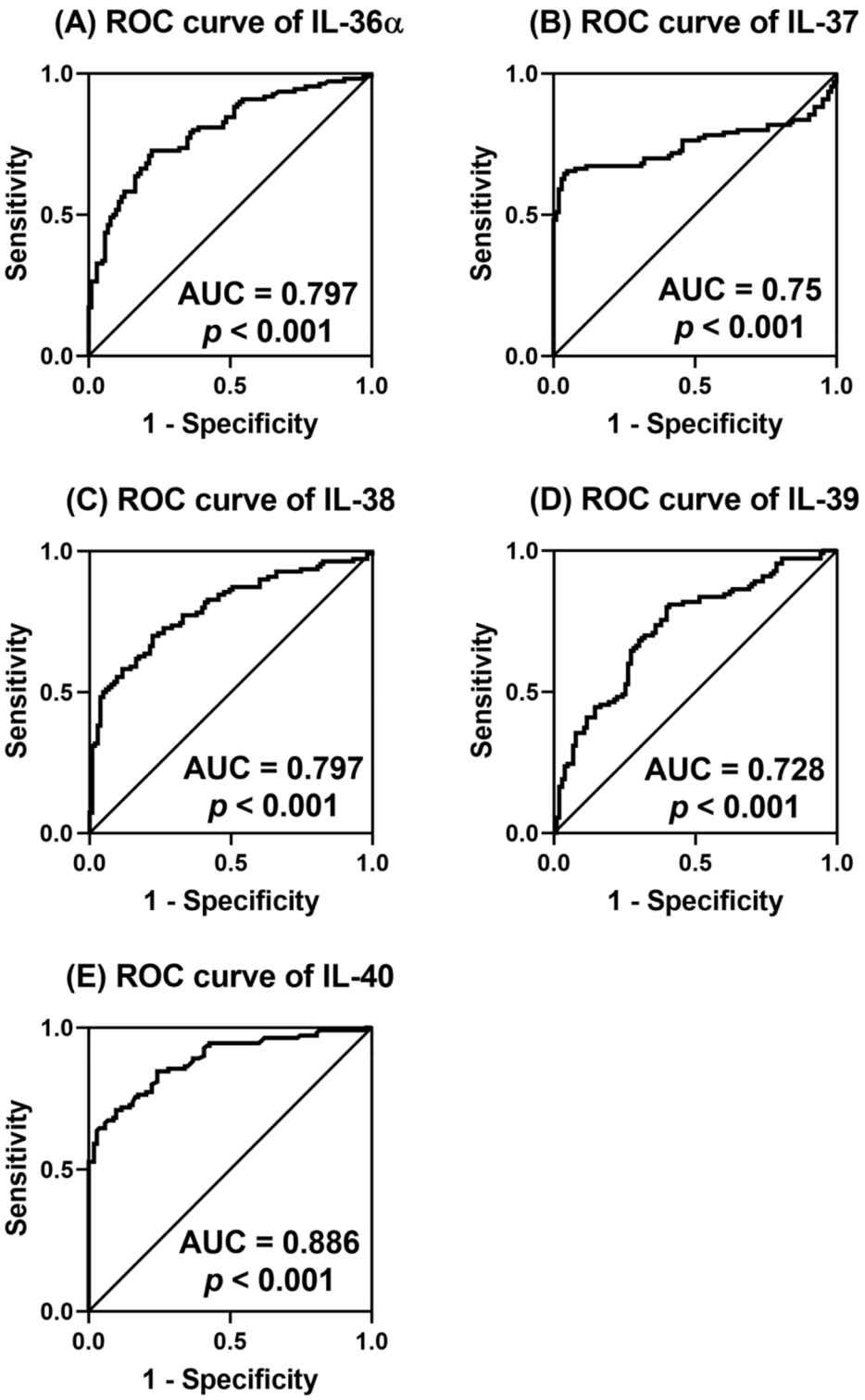

Fig. 2. ROC curve analysis of IL-36α (A), IL-37 (B), IL-38 (C), IL-39 (D) and IL-40 (E) in ankylosing spondylitis patients versus healthy controls showing area under the curve (AUC) and probability (*p*). The AUC refers to the overall performance of a diagnostic marker to distinguish between cases with and without the disease or a condition-based test. An AUC of 0.50–0.59 suggests no discrimination, 0.60–0.69 indicates poor discrimination, 0.70–0.79 is considered acceptable, 0.80–0.89 is considered excellent and ≥ 0.9 is considered outstanding.

and autoantibody titers [31]. Further, patients with neuromyelitis optica spectrum disorder, which is an inflammatory demyelinating autoimmune disorder, showed significantly higher serum levels of IL-39 compared to controls [32]. On the contrary, serum IL-39 levels were significantly lower in patients with autoimmune thyroid disease than in controls, but were positively correlated with two indicators of inflammation, C-reactive protein and leukocyte count [33]. The current study results share the pro-inflammatory potential of IL-39 and suggest its involvement in the pathogenesis of AS, especially if we consider that AS is driven by up-regulated production of pro-inflammatory cytokines [5].

Regarding IL-40, the results of the present study showed that this cytokine was significantly up-regulated in the serum of AS patients, and its diagnostic performance was excellent as indicated by an AUC value of 0.886. These data point out that IL-40 can be considered as a novel biomarker of susceptibility to AS. It has recently been reported that serum and synovial IL-40 levels were up-regulated in RA patients and positively correlated with rheumatoid factor-IgM and anti-cyclic citrullinated peptides. Further, an overexpression of IL-40 was observed in

Table 2
Serum IL-36α, IL-37, IL-38, IL-39 and IL-40 levels classified according to characteristics of ankylosing spondylitis patients.

Characteristic	Median (IQR)				
	IL-36α; pg/mL	IL-37; ng/L	IL-38; pg/mL	IL-39 ng/L	IL-40 ng/L
Age group; year					
< 45	89.7 (49.8–166.2)	151.2 (62.8–228.3)	137.9 (78.2–212.0)	58.5 (36.2–96.1)	4.17 (3.12–6.50)
≥ 45	113.4 (65.1–171.4)	173.2 (56.2–300.8)	134.5 (78.1–227.1)	53.8 (32.8–90.8)	3.65 (2.80–6.09)
p-value (*pc*)	0.477 (1.0)	0.277 (1.0)	0.503 (1.0)	0.459 (1.0)	0.299 (1.0)
Onset age group; year					
17–24	119.7 (75.1–175.0)	145.8 (63.0–225.9)	150.5 (78.2–220.5)	66.8 (33.1–106.0)	3.65 (2.72–5.30)
25–39	76.5 (46.9–133.6)	159.0 (46.1–241.6)	127.3 (79.4–204.6)	56.8 (33.5–92.3)	4.48 (3.12–6.60)
≥ 40	118.8 (73.8–174.0)	170.0 (67.9–252.4)	135.8 (78.0–227.0)	58.8 (36.0–87.7)	4.17 (2.84–6.45)
p-value (*pc*)	**0.05** (0.25)	0.954 (1.0)	0.768 (1.0)	0.782 (1.0)	0.355 (1.0)
Disease duration group; year					
≤ 1	93.4 (64.8–134.3)	186.8 (121.1–231.9)	150.5 (81.7–191.3)	46.4 (32.6–58.5)	3.85 (3.12–5.30)
2–5	88.7 (61.6–149.9)	142.6 (66.4–212.1)	134.2 (92.6–220.5)	64.0 (39.1–102.9)	4.43 (2.96–6.42)
6–10	93.3 (42.9–190.7)	167.2 (29.5–261.9)	101.8 (70.6–176.5)	71.1 (35.6–95.2)	4.76 (2.84–6.86)
> 10	87.3 (48.5–166.1)	169.7 (56.2–241.6)	157.3 (65.7–221.7)	39.6 (33.1–94.8)	3.65 (3.16–6.60)
p-value (*pc*)	0.992 (1.0)	0.755 (1.0)	0.418 (1.0)	0.283 (1.0)	0.957 (1.0)
BASDAI group					
< 4.0	94.3 (55.8–166.1)	146.7 (62.6–221.8)	134.2 (85.9–220.5)	59.3 (34.1–90.8)	3.94 (2.99–6.42)
≥ 4.0	87.0 (45.9–171.8)	182.8 (69.6–245.2)	141.6 (74.6–208.3)	57.7 (33.3–94.3)	3.76 (2.97–6.19)
p-value (*pc*)	0.5 (1.0)	0.434 (1.0)	0.789 (1.0)	0.965 (1.0)	0.886 (1.0)
BASFI group					
< 4.0	96.1 (73.9–175.0)	159.0 (62.8–248.7)	133.8 (78.1–214.1)	59.1 (34.1–93.8)	3.98 (3.01–6.19)
≥ 4.0	72.7 (42.2–123.7)	167.0 (63.5–217.4)	152.7 (92.4–212.0)	57.0 (34.9–92.0)	3.88 (2.88–6.18)
p-value (*pc*)	**0.018** (0.09)	0.663 (1.0)	0.538 (1.0)	0.826 (1.0)	0.739 (1.0)
Medication					
Anti-TNF	91.8 (53.3–171.4)	153.3 (62.8–228.3)	134.5 (78.2–214.4)	58.5 (34.9–93.8)	3.89 (2.99–6.19)
Newly diagnosed cases	88.0 (73.9–117.1)	229.0 (209.8–291.9)	146.0 (107.1–178.8)	57.0 (29.4–67.0)	3.86 (3.17–6.08)
p-value (*pc*)	0.887 (1.0)	0.063 (0.315)	0.531 (1.0)	0.362 (1.0)	0.927 (1.0)
HLA-B27					
Positive	77.2 (39.0–179.6)	163.7 (46.1–241.6)	210.5 (78.1–260.5)	59.1 (34.1–83.8)	3.89 (2.81–6.96)
Negative	94.8 (84.2–215.3)	176.5 (74.9–217.4)	146.0 (78.2–204.6)	36.3 (20.8–106.0)	3.64 (2.76–4.75)
p-value (*pc*)	0.173 (0.865)	0.432 (1.0)	0.092 (1.0)	0.936 (1.0)	0.413 (1.0)

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; TNF: Tumor necrosis factor; IQR: Interquartile range; *p*: Probability of Mann-Whitney *U* test (to compare two groups) or Kruskal-Wallis test (to compare more than two groups); *pc*: Corrected probability (Bonferroni correction). Significant *p*-value is indicated in bold.

Table 3
Spearman's rank order correlation analysis of IL-36α, IL-37, IL-38, IL-39 and IL-40 in relation to study variables among ankylosing spondylitis patients.

Variable	Statistics	IL-36α	IL-37	IL-38	IL-39	IL-40
Age	r_s	0.008	0.043	−0.088	−0.071	−0.021
	p-value	0.933	0.653	0.363	0.460	0.827
Onset age	r_s	0.030	0.011	−0.026	−0.085	0.038
	p-value	0.759	0.906	0.788	0.379	0.697
Disease duration	r_s	0.031	−0.005	−0.041	0.083	−0.035
	p-value	0.750	0.956	0.669	0.390	0.720
BASDAI	r_s	−0.088	0.021	0.049	0.026	−0.049
	p-value	0.362	0.825	0.610	0.790	0.611
BASFI	r_s	−0.137	0.030	0.116	−0.021	−0.074
	p-value	0.153	0.753	0.226	0.826	0.439
WBC	r_s	−0.039	0.108	0.102	−0.074	0.077
	p-value	0.690	0.264	0.293	0.445	0.429
ESR	r_s	−0.144	−0.156	−0.066	0.033	0.066
	p-value	0.133	0.104	0.495	0.732	0.496

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; WBC: White blood cell count; ESR: Erythrocyte sedimentation rate; r_s: Correlation coefficient; *p*: Two-tailed probability.

the synovial tissue of RA patients. It was also interesting to note that synovial fibroblasts produced significantly higher levels of CXCL8, monocyte chemoattractant protein-1 and matrix metalloproteinase-13when exposed to IL-40 compared to unexposed cells [34]. Catalan-Dibene and colleagues, the researchers who identified IL-40, conducted further studies to explore the immunological aspects of IL-40. They reported that human B cells show up-regulated expression of IL-40 when activated by anti-CD40 and anti-IgM antibodies, as well as IL-4. Further, IL-40-deficient mice showed a general IgA deficiency and the lack of IL-40 was associated with a dysregulated gut microbiota [12,35]. With regard to AS, the latter observation is important because it is suggested that the gut microbiota may have a role in the development of AS [36].

The current study data suggest the contribution of IL-36α, IL-37, IL-38, IL-39 and IL-40 to the pathogenesis of AS likely through their pro-inflammatory and anti-inflammatory effects. However, the five cytokines did not show any significant correlation with AS variables and were not influenced by these variables (age, onset age, disease duration, BASDAI, BASFI, WBC and ESR). On the other hand, some of these cytokines were significantly associated in AS patients and this may reflect the pattern of interactions between these cytokines during the pathogenic mechanisms involved in AS. However, these insights of interplay between IL and 36α, IL-37, IL-38, IL-39 and IL-40 on AS driving mechanisms require empirical evidence, which could improve our understanding of their effect and efficacy in the initiation and progression of disease. To enrich these insights, all other IL-1 and IL-12 cytokine family members should be included, and this may represent an important limitation faced by the current study.

5. Conclusions

The levels of IL-36α, IL-37, IL-38, IL-39 and IL-40 were up-regulated in the serum of AS patients regardless of age, age at disease onset, disease duration, disease activity or HLA-B27.

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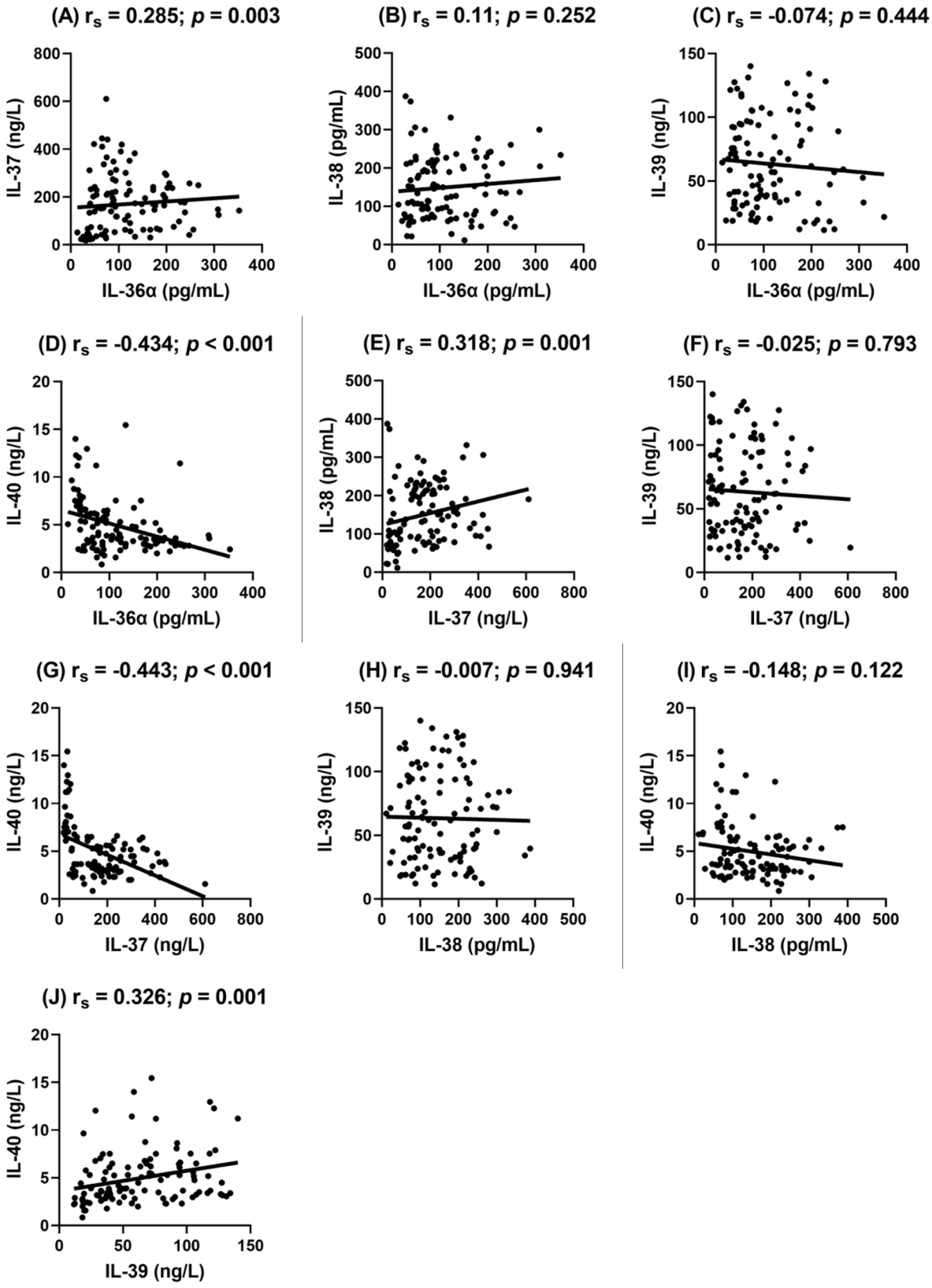

Fig. 3. Correlation between cytokine levels in serum from 110 ankylosing spondylitis (AS) patients. Correlations among levels of IL-36α, IL-37, IL-38, IL-39 and IL-40 were analyzed using Spearman's rank order correlation for all possible combinations. Correlation coefficient (r_s) and probability (p) are shown. Among the ten combinations, five showed statistical significance; three positive correlations (**A**: IL-36α with IL-37 [$r_s = 0.285$; $p = 0.003$], **E**: IL-37 with IL-38 [$r_s = 0.318$; $p = 0.001$], and **J**: IL-39 with IL-40 [$r_s = 0.326$; $p = 0.001$]) and two negative correlations (**D**: IL-36α with IL-40 [$r_s = -0.434$; $p < 0.001$] and **G**: IL-37 with IL-40 [$r_s = -0.443$; $p < 0.001$]).

CRediT authorship contribution statement

Adhraa S. Jaber: Conceptualization, Visualization, Methodology, Investigation, Validation, Writing – review & editing. **Ali H. Ad'hiah:** Conceptualization, Visualization, Methodology, Investigation, Supervision, Software, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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